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EXTRATOS VEGETAIS: ATIVIDADE BIOLÓGICA, COMPOSIÇÃO E APLICAÇÃO

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"É fazendo que se aprende a fazer aquilo que se deve aprender a fazer."
(Aristóteles)

"Todo homem que se vende recebe mais do que vale."
(Barão de Itararé)

"Precisamos dar um sentido humano às nossas construções. E, quando o amor ao dinheiro, ao sucesso nos estiver deixando cegos, saibamos fazer pausas para olhar os lírios do campo e as aves do céu."
(Érico Veríssimo)

"Há três coisas na vida que nunca voltam atrás: a flecha lançada, a palavra pronunciada e a oportunidade perdida."
(provérbio chinês)

"Aquele que pergunta, pode ser um tolo por cinco minutos. Aquele que deixa de perguntar, será um tolo para o resto da vida."
(provérbio chinês)

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RESUMO GERAL

No presente trabalho foram avaliados alguns extratos vegetais com relação à atividade biológica (antioxidante e antimicrobiana) e sua composição. Do carrapicho (*Xanthium strumarium*) foram estudados 18 extratos diferentes obtidos com extração supercrítica, hidrodestilação, maceração, maceração dinâmica e soxhlet com solventes de diferentes polaridades, incluindo metanol, etanol, acetato de etila e clorofórmio/diclorometano (1/1), enquanto que das plantas cravo-da-índia (*Caryophillus aromaticus L.*), citronela (*Cymbopogon winterianus*) e palmarosa (*Cymbopogon martinii*) apenas o óleo essencial. Também foram avaliadas as frações isoladas eugenol e eucaliptol. Para determinação da atividade antioxidante foi desenvolvido um novo índice utilizando o radical DPPH (2,2-difenil-1-picril-hidrazila), chamado de Índice de Atividade Antioxidante (IAA), através do qual foi possível comparar a capacidade antioxidante dos diferentes extratos vegetais com compostos de referência. A determinação da atividade antimicrobiana foi realizada através da determinação da concentração inibitória mínima (CIM), através da qual a ação antimicrobiana dos extratos foi comparada com a ação de alguns antibióticos frente aos microrganismos *Staphylococcus aureus, Escherichia coli, Salmonella thyphimurium*, *Pseudomonas aeruginosa* e *Clostridium perfringens*. Na avaliação da composição das frações voláteis, foi utilizada a cromatografia a gás com detector de massas para a identificação e com detector de ionização em chama para a quantificação dos compostos. Nos extratos fixos, foi utilizada a cromatografia líquida com detector de arranjo de diodos e detector de massas para a confirmação da identidade dos compostos fenólicos avaliados. Os resultados mostraram que os extratos hidrodestilados e extratos supercríticos de *X. strumarium* apresentaram uma composição qualitativa muito semelhante, entretanto, foram diferentes quantitativamente, pois no extrato hidrodestilado o

β-guaieno foi encontrado com 79,6% da composição, enquanto que nos extratos supercríticos a concentração foi de 0,2 a 0,5%. Os extratos hidrodestilados e os extratos supercríticos de *X. strumarium* apresentaram ação antioxidante muito fraca, porém forte ação antimicrobiana, entretanto, os extratos fixos de *X. strumarium* apresentaram forte ação antioxidante e forte ação antimicrobiana. Nos extratos fixos de *X. strumarium* foram identificados dois compostos fenólicos majoritários, o ácido ferúlico e o ácido clorogênico, sendo que esses compostos foram encontrados em maiores quantidades nos extratos metanólicos e etanólicos do que nos extratos obtidos com acetato de etila e diclorometano/clorofórmio. Além disso, foi encontrado um alto grau de correlação entre o teor desses compostos com a atividade antioxidante dos extratos. Foi encontrado nos cotilédones e nas sementes de *X. strumarium* o composto hepatotóxico carboxiatractilosideo, mas não foi encontrado nas folhas em estádio adulto, sendo assim, o uso medicinal dessa planta deve ser restrito apenas as folhas na fase adulta. Tanto o óleo de cravo quanto o eugenol apresentaram atividade antioxidante muito forte e ação antimicrobiana forte a moderada sobre os microrganismos avaliados. Os óleos de citronela e palmarosa e o eucaliptol apresentaram fraca ação antioxidante, porém forte a moderada ação antimicrobiana. O eugenol é o componente majoritário do óleo de cravo seguido pelo β-cariofileno, enquanto que nos óleos de citronela e palmarosa, o β-citronelal e o geraniol foram os componentes majoritários, respectivamente. Considerando que o eucaliptol e o eugenol apresentaram boa atividade antimicrobiana, e o fato de serem frações isoladas, o que facilita o controle de qualidade e a aquisição de lotes homogêneos de amostras, os dois compostos foram escolhidos para serem testados como alternativa aos antibióticos utilizados como promotores de crescimento em frangos de corte no período de 1 a 21 dias.

No experimento, foram utilizados 392 pintos machos distribuídos em 7 tratamentos onde foram utilizados o eucaliptol e o eugenol microencapsulados e o antibiótico avilamicina. Os resultados mostraram que a associação dos dois compostos afetou negativamente o desempenho das aves, entretanto, a utilização de eucaliptol (500 mg/kg) ou eugenol (500 mg/kg) melhorou o desempenho das aves em relação ao controle. Não foram verificadas diferenças significativas com o tratamento de 10 mg/kg de avilamicina, portanto, é possível substituir a avilamicina pelos extratos vegetais testados sem comprometer o desempenho das aves na fase inicial.

Palavras-chave: extratos vegetais, atividade biológica, cromatografia, espectrometria de massas, desempenho de frangos.

GENERAL ABSTRACT

In this work the biological activity (antioxidant and antimicrobial), as well as the composition of several plant extracts were studied. For cocklebur (*Xanthium strumarium*), 18 different extracts were studied, including supercritical extraction, hydrodistillation, maceration, dynamic maceration and soxhlet with different extracting solvents, including methanol, ethanol, ethyl acetate and chloroform/dichloromethane (1/1), while clove (*Caryophillus aromaticus L.*), citronella (*Cymbopogon winterianus*) and palmarosa (*Cymbopogon martinii*) only the essential oil. The isolated fractions eucalyptol and eugenol were analyzed too. To the antioxidant activity evaluation, a new Antioxidant Activity Index (AAI) using the DPPH radical (2,2-diphenyl-1-picrylhydrazyl) was proposed, that use was possible to compare the antioxidant strength between plant extracts, as well as between pure compounds. The antimicrobial activity of samples was evaluated by the determination of the minimal inhibitory concentration (MIC), by the way the extracts were compared with antibiotics against the microorganisms *Staphylococcus aureus, Escherichia coli, Salmonella thyphimurium*, *Pseudomonas aeruginosa* e *Clostridium perfringens*. The composition of the volatile fractions was carried out using a gas chromatography coupled with mass spectrometer detector for the identification and coupled with flame ionization detector (FID) for the quantification of the compounds. For non-volatile fractions, a liquid chromatography coupled with diode array detector and coupled with mass spectrometer detector was used in the phenolic compounds analysis. The results showed that the composition of the *X. strumarium* extracts obtained by supercritical extraction and by hydrodistillation presented little qualitative difference, but they did differ quantitatively, 79.6% of β-guaiene was found in the hydrodistilled, while as 0.2 to 0.5% in the

supercritical extracts. Hydrodistilled and supercritical extracts of *X. strumarium* presented poor antioxidant activity, but strong antimicrobial activity, however, the non-volatile fractions exhibit strong antioxidant and antimicrobial activities. In the non-volatile fractions, two phenolic compounds was indentified, the ferulic and chlorogenic acids, since that the concentrations were higher in methanolic and ethanolic extracts than the ethyl acetate and chloroform/dichloromethane extracts, moreover, high correlation between these compounds and antioxidant activity was found. The results show that carboxyatractyloside, a high liver toxic compound, is indeed present in the seeds and in the cotiledonary stage (two leaves stage) but not in adult leaves, therefore for medicinal use, only the adult leaves should be used. The clove oil and the eugenol presented very strong antioxidant activity and strong to moderate antimicrobial activity against tested microorganisms. The citronella and palmarosa oils and the eucalyptol showed poor antioxidant activity and strong to moderate antimicrobial activity. The eugenol was found as the majority compound in the clove oil followed by β-caryophyllene, while as in the citronella and palmarosa oils the β-citronellal and geraniol were the majority compounds, respectively. Considering that the eucalyptol and eugenol showed good antimicrobial activity and are isolated compounds, that make easy the control quality and the acquisition of the homogeneous samples batches, they are chosen compounds to be tested as alternative growth promoters in broilers from 1 to 21 days of age. In this experiment were used 392 male broilers (Ross), distributed in 7 treatments where used eucalyptol and eugenol microencapsulated and the antibiotic avilamicyn. The results showed that the association of the two compounds had negative effect in the broilers performance, however, when the eucalyptol (500 mg/kg) or eugenol (500 mg/kg) was used, the broilers performance were better than control group, and no

significant difference to 10 mg/kg of avilamicyn was observed. So, the avilamycin can be substituted by the tested plant extracts without to lose the performance of birds in the growing phase.

Key words: plant extracts, biological activity, chromatography, mass spectrometry, broilers performance.

SUMÁRIO

INTRODUÇÃO GERAL

A grande extensão territorial e latitudinal, aliadas e a diversidade climática do Brasil explicam a extraordinária riqueza vegetal que o país possui. A quantidade de espécies vegetais nativas e exóticas de importância econômica, conhecidas e descritas em trabalhos científicos, representa apenas uma amostra das que provavelmente existem. O Brasil possui a maior biodiversidade vegetal do planeta, com mais de 55 mil espécies de plantas superiores e cerca de 10 mil de briófitas, fungos e algas, um total equivalente a quase 25% de todas as espécies de plantas existentes. A diversificada flora brasileira é amplamente utilizada pela população, embora pouco se conheça cientificamente sobre seu uso. É provável que muitas espécies de plantas brasileiras tenham uso terapêutico ainda desconhecido. Esse conhecimento, entretanto, está ameaçado pelo desmatamento e pela expansão das terras agropecuárias (Brasil Enciclopédia, 1998).

Muitas plantas produzem compostos com propriedades biológicas, podendo ser utilizadas para síntese de novos medicamentos, ou mesmo serem utilizadas como substitutos de antibióticos com o objetivo de reduzir a resistência microbiana, ou ainda substituir aditivos sintéticos em formulações alimentícias (Ahmad & Beg, 2001). O uso de extratos vegetais como ingredientes funcionais em formulações alimentícias, cosméticas ou ainda em formulações sanitizantes, tem despertado grande interesse das indústrias devido a grande aceitação dos consumidores por produtos naturais.

Segundo a Resolução RDC nº 48, de 16 de março de 2004 da Agência Nacional de Vigilância Sanitária (ANVISA, 2004), a definição de droga vegetal é: “a planta medicinal ou suas partes, após processos de coleta, estabilização e secagem, podendo ser íntegra, rasurada, triturada ou pulverizada”, enquanto que derivado de droga vegetal é: “produtos de

extração da matéria prima vegetal: extrato, tintura, óleo essencial, cera, exsudato, suco, e outros".

A literatura apresenta inúmeros relatos sobre atividade biológica de extratos vegetais, como ação antifúngica (Mishra & Dubey 1994; Ozcan 1998; Aligiannis et al., 2001; Duarte et al., 2005; Hamza, et al., 2006; Alanís-Garza et al., 2007; Korukluoglu, Sahan & Yigit, 2008), ação antibacteriana (Weigenand et al., 2004; Dakir et al., 2005; Taguri, Tanaka & Kouno, 2006; Zhang et al., 2007; Ai-Turki, Ei-Ziney & Abdel-Salam, 2008), atividade anti-tumoral (Leal et al., 2003, Harmsma et al., 2004; Heo et al., 2005; Jiménez-Medina et al., 2006; Kaileh et al., 2007), ação antiinflamatória e analgésica (Asongalem et al., 2004; Khayyal et al., 2005; Santos et al., 2005; Daud, Habib & Riera, 2006; Bose et al., 2007; Díaz-Viciedo et al., 2008) atividade antioxidante (Shui, Wong & Leong, 2004**;** Cai et al., 2005; Fiorentino et al., 2006; Ferreres et al., 2007; Starzyńska-Janiszewska, Stodolak & Jamróz, 2008), dentre outras. Ceylan e Fung (2004) publicaram uma ampla revisão bibliográfica sobre a atividade antimicrobiana de diversos temperos e plantas medicinais utilizadas na culinária. Nessa revisão os autores apresentam mais de 30 temperos e plantas com atividade contra microrganismos específicos, mostrando que alguns temperos possuem amplo espectro de ação como o alecrim e tomilho, enquanto que outros são ativos contra um pequeno número de microrganismos, como a cebola.

Existe um consenso de opinião de que radicais livres induzem a danos oxidativos em biomoléculas. Esses danos podem causar aterosclerose, envelhecimento, câncer, e uma série de outras doenças (Aruoma, 1998). Além disso, os radicais livres contribuem para oxidação de lipídeos em alimentos, a qual é responsável pela formação de odores indesejáveis, além de reduzir a qualidade nutricional do alimento. Por essa razão, antioxidantes sintéticos, como o BHA (butil hidroxianisol), BHT (butil hidroxitolueno) e

TBHQ (terc-butilhidroquinona) são amplamente utilizados em indústrias de alimentos como potenciais inibidores da oxidação lipídica. Entretanto, estudos demonstraram que o BHA e o BHT se acumulam no organismo causando danos ao fígado e carcinogênese (Ito et al., 1986; Whysner et al., 1994).

O interesse em fontes naturais de antioxidantes tem aumentado e despertado o interesse das indústrias de alimentos, bebidas e cosméticos, resultando em um grande campo de pesquisa nos últimos anos. Antioxidantes naturais extraídos de plantas possuem elevada atividade antioxidante e possuem diversas aplicações na área de alimentos. Dentre as substâncias, os compostos fenólicos possuem habilidade de seqüestrar radicais livres (Hirano et al., 2001). Vários estudos têm reportado atividade antioxidante de extratos de plantas e a relação com o conteúdo de compostos fenólicos (Aaby, Hvattum, & Skrede, 2004; Silva et al., 2005; Sun & Ho, 2005; Yuan, Bone, & Carrington, 2005; Singh et al., 2007). Stratil, Klejdus e Kublán (2006) encontraram uma alta relação entre o conteúdo de compostos fenólicos totais e a atividade antioxidante de extratos vegetais.

Dentre as diversas plantas utilizadas para fins terapêuticos, as do gênero *Xanthium* são freqüentemente usadas pela população, como a *Xanthium cavanillesii,* também conhecida como *X. strumarium*. Estas plantas distribuem-se por inúmeros países, em regiões de clima temperado ou subtropical. O gênero X*anthium* é muito usado pela população como diurético, contra a pneumonia, problemas gastrintestinais e ulcerações cutâneas. Também é utilizada em lesões cutâneas como antimicrobiano. Nieves et al. (1999) encontraram uma ação diurética nos extratos aquosos de *X. cavanillesii* superior a ação diurética da furosemida.

Duarte et al. (2005) avaliaram a atividade anti-*Candida albicans* de 35 plantas medicinais brasileiras. O óleo essencial de 13 plantas mostraram boa atividade contra esse

fungo, inculindo *Cymbopogon martini* (citronela) e *Cymbopogon winterianus* (palmarosa), concordando com Yonzon et al. (2005). O cravo-da-índia (*Caryophillus aromaticus*) é uma planta amplamente utilizada para as mais diversas aplicações, como por exemplo, anestesia para peixes durante o transporte ou para minimizar o estresse antes do abate (Roubach et al., 2005), ou ainda como anestésico tópico (Alqareer et al., 2006), tratamento de candidíase vaginal (Ahmad et al., 2005), além de ser muito utilizado na culinária. As plantas citronela (*C. winterianus*) e palmarosa (*C. martinii*) têm sido estudadas em relação a sua atividade repelente de insetos (Makhaik et al., 2005; Kumar, Srivastava & Dubey, 2007) e ação antifúngica (Duarte et al., 2005), entretanto não há relatos sobre a atividade antioxidante e antimicrobiana contra os principais microrganismos causadores de enfermidades ou deteriorantes de alimentos.

A aplicação de extratos vegetais em alimentos ainda é pouco estudada. Suhr e Nielsen (2003) estudaram o efeito de óleos essenciais e extratos sobre a deterioração de pão de centeio por fungos. Os resultados mostaram que extratos de capim-limão, canela, cravo, mostarda e tomilho apresentaram um efeito positivo na preservação do pão. Nadarajah, Han e Holley (2005) utilizaram farinha de mostarda produzida a partir de sementes em níveis de 5, 10 e 20% em carne moída armazenada sob refrigeração para inibir o crescimento de *E. coli*, os resultados mostraram que foi possível eliminar *E. coli* utilizando níveis de 5-10%. Mytle et al. (2006) descreveram que a aplicação de óleo de cravo na proporção de 1 e 2% reduziu a contagem de *Listeria monocytogenes* em salsicha alemã.

Algumas cepas enterotoxigênicas de *Escherichia coli* assim como outras bactérias são responsáveis por grandes perdas econômicas na suinocultura e na avicultura em várias partes do mundo (Ferreira & Knöbl, 2000; White et al., 2003). Tanto em aves, como em suínos, a infecção causada por *E. coli* é chamada de colibacilose. Em aves, o trato

respiratório é a principal entrada do microrganismo onde forma colônias na traquéia causando quadros como peritonite, pneumonia e doença respiratória crônica complicada (DRCC) entre outros quadros clínicos (Ferreira & Knöbl, 2000).

Devido à alta incidência de problemas relacionados com microrganismos nos aviários e na suinocultura, o uso de promotores de crescimento (doses preventivas de antimicrobianos e quimioterápicos) tem sido uma medida muito utilizada para a redução dos prejuízos e melhoramento do desempenho dos animais (Freed et al., 1993). O efeito benéfico pode ser melhor expresso em animais jovens, devido sua proteção imunológica ser deficiente (Rutz & Lima, 2001). Há um consenso geral de que os efeitos benéficos dos promotores de crescimento devem-se ao controle da microflora intestinal no animal. Mas, o uso de antibióticos tem sido questionado, tanto sobre a quantidade de resíduos que estes podem deixar nos produtos em que consumimos (Vassalo et al., 1997), bem como ao surgimento de bactérias resistentes a estes medicamentos. Baccaro et al. (2002) avaliaram resistência de amostras de *E. coli* em fezes isoladas leitões lactentes com diarréia frente a diversos antibióticos. Os autores descrevem a existência de elevada resistência às drogas testadas (ampicilina 86%, neomicina 57,3%, gentamicina 86%, norfloxacino 92%, sulfadiazina-trimetropina 87,4%, penicilina 80%, entre outras), além de múltipla resistência aos principais antimicrobianos utilizados no tratamento de diarréias em leitões. Blanco et al. (1997) e Cardoso et al. (2002) encontraram uma alta resistência microbiana de amostras de *E. coli* isoladas de frango frente aos principais antibióticos utilizados, como sulfadiazina-trimetropina, tetraciclina, ampicilina e outros. A alta resistência microbiana tem assustado tanto a suinocultura quanto a avicultura mundial (Baccaro et al., 2002, Cardoso et al., 2002; White et al., 2003). Ela é transmitida por diversos mecanismos em

uma mesma população ou em populações diferentes, como da microbiota animal para a humana e vice-versa (Nijsten et al., 1993; Casewell et al., 2003).

Devido a opiniões públicas, políticas e o conceito científico de que a resistência de animais pode ser transmitida para os humanos, a União Européia (EU) vem banindo gradativamente o uso de alguns promotores de crescimento, como recentemente a avoparcina e espiramicina (Casewell et al., 2003), sendo que a Suécia baniu os antibióticos promotores de crescimento desde 1986 (Butolo, 1999) e a Dinamarca desde 2000. Segundo Gadd (1998, appud Butolo, 1999) passados alguns anos após a proibição na Suécia, houve um aumento no custo de produção, redução em torno de 5% no ganho de peso dos animais e 4% a mais no uso de antibióticos para o tratamento de doenças. No Brasil, muitos antibióticos já foram banidos, como a avoparcina pelo ofício Circular DFPA Nº 047/98, penicilina, tetraciclinas e sulfonamidas sistêmicas pela Portaria 193 de 12/05/1998, o cloranfenicol e os nitrofuranos pela Instrução Normativa 09, de 27/06/2003, o olaquindox pela Instrução Normativa 11, de 24/11/2004 e o carbadox pela Instrução Normativa 35, de 14/11/2005 (Brasil, Ministério da Agricultura, Pecuária de Abastecimento).

Apesar de pequeno número, alguns estudos com fitoterápicos foram descritos na tentativa de encontrar alternativas aos promotores de crescimento. Em estudo prévio, foram avaliados os efeitos de um agente antimicrobiano, probiótico, pré-biótico e um extrato vegetal misto (500 mg/kg - alho, cravo, canela, pimenta, tomilho, cinamaldeído e eugenol) sobre o desempenho de suínos. Os resultados mostraram que o prebiótico proporcionou desempenho equivalente ao tratamento antimicrobiano nos primeiros 14 dias experimentais, mas não melhorou a conversão alimentar. Entretanto, o probiótico e o extrato vegetal não promoveram benefício ao desempenho dos animais (Utiyama, et al., 2006). O mesmo grupo publicou outro trabalho utilizando maiores concentrações de extratos vegetais

microencapsulados na ração de suínos (700, 1.400 e 2.100 mg/kg). O extrato vegetal foi composto de óleo essencial de cravo acrescido de eugenol, tomilho e orégano acrescido de carvacrol, sendo que a composição final do produto encapsulado consistiu de 20% da mistura, sendo 3,33% de óleo essencial de cravo, 3,33% de eugenol, 3,33% de óleo essencial de orégano, 3,33% de carvacrol e 6,66% de óleo essencial de tomilho. Os extratos passaram por um processo de microencapsulação com o objetivo de minimizar o sabor dos óleos na dieta e liberá-los, de forma controlada, no estômago do animal. Os resultados mostraram que a freqüência de diarréia dos animais que receberam antimicrobianos, no entanto, foi estatisticamente inferior à do tratamento controle ou com extratos vegetais no período de 1 a 35 dias de experimentação. Os extratos vegetais apresentaram resultados intermediários de freqüência de diarréia entre o tratamento controle e com antimicrobianos (Oetting et al., 2006).

Em outro estudo, foram avaliados a adição de óleo de cravo acrescido de eugenol, óleo de orégano acrescido de carvacrol, e a mistura de ambos na ração de suínos em comparação com promotores de crescimento. Os agentes antimicrobianos, em uma combinação de 75 mg/kg de colistina e 75 mg/kg de tiamulina, proporcionaram os melhores desempenhos de leitões em fase de creche. Os extratos vegetais de cravo e orégano, individualmente, apresentaram efeitos ligeiramente redutores sobre o desempenho dos animais. Por outro lado, a combinação dos extratos de cravo e orégano (105 mg/kg de óleo essencial de cravo + 105 mg/kg de eugenol e 105 mg/kg de óleo essencial de orégano + 105 mg/kg de carvacrol) promoveu desempenho muito próximo ao obtido com os antimicrobianos, demonstrando ser uma alternativa promissora como promotor (Costa, Tse & Miyada, 2007).

Botsoglou et al. (2002) avaliaram dois níveis de óleo essencial de orégano adicionados à dieta de frangos de corte (50 e 100 mg/kg), sendo que não foi observado uma melhora no desempenho dos animais, em comparação aos alimentados com a dieta controle. Segundo os autores, uma vez que o orégano possui ação antimicrobiana e antifúngica, era previsível uma melhora no desempenho dos frangos que receberam óleo essencial de orégano na dieta, em relação àqueles alimentados com a dieta isenta de promotor de crescimento. Segundo Zuanon et al. (1998) a ação antimicrobiana dos extratos vegetais sobre a microbiota intestinal pode controlar ou inibir o crescimento dos patógenos e, conseqüentemente, proporcionar crescimento dos microrganismos benéficos, favorecendo o desempenho animal.

Considerando que estudo de substitutos aos antibióticos como promotores de crescimento é uma necessidade atual e mundial, o objetivo do presente trabalho foi avaliar a atividade antioxidante e a atividade antimicrobiana, bem como determinar a composição de vários extratos vegetais, incluindo frações fixas e voláteis, para dessa forma, encontrar extratos brutos ou frações isoladas que possuam alguma atividade biológica que possa ter aplicações na área de alimentos, como por exemplo, alternativa aos promotores de crescimento.

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Capítulo 1

Antioxidant activity index (AAI) by 2,2-diphenyl-1-picrylhydrazyl method.

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Antioxidant activity index (AAI) by the 2,2-diphenyl-1-picrylhydrazyl method

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Abstract

The aim of this work was to propose a new antioxidant activity index (AAI) using the DPPH$^{\bullet}$ method. Antioxidant activity was expressed as the antioxidant activity index (AAI) calculated as follows: AAI = final DPPH$^{\bullet}$ concentration (μg.mL^{-1})/IC$_{50}$ (μg.mL^{-1}). The compounds, BHA, chlorogenic acid, ferulic acid, gallic acid, caffeic acid, quercetin, rutin, protocatechuic acid and *trans*-cinnamic acid were used, as well as the samples clove essential oil, eugenol and *Xanthium strumarium* extract. Three concentrations of DPPH$^{\bullet}$ were used and no significant difference in the AAI for each compound tested was observed, indicating that the AAI found was appropriate to compare the antioxidant strength of plant extracts, as well as of pure compounds. Gallic acid showed the higher AAI value (27) followed by protochatechuic acid (20) and quercetin (15). Clove essential oil showed very strong antioxidant activity (9) while the *Xanthium strumarium* extract presented strong antioxidant activity (1.6).

Keywords: DPPH; AAI; phenolic compounds; antioxidant;

1. Introduction.

There is consensus of opinion that free radicals induce oxidative damage to biomolecules. This damage causes atherosclerosis, aging, cancer and several other diseases (Aruoma, 1998). Moreover, free radicals are known to take part in lipid peroxidation in foods, which is responsible for rancid odors and flavors, which decrease the nutritional quality. Therefore, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butyhydro-quinone (TBHQ) are widely used in the food industry as potential inhibitors of lipid peroxidation. However, previous studies have been demonstrated that BHA and BHT accumulate in the body and result in liver damage and carcinogenesis (Ito, Hirose, Fukushima, Tsuda, Shirai, & Tatematsu 1986; Whysner, Wang, Zang, Iatropoulos, & Williams, 1994).

Interest in natural sources of antioxidant molecules for use in the food, beverage and cosmetic industries has resulted in a large body of research in recent years. It is well known that natural antioxidants extracted from herbs and spices have high antioxidant activity and are used in many food applications. Of these substances, the phenolic compounds, which are widely distributed, have the ability to scavenge free radicals by single-electron transfer (Hirano, Sasamoto, Matsumoto, Itakura, Igarashi, & Kondo, 2001). Several studies have reported the antioxidant activity of plant extracts and their relationship with the phenolic compound content (Aaby, Hvattum, & Skrede, 2004; Silva, Ferreres, Malva, & Dias, 2005; Sun & Ho, 2005; Yuan, Bone, & Carrington, 2005; Singh, Singh, Kumar, & Arora, 2007). Stratil, Klejdus & Kublán (2006) found high correlation between the content of phenolic substances and the total antioxidant activity of sets of samples.

Several methods have been proposed to measure the antioxidant activity of pure compounds and plant extracts, such as FRAP (Ferric Reducing Antioxidant Power), ORAC

(Oxygen Radical Absorbance Capacity), ESR (Electron Spin Resonance), ABTS (2,2-azinobis (3-ethyl-benzothiazoline-6-sulphonate) and DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl). The DPPH$^{\bullet}$ method is used worldwide in the quantification of free radical scavenging activity. The reaction is based on the color decrease occurring when the odd electron of the nitrogen atom in DPPH$^{\bullet}$ is reduced by receiving a hydrogen atom from antioxidant compounds. DPPH$^{\bullet}$ is know as a stable free radical, but is sensitive to light, oxygen, pH and the type of solvent used (Ozcelik, Lee & Min, 2003).

Several methods for the DPPH$^{\bullet}$ assay have been reported, including different initial concentrations of the DPPH$^{\bullet}$ solution such as 0.025 mM (Baydar, Özkan & Yasar, 2007), 0.06 mM (Prakash, Singh & Upadhyay, 2007) 0.1 mM (Sharififar, Moshafi, Mansouri, Khodashenas & Khoshnoodi, 2007), 0.2 mM (Xu, Chen & Hu, 2005), 0.3 mM (Umamaheswari, Asokkumar, Rathidevi, Sivashanmugam, Subhadradevi & Ravi, 2007) and 0.5 mM (Elzaawely, Xuan & Tawata, 2007). Moreover, different aliquots of the extracts and the DPPH$^{\bullet}$ solutions have been reported, resulting in different final concentrations of plant extract or pure compound and of the DPPH$^{\bullet}$. Reaction times (in the dark) of 10 min (Cui, Kim & Park, 2005), 15 min (Meda, Lamien, Romito, Millogo & Nacoulma, 2005), 20 min (Chung, Chen, Hsu, Chang & Chou, 2005), 30 min (Tepe, Sokmen, Akpulat & Sokmen, 2005), 60 min (Akowuah, Ismail, Norhayati & Sadikun, 2005), 100 min (Yuan, Bone & Carrington, 2005) and 120 min (Sun & Ho, 2005) have been reported.

The results of the DPPH$^{\bullet}$ assays have been presented in many ways, such as inhibition of the free radical DPPH$^{\bullet}$ in percent (I%) calculated in the following way: I% = $[(Abs_0 - Abs_1)/Abs_0] \times 100$, where Abs_0 was the absorbance of the control and Abs_1 was

the absorbance in the presence of the test compound (Guerrero, Guirado, Fuentes & Pérez, 2006), percentage of residual $DPPH^{\bullet}$ ($DPPH^{\bullet}_{R}$) calculated as follows: % $DPPH^{\bullet}_{R}$ = [$(DPPH^{\bullet})_{t}$ / $(DPPH^{\bullet})_{t=0}$] x 100, where $DPPH^{\bullet}_{t}$ was the concentration of $DPPH^{\bullet}$ at steady-state and $DPPH^{\bullet}_{t=0}$ was the concentration of $DPPH^{\bullet}$ at zero time (initial concentration) (Siddhuraju & Becker, 2007), antiradical activity calculated according to the formula: antiradical activity = 100 x (1 – absorbance of sample/absorbance of control) (Baydar, Özkan & Yasar, 2007), ascorbic acid (AA) equivalent antioxidant capacity (AEAC) using the following equation: AEAC (mgAA/100g) = $(A_{control} - A_{sample})/(A_{control} - A_{(AA)})$ x conc. AA (mg/mL) x vol. extract (mL) x 100/g sample (Lim, Lim and Tee, 2007). The majority of the studies express the results as the IC_{50} value defined as the amount of antioxidant necessary to decrease the initial $DPPH^{\bullet}$ concentration by 50%, calculated using the graph by plotting inhibition percentage against extract concentration (Sokmen et al., 2004; Tepe, Sokmen, Akpulat & Sokmen, 2005; Ani, Varadaraj & Naidu, 2006; Elzaawely, Xuan & Tawata, 2007).

Despite the worldwide used of the $DPPH^{\bullet}$ method, the lack of standardization of the results makes it difficult to compare the antioxidant strength of different plant extracts as well as of pure compounds. Up to the present, no paper in the literature has proposed a universal index for the $DPPH^{\bullet}$ assay. For plant extracts or pure compounds the data presented, such as I% or the IC_{50} value, change according to the final concentration of the $DPPH^{\bullet}$ used. Therefore, the aim of this work was to propose a new antioxidant activity index (AAI) using the $DPPH^{\bullet}$ method.

2. Material and methods

2.1. Standards and reagents

Methanol (Ecibra, Brazil) and 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) (Sigma, USA), were used. The synthetic antioxidant butylated hydroxyanisole (BHA) and the compounds chlorogenic acid, ferulic acid, gallic acid, caffeic acid, quercetin, rutin, protocatechuic acid and *trans*-cinnamic acid were purchased from Sigma (USA).

2.2. Samples

The *Xanthium strumarium* used in this work was cultivated on the experimental farm of the Faculty of Agricultural Engineering (FEAGRI) of the State University of Campinas (UNICAMP, Campinas, São Paulo, Brazil). Voucher specimens were deposited at the State University of Campinas Herbarium denominated as 134865, and identified by Dr. Washington M. F. Neto (curator). The leaves were separated and dried in a tray drier with air circulation at 45°C (Marconi, model 035, Piracicaba, SP, Brazil), packed in dark plastic bags and stored in a domestic freezer at −20°C until the extractions. Before being submitted to extraction, the leaves were triturated in a domestic food processor (Wallita, model Master, São Paulo, SP) and the particles from 24 - 48 mesh selected using a magnetic agitator (Bertel, Model 1868, Caieiras, SP). The selected leaves were extracted (n = 3) with 80% methanol (20 g per 100 mL) for 7 days with periodic agitation. The extract was then filtered through filter paper and the residue resubmitted to agitation for 10 min with 100 mL of 80% methanol and filtered again. Both filtrates were mixed and the solvent evaporated to dryness under vacuum at 38°C. The dry extract was stored in a freezer at –20°C until assayed. The clove essential oil and eugenol were purchased from Dierberger Essential Oils S.A. (Brazil).

2.3. Antioxidant Activity

The antioxidant activity of the samples and standards was determined by way of the radical scavenging activity method using 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$). A 0.1 mL aliquots of methanolic solutions of the samples or standards at different concentrations were each added to 3.9 mL of a $DPPH^{\bullet}$ methanolic solution. Three $DPPH^{\bullet}$ solutions were tested, 0.2000, 0.1242 and 0.0800 mM, prepared by dissolving 39.4, 24.5 or 15.8 mg in 500 mL of methanol, respectively. These concentrations were selected due the linearity range of $DPPH^{\bullet}$ solution, above 0.2 mM the concentration is very high and may be able to have a mistake due Beer's law, and below to 0.5 mM the color is very weak with limited range of absorbance reading. The blank sample consisted of 0.1 mL of methanol added to 3.9 mL of $DPPH^{\bullet}$. The tests were carried out in triplicate. After a 90 min incubation period at room temperature in the dark, the absorbance was measured at 517 nm. The radical scavenging activity was calculated as follows: $I\% = [(Abs_0 - Abs_1)/Abs_0] \times 100$, where Abs_0 was the absorbance of the blank and Abs_1 was the absorbance in the presence of the test compound at different concentrations. The IC_{50} (concentration providing 50% inhibition) was calculated graphically using a calibration curve in the linear range by plotting the extract concentration vs the corresponding scavenging effect. The antioxidant activity was expressed as the antioxidant activity index (AAI), calculated as follows as:

$$AAI = \frac{\text{final concentration of } DPPH^{\bullet} \ (\mu g.mL^{-1})}{IC_{50} \ (\mu g.mL^{-1})} \qquad \text{(Equation 1).}$$

Thus, the AAI was calculated considering the mass of $DPPH^{\bullet}$ and the mass of the tested compound in the reaction, resulting in a constant for each compound, independent of the

concentration of DPPH$^{\bullet}$ and sample used. In this work we considered the plant extracts to show poor antioxidant activity when AAI < 0.5, moderate antioxidant activity when AAI between 0.5 – 1.0, strong antioxidant activity when AAI between 1.0 – 2.0, and very strong when AAI > 2.0. The assays were carried out in triplicate and all the samples and standard solutions, as well as the DPPH$^{\bullet}$ solutions, were prepared daily.

2.4. Statistical analysis

The data obtained were analyzed using ANOVA/Tukey (p<0.05). The statistical package used was Statistica^{TM} 6.0 data analysis software by Statsoft, Inc, USA.

3. Results and discussion

Table 1 shows the results of the extracts and the pure compounds. For the IC_{50} determination, it is very important that this be carried out in the linear range for each compound. Therefore, every day the analysis was carried out a calibration curve was performed for all the compounds tested, and a good linear range was observed (Table 1). The stability and linearity ranges of the DPPH$^{\bullet}$ solutions were evaluated and the results presented in Figure 1. No difference in absorbance was observed between 0 and 90 min for any of the concentrations tested and good linear ranges were observed. A previous study reported that the absorbance of DPPH$^{\bullet}$ at 517 nm in methanol and in acetone decreased by 20 and 35% respectively at 25°C in the light, however, in the dark, no significant change was observed during 150 min (Ozcelik, Lee & Min, 2003).

The AAI was determined using equation 1, where the final concentrations of DPPH$^{\bullet}$ solutions were 76.89, 47.75 and 30.75 μg.mL^{-1} for the 0.2, 0.1242 and 0.08 mM solutions. Gallic acid showed the highest AAI value followed by protochatechuic acid and quercetin. No significant differences were observed between eugenol, chlorogenic acid, clove

essential oil, caffeic acid and BHA, which all showed higher AAI values than ferulic acid and rutin, which were similar to each other (Table 1). *Trans*-cinnamic acid presented no ability to reduce the $DPPH^{\bullet}$ even when tested at a higher concentration (200 $\mu g.mL^{-1}$ final concentration). The *Xanthium strumarium* extract showed a high AAI value and exhibited strong antioxidant activity. A previous study reported the presence of phenolic compounds, such as chlorogenic and ferulic acids in the *Xanthium strumarium* extracts (Han, Li, Zhang, Zheng & Qin 2006). Clove essential oil had very strong antioxidant activity due the presence of eugenol, which was reported as the majority compound (Tomaino et al., 2005; Jirovetz, Buchbauer, Stoilova, Stoyanova, Krastanov & Schmidt, 2006).

Structurally, phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerised compounds (Bravo, 1998). The structure of phenolic compounds is a key determinant of their radical scavenging and metal chelating activity, and this is referred to as structure activity relationships (Balasundram, Sundram & Samman, 2006). The antioxidant activity of phenolic acids increase with increasing degree of hydroxylation, as is the case of gallic acid (trihydroxylated) and protochatechuic acid (dihydroxylated) which show high AAI values. The substitution of the hydroxyl group in the aromatic ring with a methoxyl group, as in the case of caffeic acid and ferulic acid (Figure 2) reduced the AAI value (Table 1), in agreement with Rice-Evans, Miller and Paganga (1996) who reported that the substitution of hydroxyl by methoxyl groups reduced the activity. This can be explained by the reduction in the hydrogen atom donating capacity of the molecule. The absence of a hydroxyl group in the aromatic ring of *trans*-cinnamic acid (non phenolic) could maybe explain its incapacity to reduce $DPPH^{\bullet}$. The structure–activity relationships (SAR) of

flavonoids are generally more complicated than those of hydroxybenzoic and hydroxycinnamic acids due to the relative complexity of the flavonoid molecules. Van Acker et al. (1996) reported that the degree of hydroxylation as well as the position of the hydroxyl groups, and a double bond combined with a R1 = OH (Figure 3), increased the radical scavenging capacity of flavonoids. For this reason, quercetin has a higher AAI value than rutin (Table 1), in which the hydroxyl group is substituted by rutinose in the R1 position (Figure 3).

When different $DPPH^{\bullet}$ solutions were used for the same extract or pure compound, the IC_{50} value varied, although the AAI value remained constant, since no significant differences between the AAI values found for each compound tested were observed (Table 1). Meda, Lamien, Romito, Millogo & Nacoulma (2005) evaluated the antioxidant activity of honey by the $DPPH^{\bullet}$ method and used quercetin as the control. The authors reported that the IC_{50} of quercetin was 0.87 $\mu g.mL^{-1}$ when a final $DPPH^{\bullet}$ concentration of 0.0338 mM was used. However, as cited before, the IC_{50} value varies with the final concentration of $DPPH^{\bullet}$ used. So, applying equation 1 proposed in this study, the AAI for quercetin in the study of Meda, Lamien, Romito, Millogo & Nacoulma (2005) was 15.32, in agreement with the results of the present study. The AAI values for samples of honey (27) ranged from 0.45 to 9.8, since that 5 samples presented AAI < 1.0 and 12 samples presented AAI between 1.0 and 2.0. Singh, Singh, Kumar & Arora (2007) evaluated the antioxidant activity of *Acacia auriculiformis*, the values of IC_{50} were 35.4 and 51.3 $\mu g.mL^{-1}$ for water and ethyl acetate fractions, so that the AAI values (equation 1) were 0.95 and 0.66, respectively. Sharififar, Moshafi, Mansouri, Khodashenas & Khoshnoodi (2007) reported IC_{50} values of 11.7 and 16.2 $\mu g.mL^{-1}$ for non-polar and polar fractions of endemic *Zataria*

multifolra Boiss, applaing the equation 1, the AAI values were 3.33 and 2.4, respectively. Elzaawely, Xuan & Tawata (2007) studied the antioxidant activity of leaves and rhizomes of *Alpinia zerumbert*, the author's related values of IC_{50} from 70 to 700 $\mu g.mL^{-1}$, according to the equation 1, the AAI values were 0.09 to 0.93 and 3.8, respectively.

As it was mentioned, there is a deficiency to compare the antioxidant potential between extracts due the several ways that of the results are presented. The $DPPH^{\bullet}$ index (I%) only shows the capacity of the sample, in a fixed concentration, to reduce or not the $DPPH^{\bullet}$ radicals, in which many cases, increasing the extract concentration the I% will be increased. The IC_{50} shows the extract concentration necessary to decrease the initial $DPPH^{\bullet}$ concentration by 50%, however, using different $DPPH^{\bullet}$ concentration the results will be different for the same sample. So, the AAI relate the $DPPH^{\bullet}$ concentration used in the assay with IC_{50} of the sample, resulting in a constant data for each compound or plant extract, since has been obtained at the same conditions, because there is a consensus that different extraction procedure or different places of harvest, could be give different results.

Conclusions

The proposed antioxidant activity index (AAI) was shown to be appropriate for the comparison of the antioxidant strength between plant extracts and essential oils, as well as between pure compounds, since no significant difference in AAI was observed when different solutions of $DPPH^{\bullet}$ and different concentrations of tested compound, were used. Gallic acid, protochatechuic acid and quercetin showed higher AAI values. The clove essential oil presented very strong antioxidant activity, and no significant difference from the AAI of eugenol was observed. *Xanthium strumarium* extract presented strong antioxidant activity.

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Table 1: Values for the antioxidant activity index (AAI) with different final concentrations of DPPH•.

	I			II			III					
DPPH• 76.89 μg.mL^{-1}	**r^2**	***IC_{50}**	**AAI**	**r^2**	**IC_{50}**	**AAI**	**r^2**	**IC_{50}**	**AAI**	**Mean IC_{50}**	**Mean AAI**	**SD AAI**
Gallic acid	0.9984	2.90	26.51	0.9996	2.76	27.86	0.9999	2.84	27.07	2.83	27.15^{a}	0.68
Protochatechuic acid	0.9987	3.80	20.23	0.9989	3.97	19.37	0.9938	3.68	20.89	3.82	20.17^{b}	0.77
Quercetin	0.9995	5.48	14.03	0.9978	4.74	16.22	0.9997	4.39	17.51	4.88	15.92^{c}	1.76
Eugenol	0.9987	7.35	10.46	0.9939	6.78	11.34	0.9985	6.83	11.26	6.99	11.02^{d}	0.49
Chlorogenic acid	0.9999	7.56	10.17	0.9956	7.32	10.50	0.9991	7.43	10.35	7.44	10.34^{d}	0.17
Clove essential oil	0.9988	8.15	9.43	0.9986	7.08	10.86	0.9999	8.27	9.30	7.83	9.86^{d}	0.87
Caffeic acid	0.9937	8.00	9.61	0.9925	8.07	9.53	0.9986	8.57	8.97	8.21	9.37^{d}	0.35
BHA	0.9976	7.83	9.82	0.9948	8.64	8.90	0.9963	8.22	9.35	8.23	9.36^{d}	0.46
Rutin	0.9992	12.09	6.36	0.9993	11.39	6.75	1.0000	12.78	6.02	12.09	6.38^{e}	0.37
Ferulic acid	0.9994	14.68	5.24	0.9990	13.70	5.61	0.9996	14.96	5.14	14.45	5.33^{e}	0.25
X. strumarium	0.9953	44.70	1.72	0.9965	43.93	1.75	0.9960	46.81	1.64	45.15	1.70^{f}	0.06
Trans-cinnamic acid	-	-	-	-	-	-	-	-	-	-	-	-
DPPH• 47.75 μg.mL^{-1}	**r^2**	**IC_{50}**	**AAI**	**r^2**	**IC_{50}**	**AAI**	**r^2**	**IC_{50}**	**AAI**	**Mean IC_{50}**	**Mean AAI**	**SD AAI**
Gallic acid	0.9992	1.83	26.09	1.0000	1.84	25.93	0.9998	1.96	24.36	1.89	25.46^{a}	0.96
Protochatechuic acid	1.0000	2.35	20.30	0.9988	2.47	19.33	0.9993	2.23	21.44	2.35	20.36^{b}	1.05
Quercetin	0.9998	3.22	14.83	0.9939	2.83	16.85	0.9997	3.05	15.66	3.03	15.78^{c}	1.02
Eugenol	0.9994	4.45	10.73	0.9998	4.71	10.14	0.9991	4.66	10.25	4.61	10.37^{d}	0.32
Chlorogenic acid	0.9995	4.89	9.76	0.9987	4.77	10.01	0.9986	4.57	10.45	4.74	10.08^{d}	0.35
Clove essential oil	0.9992	4.98	9.59	0.9992	4.99	9.57	0.9996	4.79	9.97	4.92	9.71^{d}	0.23
Caffeic acid	0.9983	4.60	10.38	0.9969	4.57	10.45	0.9966	4.25	11.23	4.47	10.69^{d}	0.47
BHA	0.9987	5.28	9.04	0.9991	5.27	9.06	0.9969	5.15	9.27	5.23	9.12^{d}	0.13
Rutin	0.9998	8.17	5.85	0.9997	7.30	6.54	0.9996	7.45	6.41	7.64	6.27^{e}	0.37
Ferulic acid	0.9997	8.27	5.77	0.9998	8.93	5.34	0.9992	9.64	4.95	8.95	5.36^{e}	0.41
X. strumarium	0.9973	29.31	1.63	0.9996	30.47	1.57	0.9958	29.99	1.59	29.92	1.60^{f}	0.03
Trans-cinnamic acid	-	-	-	-	-	-	-	-	-	-	-	-
DPPH• 30.75 μg.mL^{-1}	**r^2**	**IC_{50}**	**AAI**	**r^2**	**IC_{50}**	**AAI**	**r^2**	**IC_{50}**	**AAI**	**Mean IC_{50}**	**Mean AAI**	**SD AAI**
Gallic acid	0.9997	1.17	26.38	0.9967	1.10	27.73	0.9967	1.11	27.66	1.12	27.25^{a}	0.76
Protochatechuic acid	0.9893	1.41	21.86	0.9885	1.48	20.71	0.9908	1.48	20.80	1.45	21.12^{b}	0.64
Quercetin	0.9968	1.98	15.56	0.9882	1.76	17.92	0.9862	1.97	15.60	1.90	16.36^{c}	1.35
Eugenol	0.9982	3.02	10.19	0.9932	2.65	11.58	0.9842	2.83	10.86	2.83	10.88^{d}	0.70
Chlorogenic acid	0.9989	2.82	10.92	0.9998	2.69	11.41	0.9999	2.73	11.28	2.75	11.20^{d}	0.25
Clove essential oil	0.9938	3.28	9.36	0.9874	2.94	10.44	0.9928	2.94	10.45	3.05	10.08^{d}	0.63
Caffeic acid	0.9957	2.89	10.64	0.9923	2.94	10.45	0.9996	2.78	11.07	2.87	10.72^{d}	0.31
BHA	0.9994	3.51	8.77	0.9816	3.03	10.14	1.0000	2.69	11.44	3.08	10.12^{d}	1.34
Rutin	0.9975	5.12	6.00	0.9940	4.28	7.18	0.9894	3.95	7.78	4.45	6.99^{e}	0.91
Ferulic acid	0.9966	6.77	4.54	0.9992	5.66	5.43	0.9992	5.08	6.06	5.84	5.34^{e}	0.76
X. strumarium	0.9923	20.23	1.52	0.9899	19.02	1.61	0.9991	18.12	1.69	19.12	1.60^{f}	0.08
Trans-cinnamic acid	-	-	-	-	-	-	-	-	-	-	-	-

I, II and III: different days of analysis; r^2: linearity coefficient; IC_{50}: concentration providing 50% inhibition; *: μg.mL^{-1}; SD: standard deviation; -: not found. Different letters correspond to significant difference (P<0.05).

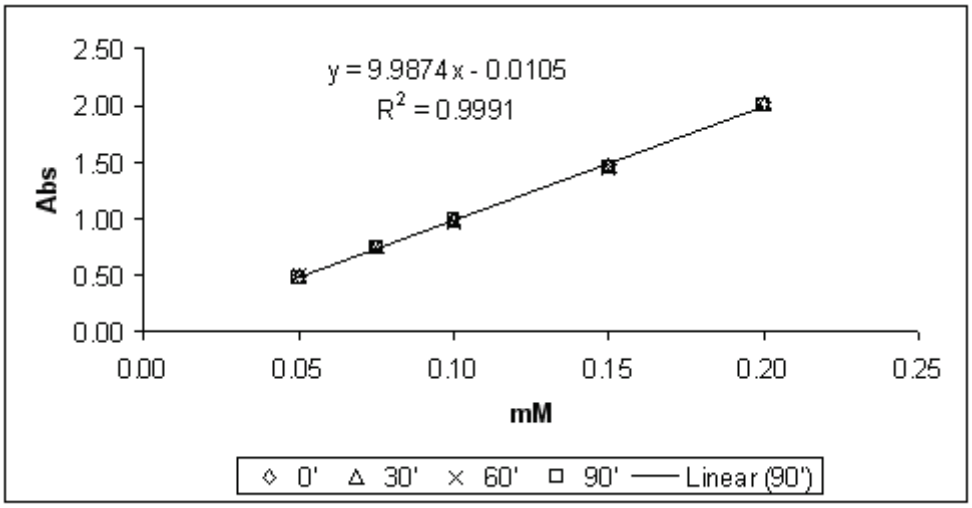

Figure 1: calibration curve and stability evaluation of the DPPH$^{\bullet}$ solutions. The assays were carried out in triplicate with 3.9 mL of DPPH$^{\bullet}$ solution plus 0.1 mL of methanol.

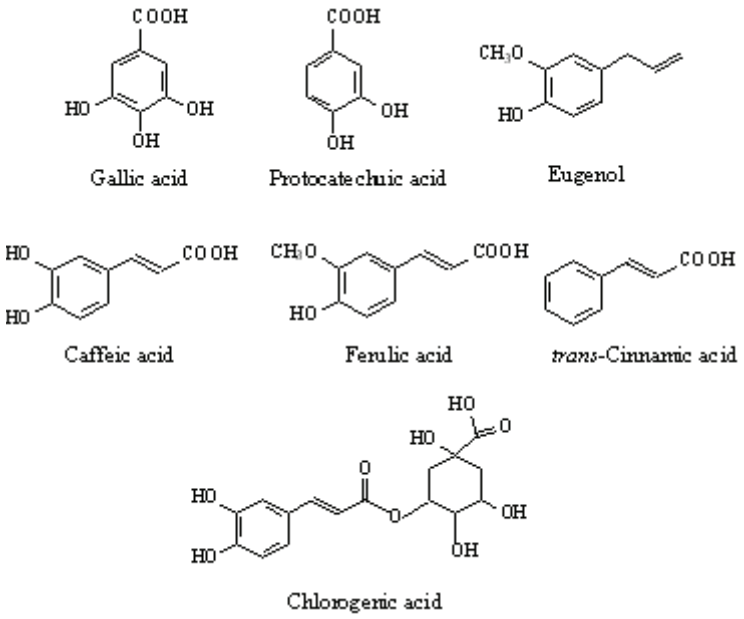

Figure 2: Structure of some of the compounds used in the assays.

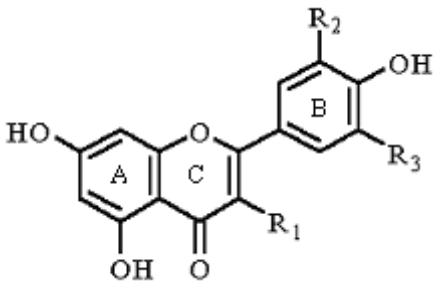

Figure 3: Structure of the flavonoid molecule. Quercetin: R1 and R2 = OH, and R3 = H; rutin is a glycoside of quercetin where R1 = disaccharide, rutinose (β-1-L-rhamnosido-6-o-glucose).

Capítulo 2

Biological Activity and Chemical Composition of Hydrodistilled and Supercritical Extracts of *Xanthium strumarium* leaves

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Biological Activity and Chemical Composition of Hydrodistilled and Supercritical Extracts of *Xanthium strumarium* L. leaves

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Abstract

The antimicrobial and antioxidant activities, and the composition of the essential oil and of the supercritical extracts of *Xanthium strumarium* were studied. The best yields were observed in the supercritical extracts (SFE). The composition of the extracts obtained by SFE and by hydrodistillation presented little qualitative difference, but they did differ quantitatively. The essential oil contained a high content of β-guaiene (79.6%), while as the major compounds in the supercritical extracts were unidentifiable compound with Kovats Index of 2303 and xanthinin. All the *X. strumarium* extracts showed strong antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Salmonella thyphimurium*, *Pseudomonas aeruginosa* and *Clostridium perfringens*, however no differences were observed between the extracts. Poor antioxidant activity was found for all the *Xanthium strumarium* extracts.

KEYWORDS: common cocklebur, *Xanthium strumarium*, Asteraceae, essential oil composition, supercritical extract composition

INTRODUCTION

Cocklebur (*Xanthium strumarium* L.) is a herbaceous annual plant with a worldwide distribution. The plant is erect, up to 2.5 m tall, with blotched purple stems. The leaves are dark green on the upper surface, similar in shape to grape leaves, 15 cm in diameter and roughly textured with minute bristles. The flowers are inconspicuous, both male and female, occurring in leaf axils towards the end of the branches. The flowers develop into hard, woody, spiny burrs (fruits), 1.2 cm to 2 cm long with numerous hooked spines. *X. strumarium* is extremely competitive with other agronomic crops. It has long been regarded as one of the worst weeds in soybean plantations (1).

Despite its medicinal use (2,3,4), some investigations have been reported that *X. strumarium* induced intoxication and could be lethal to cattle (5), sheep (6), pigs (7) and humans (8). The results have shown that consumption of the fruits (burs) and the cotyledonary stage (two-leaf stage) induces hepatic necrosis and myocardial injury in humans. The toxic principle in the poison of *X. strumarium* was isolated and identified as carboxyatractyloside (CAT) (9), a highly selective inhibitor of oxidative phosphorylation (10). The presence of CAT in adult and cotyledonary stage dried leaves, seeds (inside the burr) and shell of the burr was investigated via ESI-MS/MS, and CAT was found in the extracts from the seed and plants in the cotyledonary stages, but not in the adult leaves or the shell of the burr, so the medicinal use of *X. strumarium* should be restricted to the adult leaves (11).

Several investigations of the biological properties of *X. strumarium* have been reported, such as anti-ulcerogenic (12), antitrypanosomal (13), anti-helmintic (14), anti-inflammatory (15) and antimicrobial activities (16), a diuretic action (17), anti-leishmanial and antifungal activities (18) and a hypoglycemic action (19). In addition, the chemical

composition of *X. strumarium* has been found to include phenolic compounds, such as chlorogenic and ferulic acids (20), triterpenoid saponin (15), carboxyatractyloside (CAT) (9) and xanthanolide sesquiterpene lactones (8-epi-xanthatin and 8-epi-xanthatin epoxide). These compounds have demonstrated significant inhibition of the proliferation of cultured human tumor cells (21), several xanthanolides (22), beta-sitosterol (23) and strumasterol (C-24 epimer of stigamsterol) (24).

The composition and physicochemical properties of the steam distilled oil of the aerial parts of *X. cavanillesii* from Argentina (synonym of *X. strumarium*) have been reported. Organic acids, phenols and mainly terpenes were found and identified, including limonene (43.6%), myrcene (5.4%), alpha-cubebene (5.1%) (25). In the leaf oil of *X. strumarium* from Iran, 28 compounds were identified, representing 85.2% of the whole oil composition. The main compounds were limonene (24.7%), borneol (10.6%), alpha-cubebene (6.3%), bornyl acetate (5.9%) and sabinene (4.2%) (26). The composition and concentration of the compounds in the oils from the same plant species may vary due ecological and plant growth factors (27).

However, there are no reports about the biological properties such as the antioxidant and antimicrobial activities of the oil of *X. strumarium*, and little data about the Brazilian plant. Therefore the aim of this work was to investigate the antioxidant and antimicrobial activities as well as the chemical composition of hydrodistilled oil and supercritical fluid CO_2 extracts of *X. strumarium* leaves from Brazil.

EXPERIMENTAL

Reagents and Standards. Anhydrous sodium sulphate (Dinâmica, Brazil), ethanol, diethyl ether and methanol (Ecibra, Brazil), hexane (Mallinckrodt, USA), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) (Sigma, Germany), nutrient agar (Merck, Germany),

dimethyl sulfoxide (DMSO) (Sigma, USA), triphenyl tetrazolium chloride (TTC) (Merck, Germany), culture medium (Mueller-Hinton 2.1%, Merck, Germany) were used. The CO_2 used for the supercritical extractions was obtained from Gama Special Gases (99.0%, Campinas, Brazil). The antibiotics amoxicillin, avilamycin, enrofloxacin, erythromycin, halquinol, tetracycline, tiamulin, tylosin, oxytetracycline, penicillin G, spiramycin, sulfadiazine and sulfanilamide were from Sigma (USA) or Fluka (Germany). The synthetic antioxidant butylated hydroxyanisole (BHA) and the phenolic compounds caffeic acid, chlorogenic acid, ferulic acid, gallic acid, quercetin, rutin, protocatechuic acid (3,4 hydroxybenzoic acid), resveratrol and (E)-cinnamic acid were purchased from Sigma (USA). Eugenol was purchased from Dierberger Essential Oils S.A. (Brazil).

Plant Material. The *X. strumarium* used in this work was cultivated at the experimental farm of the Faculty of Agricultural Engineering (FEAGRI) of the State University of Campinas (UNICAMP, Campinas, São Paulo, Brazil). A voucher specimen was deposited at the Campinas State University Herbarium as number 134865, and identified by Dr. Washington M. F. Neto (curator). The leaves were separated and dried in a tray drier with air circulation at 45 °C (Marconi, model 035, Piracicaba, Brazil), packed in dark plastic bags and stored in a domestic freezer at −20 °C until the either distilled or extracted.

Extracts. Before extraction the dry leaves were triturated in a domestic food processor (Wallita, model Master, São Paulo, Brazil) and particles with sizes from 24 - 48 mesh were selected using a magnetic stimer (Bertel, Model 1868, Caieiras, Brazil). Hydrodistillation (HD) was accomplished using a commercial Clevenger apparatus, using 400 g in 500 mL of water for 3 h. The aqueous phase was extracted with diethyl ether, dried over anhydrous sodium sulphate, filtered and the solvent evaporated to dryness. The oil

obtained was denominated as HD and stored at −20 °C until use. For the supercritical fluid extraction (SFE), the effects of extraction pressures (150, 250 and 350 Bar) and temperature (30 °C and 50 °C) on the overall yield of the extracts from *X. strumarium* were evaluated. The extracts were denominated as follow: SFE1 – 150 bar/50 °C, SFE2 – 250 bar/50 °C, SFE3 – 350 bar/50 °C, SFE4 – 150 bar/30 °C, SFE5 – 250 bar/30 °C and SFE6 – 350 bar/30 °C. The SFE unit used was built in the Technical University of Hamburg-Harburg (TUHH, Hamburg, Germany) (28). The raw material packed bed was prepared by manual packing of the ground particles *X. strumarium* (7 g) inside the extraction column. A glass wool plug was placed at both ends of the extraction column in order to avoid the drag of small particles by the solvent. The CO_2 mass flow rate was 5 g/min. The extract was collected in a cooled glass flask to reduce the amount of volatile compounds in the outlet gas stream. The CO_2 was allowed to flow into the extraction column until there was no noticeable extract leaving the system (1 h). The tubing line after the extraction column was washed with ethanol to recover the extract deposited on it. The solvent was separated from the extract using a rotatory evaporator. The total extract mass was determined by the sum of the extract obtained during the extraction and the one obtained by the cleaning process.

Gas Chromatographic Analysis. A Shimadzu 17A gas chromatograph coupled with a Shimadzu QP-5000 quadrupole mass spectrometer (identification) and Varian 3800 gas chromatograph coupled with FID detector (quantification) instrument, equipped a silica capillary column (DB-5; 30 m x 0.25 mm; 0.25 μm film thickness). Helium was the carrier gas at a flow rate of 1.0 mL/min. The analyses were performed using splitless injection (1 μL), with the injector set at 230 °C. The oven temperature program used was 60 - 240 °C at 3 ºC/min and the final temperature was held for 7 min. The GC-MS interface and FID

detector were maintained at 240 ºC and 250 °C, respectively. The extracts were dissolved in hexane for the analyses. The MS data were obtained in the scan mode (35 – 400 m/z). Retention Indices (RI) were determined by injection of standard hydrocarbon solutions. The components were identified by comparison with data from the literature (29), the profiles from the NIST 98 libraries, and by injection of authentic standards, when available.

Antioxidant Activity. The antioxidant activity of the oil and extracts and standards was determined by way of the radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) according to Scherer and Godoy (30). A 0.1 mL aliquot of a methanolic solution of the extracts or standards in different concentrations was added to 3.9 mL of DPPH (0.2 mM) prepared daily. The blank sample consisted of 0.1 mL of methanol added to 3.9 mL of DPPH (0.2 mM). The tests were carried out in triplicate. After a 90 min incubation period at room temperature in the dark, the absorbance was measured at 517 nm. The radical scavenging activity was calculated as follows: scavenging effect (%) = [(Abs_0 – Abs_1)/Abs_0] x 100, where Abs_0 was the absorbance of the blank and Abs_1 was the absorbance in the presence of the test compound at different concentrations. The IC_{50} (concentration providing 50% inhibition) was calculated graphically using a calibration curve in the linear range by plotting the extract concentration vs the corresponding scavenging effect. The antioxidant activity was expressed as antioxidant activity index (AAI) calculated as follows: AAI = concentration of DPPH ($\mu g.mL^{-1}$)/IC_{50} ($\mu g.mL^{-1}$). In the present study, the plant extracts were considered to show poor antioxidant activity when the AAI < 0.5, moderate antioxidant activity when the AAI was between 0.5 – 1.0, and strong antioxidant activity when the AAI was between 1.0 – 2.0 and very strong activity when the AAI > 2.0.

Antimicrobial activity. The minimal inhibitory concentration (MIC) tests were carried out according to the NCCLS (National Committee of Laboratory Standards, 2003) (31) using a tissue culture test plate (96 wells). *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (isolated from swine), *Salmonella typhimurium* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 13388) and *Clostridium perfringens* (ATCC 1324) were obtained from the CBMAI (Brazilian Collection of Environmental and Industrial Microorganisms, CPQBA/UNICAMP, Brazil). The microorganisms were grown overnight at 36 °C in nutrient agar plates in aerobiosis, except for *Clostridium perfringens*, cultivated in thioglycolate broth in anaerobiosis. The inoculum for the assays was prepared by diluting scraped cell mass in 0.85% NaCl solution, adjusted to the McFarland scale 0.5. Cell suspensions were finally diluted to 10^4 CFU/mL for use in the assays. The oil and the extracts and the antibiotics were diluted in DMSO and stored at −20 °C until used. The final concentrations of the extracts were 1.8, 1.5, 1.2, 1.0, 0.8, 0.6, 0.4 and 0.2 mg/mL, while serial dilutions of the antibiotics were prepared between 0.25 and 0.00195 mg/mL. The inoculum was added to all the wells and the plates were incubated at 36 °C for 24 h. To verify the sterility of the extracts and culture medium (Mueller-Hinton 2.1%), as well as the viability of the microorganism, a control test was made with all the test plates. Antimicrobial activity was detected by adding 0.05 mL of an aqueous 0.5% triphenyl tetrazolium chloride (TTC) solution. MIC was defined as the lowest concentration of the oil or extract that visually inhibited the growth, as indicated by the TCC staining (dead cells are not stained by TTC).

RESULTS AND DISCUSSION

The yield of oil obtained by hydrodistillation was 0.12 ± 0.01%, in agreement with Thaer et al. (25) who reported a yield of 0.12%. However, this result was lower than the

yield obtained in supercritical extractions, where the yields were more than 20 times larger. The yields (%) for the supercritical extractions were: SFE1 – 3.80, SFE2 – 3.34, SFE3 – 2.43, SFE4 – 3.47, SFE5 – 3.66 and SFE6 – 2.58. Lower yields were observed with the higher pressure (350 bar) and the condition of 150 bar/50 °C (SFE1) showed the best yield. Little variation was observed between the other extractions. Previous studies have evaluated the yield of extracts and oil obtained by SFE and HD, respectively, and all the authors reported higher yields with SFE (32,33). Steam distillation is the worldwide method for isolating essential oil from plants. Its advantage over other methods involving solvents is the absence of non-volatile compounds in the extract, such as chlorophyll and fatty acids. However, supercritical fluid extraction (SFE) has been extensively investigated as an alternative process to produce extracts from vegetable matrices, and one of the reasons is the higher yields (34).

The relative contents of the compounds identified in the oil and extracts are shown in Table I. The oil was found to contain β-guaiene (79.6%), β-caryophyllene (3.9%), α-humulene (2.1%), limonene (2.2%), and valencene (1.0%). The supercritical extracts were similar amongst themselves, but some differences with the oil were observed (Figure 1). In the supercritical extracts two major compounds were found, and both compounds presented similar mass spectra, but only xanthinin tentatively identified (*m/z* 43, 60, 91, 93, 135 and 246) and the other compound with Retention Index of 2303 (*m/z* 43, 91, 93, 135 and 246) (Table 1). They also contained moderate amounts of γ-cadinene, δ-cadinene and α-cadinene, but low contents of β-guaiene. Although the compositions of the extracts obtained by SFE and the oil by hydrodistillation were similar, they did differ quantitatively, in agreement with other previous studies (32,33).

The results of the antimicrobial assays are shown in Table II. No differences between the *X. strumarium* oil and extracts were observed except for the *P. aeruginosa*, which was more susceptible to the HD and SFE4 extracts than the others. *Staphylococcus aureus* was the most susceptible microorganism followed by *E. coli* and *P. aeruginosa,* while *S. typhimurium* and *C. perfringens* were the more resistant to the *X. strumarium* oil and extracts. Duarte et al. (35) proposed a classification for plant materials, based on the MIC results as follows, strong inhibitors when MIC below 0.5 mg/mL, moderate inhibitors when MIC between 0.6 and 1.5 mg/mL and weak inhibitors when MIC above 1.6 mg/mL. These authors have established 2.0 mg/mL as the highest concentration, so that only the oils or extracts presenting a MIC below 2.0 were considered as having potential antimicrobial activity. Thus, considering the classifications cited above, all the *X. strumarium* extracts and oil showed potential antimicrobial activity against the microorganisms evaluated (Table II). Xanthatin was tentatively found in small quantities in all the *X. strumarium* extracts (Table I). This compound was isolated from the extracts of *X. spinosum* and was active against *Colletotrichum gloesporoides, Trichothecium roseum, Bacillus cereus* and *Staphylococcus aureus* (36).

For the antibiotics, enrofloxacin seems to be the most effective against the microorganisms tested, since the MIC values were below 0.0019 mg/mL for all strains except for *S. typhimurium* (Table II). *Staphylococcus aureus* appears to be the most susceptible microorganism, since the tested antibiotics presented very low MIC values (Table II).

The results for the antioxidant activity study can be seen in Table III. All the *X. strumarium* extracts and oil showed very high IC_{50} values and consequently very low AAI values, indicating poor antioxidant activity. The supercritical extracts SFE4 and SFE5

showed the best results although with insignificant antioxidant activity. The phenolic compounds gallic acid and protocatechuic acid showed the highest AAI values followed by quercetin and chlorogenic acid, while resveratrol, ferulic acid and morin showed the lowest values, respectively (Table III). The synthetic antioxidant BHA was similar to caffeic and chlorogenic acids. The (E)-cinnamic acid showed no ability to reduce the DPPH even when a higher concentration was tested (200 μg/mL of final concentration).

The structure of phenolic compounds is a key determinant of their radical scavenging and metal chelating activities, and this is referred to as the structure activity relationships (37). The antioxidant activity of phenolic acids increases with increasing degree of hydroxylation, as is the case for gallic acid (trihydroxylated) and protocatechuic acid (dihydroxylated), which show high AAI values. The substitution of the hydroxyl group in aromatic ring with methoxyl group, in the case of caffeic acid to ferulic acid, reduced the AAI value (Table III), in agreement with Rice-Evans et al. (38) who reported that the substitution of the hydroxyl by the methoxyl group reduced the activity. This can be explained by the reduction of the hydrogen atom donating capacity of the molecule. The absence of a hydroxyl group in the aromatic ring of (E)-cinnamic acid (non-phenolic) may explain its incapacity to reduce DPPH.

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Table I. Percentage composition of the oil (HD) and supercritical extracts (SFE) of *Xanthium strumarium.*

Compound	RI	RI*	HD	SFE1 (150Bar/ 50°C)	SFE2 (250Bar/ 50°C)	SFE3 (350Bar/ 50°C)	SFE4 (150Bar/ 30°C)	SFE5 (250Bar/ 30°C)	SFE6 (350Bar/ 30°C)
			%	%	%	%	%	%	%
myrcene	994	991	0.6	tr	tr	tr	tr	tr	tr
limonene	1032	1031	2.2	tr	tr	tr	tr	tr	tr
linalyl acetate	1257	1257	0.1	-	-	-	-	-	-
thujyl alcool	1287		0.2	tr	tr	tr	tr	-	-
α-cubebene	1353	1351	tr	tr	tr	tr	tr	tr	tr
eugenol	1359	1356	tr	0.1	tr	tr	tr	tr	tr
α-ylangene	1372	1373	-	tr	tr	0.1	0.1	0.1	0.1
α-copaene	1377	1376	0.1	tr	tr	0.1	tr	tr	tr
β-cubebene	1391	1390	0.3	tr	tr	tr	tr	tr	tr
β-elemene	1393	1391	0.4	-	-	-	-	-	-
β-caryophylene	1420	1418	3.9	0.6	0.5	0.7	0.5	0.6	0.7
β-gurjunene	1431	1432	0.3	tr	-	-	-	-	-
α-humulene	1455	1454	2.1	0.3	0.4	0.2	0.4	0.2	0.3
germacrene d	1478	1480	-	1.1	1.2	1.1	1.8	1.5	1.4
β-guaiene	1486	1490	79.6	0.4	0.2	0.4	0.5	0.4	0.4
valencene	1488	1491	1.0	-	-	-	-	-	-
α-muurolene	1500	1499	0.3	0.1	0.1	0.2	0.3	0.1	0.2
γ-cadinene	1515	1513	0.3	3.2	2.8	5.2	5.1	3.7	4.6
δ-cadinene	1525	1524	0.4	5.3	4.0	6.5	7.3	5.6	6.4
α-cadinene	1539	1538	0.03	2.1	1.5	2.4	2.3	1.9	1.5
xanthatin	1572		tr	0.2	0.1	0.3	0.2	0.1	0.3
spathulenol	1577	1576	tr	tr	0.1	tr	tr	tr	tr
epi-α-cadinol	1642	1640	0.5	tr	0.1	0.1	0.1	tr	tr
NI	2303		-	42.9	55.2	66.3	57.2	63.6	61.3
xanthinin	2383		-	30.4	18.9	4.9	12.7	11.7	4.1
NI	2394		-	2.7	0.3	0.6	0.4	0.26	0.5
Total			92.3	89.4	85.4	89.1	88.9	89.7	81.8

RI: Retention Index (GC-MS); *Adams (1995); NI: not identified; tr = trace = less than 0.1%; - : absent. The chromatography conditions were described in the text.

Table II. Minimal inhibitory concentration (MIC, mg/mL) of the oil (HD), supercritical extracts (SFE) of *Xanthium strumarium* and antibiotics.

	Minimal inhibitory concentration (MIC, mg/mL)				
	S. aureus	*E. coli*	*P. aeruginosa*	*S. typhimurium*	*C. perfringens*
HD	0.200-0.400	0.400-0.600	0.400-0.600	0.600-0.800	0.600-0.800
SFE1 (150 Bar /50 °C)	0.200-0.400	0.400-0.600	0.600-0.800	0.600-0.800	0.600-0.800
SFE2 (250 Bar /50 °C)	0.200-0.400	0.400-0.600	0.600-0.800	0.600-0.800	0.600-0.800
SFE2 (350 Bar /50 °C)	0.200-0.400	0.400-0.600	0.600-0.800	0.600-0.800	0.600-0.800
SFE4 (150 Bar /30 °C)	0.200-0.400	0.400-0.600	0.400-0.600	0.600-0.800	0.600-0.800
SFE5 (250 Bar /30 °C)	0.200-0.400	0.400-0.600	0.600-0.800	0.600-0.800	0.600-0.800
SFE6 (350 Bar /30 °C)	0.200-0.400	0.400-0.600	0.600-0.800	0.600-0.800	0.600-0.800
Amoxicillin	*	0.031-0.062	0.062-0.125	0.062-0.125	0.031-0.062
Enrofloxacin	*	*	*	0.004-0.008	*
Erythromycin	*	*	0.031-0.062	0.031-0.062	0.031-0.062
Oxytetracycline	*	0.008-0.015	0.062-0.125	0.062-0.125	0.062-0.125
Penicilin G	*	0.008-0.015	0.062-0.125	0.062-0.125	0.062-0.125
Spiramycin	0.002-0.004	0.004-0.008	0.062-0.125	0.062-0.125	0.062-0.125
Sulfadiazine	0.062-0.125	0.004-0.008	0.062-0.125	0.062-0.125	0.062-0.125
Sulfanilamide	0.062-0.125	0.004-0.008	0.062-0.125	0.062-0.125	0.062-0.125
Tetracycline	*	*	0.031-0.062	0.031-0.062	0.008-0.015

* < 0.0019 mg/mL.

Table III. Antioxidant activity index (AAI) of the oil and extracts from *Xanthium strumarium* and phenolic compounds.

	r^2	IC_{50} ($\mu g.mL^{-1}$)	AAI
HD	0.9889	1294	0.06
SFE1 (150 Bar /50 °C)	0.9847	1454	0.05
SFE2 (250 Bar /50 °C)	0.9949	1423	0.05
SFE3 (350 Bar /50 °C)	0.9854	1387	0.06
SFE4 (150 Bar /30 °C)	0.9991	915	0.08
SFE5 (250 Bar /30 °C)	0.9996	793	0.10
SFE6 (350 Bar /30 °C)	0.9895	1532	0.05
Gallic acid	0.9984	2.90	26.51
Protochatechuic acid	0.9987	3.80	20.23
Quercetin	0.9995	5.48	14.03
Eugenol	0.9987	7.35	10.46
Chlorogenic acid	0.9999	7.56	10.17
Caffeic acid	0.9937	8.00	9.61
BHA	0.9976	7.83	9.82
Rutin	0.9992	12.09	6.36
Resveratrol	0.9981	14.16	5.43
Ferulic acid	0.9994	14.68	5.24
(E)-cinnamic acid	-	-	-

r^2: linearity coefficient; IC_{50}: concentration providing 50% inhibition; SFE: supercritical fluid extraction.

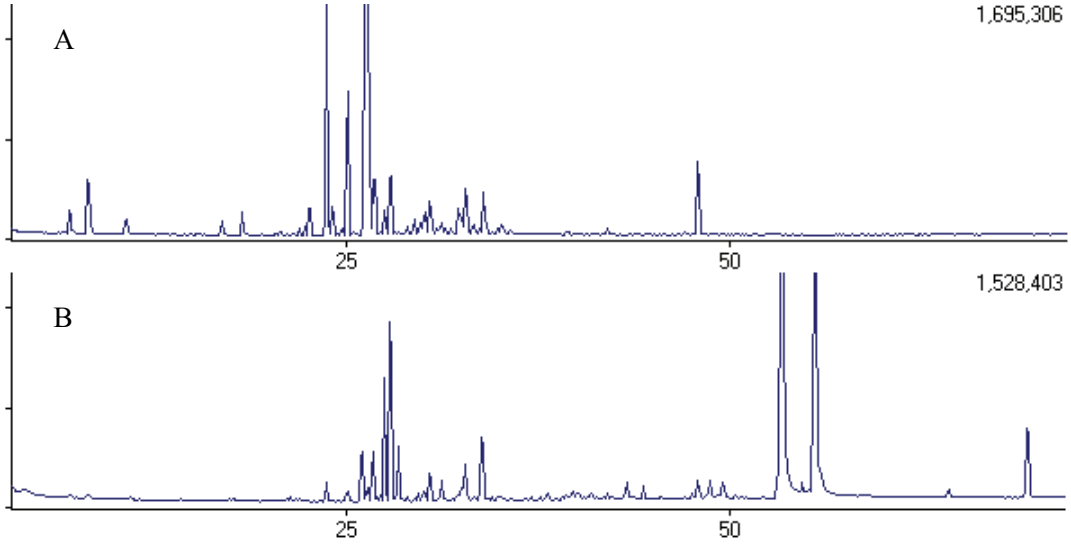

Figure 1: Chromatogram of the oil (A) and supercritical extract (SFE3 - 150 Bar/50 °C) (B) of *Xanthium strumarium* leaves (DB-5 column). The chromatography conditions were described in the text.

Capítulo 3

Antioxidant activity and related compounds of *Xanthium strumarium* L. extracts

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Antioxidant activity and related compounds of *Xanthium strumarium* L. extracts

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Graphical abstract

The *Xanthium strumarium* L. extracts presented high antioxidant activity and the phenolics ferulic and chlorogenic acids were identified in the extracts. High correlation between total phenolics content and antioxidant activity was found. Methanol and ethanol were the most appropriate solvents to analysis of phenolic compounds.

Xanthium strumarium L.

Abstract

The effect of extraction methods and extracting solvents on the overall yield, total phenolics content, antioxidant activity and on the composition of the phenolic compounds in *Xanthium strumarium* extracts were studied. The methods were maceration, dynamic maceration and soxhlet, and 80% ethanol, 80% methanol, ethyl acetate and dichloromethane/chloroform (1:1) were used as the solvents. The antioxidant activity was determined by using 2,2-diphenyl-1-picrylhydrazyl radical and the composition of the phenolics was determined by HPLC-DAD and by direct injection electrospray ionization mass spectrometry (ESI-MS). All the results were affected by the extraction method and mainly by the solvent used, the best results being obtained with methanol. The total phenolics content varied from 0.08 to 2.15 g.100 g^{-1} of leaves depending on the method and solvent used. The methanolic and ethanolic extracts exhibited strong antioxidant activity, but the ethyl acetate and chloroform/dichloromethane (1:1) extracts showed poor activity. Chlorogenic and ferulic acids were the most abundant phenolic compounds in the *X. strumarium* extracts.

KEYWORDS: *Xanthium strumarium*, Asteraceae, common cocklebur, antioxidant activity, phenolic compounds,

1. Introduction

Common cocklebur (*Xanthium strumarium* L.) has a nearly worldwide distribution between latitude 53 degrees N. and 33 degrees S. Stems are erect, ridged, rough and hairy, and frequently branched, resulting in somewhat bushy plants from 8 to 59 inches (20-150 cm) tall. It has small, green unisexual flowers occurring in separate clusters at the end of the branches and main stem. The fruit is brown, hard, woody bur from 0.4 to 0.8 inch long and covered with stout, hooked prickles. Each fruit contains two seeds. *X. strumarium* is extremely competitive with other agronomic crops, and it was reported as one of the worst weeds of soybean plantations (Bozsa and Oliver, 1993).

Xanthium species have been used as traditional herb medicines for a long time in oriental countries. The whole plant has been used to treat bacterial infections, diabetes, skin pruritus, inflammatory diseases like rhinitis and rheumatoid arthritis (Hsu et al., 1984; Evans, 2002). Despite the medicinal use, some investigations have reported that *X. strumarium* induced intoxication and could be lethal to cattle (Colodel et al., 2000), sheep (Loretti et al., 1999), pigs (Stuart et al., 1981; Masvingwe and Mavenyengwa, 1998) and humans (Turgut et al., 2005). The results showed that the consumption of the fruits (burs) and the cotyledonary stage (two-leaf stage) induced hepatic necrosis, as well as myocardial injury in humans. The toxic principle in the poison of *X. strumarium* was isolated and identified as carboxyatractyloside (CAT) (Cole et al., 1980), a highly selective inhibitor of oxidative phosphorylation (Scott et al., 1993). The presence of CAT in the adult and cotyledonary stages of the dried leaves, seeds (inside the burr) and the shell of the burr was investigated via ESI-MS/MS, and CAT was found in the extracts from the seed and plants in the cotyledonary stage, but not in the adult leaves or shell of burr, so the medicinal use of *X. strumarium* should be restricted to the adult leaves (Scherer et al., 2008).

Interest in natural sources of antioxidant molecules in the food, beverage and cosmetic industries has resulted in a large body of research in recent years. It is well known that natural antioxidants extracted from herbs and spices have high antioxidant activity and are used in many food applications. Of these substances, the phenolic compounds, which are widely distributed, have the ability to scavenge free radicals by single-electron transfer (Hirano et al., 2001).

Several investigations on the biological properties of *X. strumarium* L. have been reported, such as its anti-ulcerogenic (Favier et al., 2005), antitrypanosomal (Talakal et al., 1995), anti-helmintic (Sharma et al., 2003), anti-inflammatory (Kim et al., 2005; Yadava and Jharbade, 2007) and antimicrobial activities (Jawad et al., 1988), and also its diuretic action (Nieves et al., 1999), anti-leishmanial and antifungal activities (Lavault et al., 2005) and hypoglycemic action (Hsu et al., 2000). However, there are no reports about its antioxidant activity. Therefore, the aim of this work was to investigate the antioxidant activity of several extracts of *X. strumarium* leaves from Brazil as well as the related phenolic compounds.

2. Results and discussion

Table 1 shows the extraction yields and total phenolics contents. The extraction yield varied from 4.6% to 26.4% (w/w) depending on the extraction method and solvent. The soxhlet method with methanol as the solvent showed the highest yields followed by ethanol. Ethyl acetate and chloroform/dichloromethane (1:1) presented low yields and little difference was observed between them. These results are in agreement with previous studies that cited the extraction solvents in the following order: methanol > ethanol > ethyl acetate (Moure et al., 2000; Sun and Ho, 2005). The total phenolics content, expressed as

gallic acid equivalents, was affected by both the extraction method and the solvent used (Table 1). The best results were observed with methanol followed by, ethanol, ethyl acetate and chloroform/dichloromethane (1:1), respectively. Maceration with methanol presented the highest TPC in the extract, but the soxhlet method exhibit the highest yield and thus higher TPC per 100 g of leaves was found using the soxhlet method. The high yield can be explained on account of the higher temperature of the extracting solvent, which increased the extraction strength.

Table 2 shows the results of the AAI for the extracts and pure compounds. For the IC_{50} it is very important that the determination be done in a linear range for each compound. Therefore a calibration curve was performed for all the compounds tested, and good linear ranges were observed (Table 2). The stability and linear range of the $DPPH^{\bullet}$ solutions were evaluated in a previous study (Scherer and Godoy, 2008). The results showed there was no difference in absorbance between 0 and 90 min for any of the concentrations tested and good linear ranges were observed. AAI was determined using equation 1, where the final concentration of the $DPPH^{\bullet}$ solution was 76.89 $\mu g.mL^{-1}$. The antioxidant activity of the *X. strumarium* extracts was affected by the extraction method and the solvent used. Ethyl acetate and chloroform/dichloromethane (1:1) showed very high IC_{50} values, and consequently very low AAI, indicating poor antioxidant activity. On the other hand, the methanolic and ethanolic extracts showed strong antioxidant activities.

Gallic acid showed the highest AAI value followed by protochatechuic acid and quercetin. No significant difference between chlorogenic acid, caffeic acid and BHA was observed, but they all showed higher AAI values than ferulic acid and rutin, which was similar between themselves (Table 2). The *trans*-cinnamic acid presented no ability to

reduce DPPH$^{\bullet}$, even when higher tested at a higher concentration (200 μg.mL^{-1} of final concentration).

Phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerised compounds (Bravo, 1998). The structure of phenolic compounds is a key determinant of their radical scavenging and metal chelating activities, and this is referred to as structure activity relationships (Balasundram et al., 2006). The antioxidant activity of phenolic acids increases with increasing degree of hydroxylation, as is the case with gallic acid (trihydroxylated) and protochatechuic acid (dihydroxylated), which show high AAI values. The substitution of the hydroxyl group in the aromatic ring with a methoxyl group, as in the case of caffeic acid to ferulic acid, reduced the AAI value (Table 2), in agreement with Rice-Evans et al. (1996) who reported that the substitution of the hydroxyl by methoxyl groups reduced the activity. This can be explained by the reduction in the hydrogen atom donating capacity of the molecule. The absence of a hydroxyl group in the aromatic ring of *trans*-cinnamic acid (non phenolic) can maybe explain its incapacity to reduce DPPH$^{\bullet}$. The flavonoid structure–activity relationships are generally more complicated than that of hydroxybenzoic and hydroxycinnamic acids due to the relative complexity of the flavonoid molecules. The degree of hydroxylation as well as the position of the hydroxyl groups increases the radical scavenging capacity of the flavonoids (van Acker et al., 1996). For this reason, quercetin has a higher AAI value than rutin (Table 2), in which the hydroxyl group was substituted by rutinose.

HPLC–DAD analysis of the *X. strumarium* extracts revealed the presence of three major compounds, chlorogenic acid, ferulic acid and *trans*-cinnamic acid (Figure 1) and the

identification of these compounds was confirmed by ESI(-)-MS/MS analyses. Negative ion mode provides a sensitive and selective method for the identification of polar organic compounds with acidic sites, such as the phenolic compounds. Deprotonated forms of the compounds of interest were then selected and dissociated and their ESI-MS/MS were compared to those of standards. The results showed the chlorogenic acid with an molecular ion at *m/z* 353.0858 (-3.97 ppm) $[M - H]^-$ and fragments with *m/z* 85.03 (13), 93.03 (5), 127.04 (6) and 191.05 (100); ferulic acid with molecular ion at *m/z* 193.0512 (5.70 ppm) $[M - H]^-$ and fragments with *m/z* 134.04 (23), 149.07 (21) and 178.04 (30) and the *trans*-cinnamic acid with an ion at *m/z* 147.0457 (7.48 ppm) $[M - H]^-$ with fragments with *m/z* 77.04 (100) and 103.05 (93).

Previous study reported the presence of chlorogenic and ferulic acids in *X. strumarium* (Han et al., 2006). Table 3 shows the results of the HPLC-DAD analyses for chlorogenic, ferulic and *trans*-cinnamic acids in the *X. strumarium* extracts. The methanolic extracts showed the highest values for chlorogenic and ferulic acids, whilst the highest value for *trans*-cinnamic acid was found in the ethanolic extracts. The extracting solvents ethyl acetate and chloroform/dichloromethane (1:1) presented the lowest values for all compounds, being about 500 times less for chlorogenic and ferulic acids, and around 3 times less for *trans*-cinnamic acid.

As cited before, the methanolic extracts showed the highest total phenolics contents and the highst AAI values followed by the ethanolic extracts, and the lowest values were found in the ethyl acetate and chloroform/dichloromethane (1:1) extracts. The phenolic compounds have the ability to scavenge free radicals by single-electron transfer, and several studies have reported the antioxidant activity of plant extracts and its relationship with the content of phenolic compounds (Aaby et al., 2004; Silva et al., 2005; Sun and Ho,

2005; Yuan et al., 2005; Singh et al., 2007). In the present study, a high correlation (r^2 = 0.97) was found between the total phenolics content and the AAI, and also a high correlation between AAI and chlorogenic acid (r^2 = 0.97), ferulic acid (r^2 = 0.96) and *trans*-cinnamic acid (r^2 = 0.90). Since *trans*-cinnamic acid does not present any ability to reduce the $DPPH^{\bullet}$, the chlorogenic and ferulic acids are important compounds with respect to the antioxidant activity of *X. strumarium.*

3. Conclusion

The yield, total phenolics content and antioxidant activity were affected by the extraction method and mainly by the solvent used in the following order: methanol > ethanol > ethyl acetate > chloroform/dichloromethane (1:1). The soxhlet method with methanol showed the highest yield, but in maceration with methanol gave to highest total phenolics contents in the dried extract. Strong antioxidant activity was observed in the methanolic and ethanolic extracts, but poor antioxidant activity for the ethyl acetate and chloroform/dichloromethane (1:1) extracts. High correlation was found between the total phenolics content and the antioxidant activity, and chlorogenic and ferulic acids were the majority phenolic compounds in the *X. strumarium* extracts.

4. Experimental

4.1. General experimental procedures

Methanol, ethanol, ethyl acetate, chloroform and dichloromethane (Ecibra, Brazil), HPLC grade methanol (Mallinckrodt, USA) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) (Sigma, USA) were used. Folin-ciocalteau reagent was obtained from QEEL (São

Paulo, Brazil) and sodium carbonate was from Nuclear (São Paulo, Brazil). The synthetic antioxidant butylated hydroxyanisole (BHA) and the phenolic compounds caffeic acid, chlorogenic acid, ferulic acid, gallic acid, quercetin, rutin, protocatechuic acid (3,4 dihydroxybenzoic acid) and *trans*-cinnamic acid were purchased from Sigma (USA).

4.2. Plant material

The *X. strumarium* used in this work was cultivated at the experimental farm of the Faculty of Agricultural Engineering (FEAGRI) of the State University of Campinas (UNICAMP, Campinas, São Paulo, Brazil). A voucher specimen was deposited at the State University of Campinas Herbarium as n° 134865, and identified by Dr. Washington M. F. Neto (curator). The leaves were separated and dried in a tray drier with air circulation at 45°C (Marconi, model 035, Piracicaba, SP, Brazil), packed in dark plastic bags and stored in a domestic freezer at −20°C until the extracted.

4.3. Extractions

Before the extractions the leaves were triturated in a domestic food processor (Wallita, model Master, São Paulo, SP) and particles with sizes from 24 - 48 meshes were selected using a magnetic agitator (Bertel, Model 1868, Caieiras, SP). The extractions were carried out using 3 different extraction methods and 4 different solvents in triplicate. The methods were maceration (1), dynamic maceration (2) and soxhlet (3). 80% Ethanol (A), 80% methanol (B), ethyl acetate (C) and dichloromethane/chloroform (1:1) (D) were the solvents used. Maceration was carried out with 20 g plus 100 mL of the different solvents giving the extracts 1A, 1B, 1C and 1D. After 7 days with periodic agitation, the extracts were filtered through paper and the residue extracted again with 100 mL of the respective solvents for 10 min under agitation. Both fractions were then blended and evaporated to dryness at 38°C in a vacuum. Dynamic maceration was carried out with 20 g plus 100 mL

of the different solvents giving the extracts 2A, 2B, 2C and 2D. After 3 h of agitation, the extracts were filtered through paper and the residue extracted again with 100 mL of the respective solvents for 1 h under agitation. Both fractions were then blended and evaporated to dryness at 38°C in a vacuum. The Soxhlet method was carried out in a soxhlet apparatus with 15 g for 5 h of extraction with the solvents A, B and C, these extracts were denominated as 3A, 3B and 3C. The extracts were dried to dryness at 38°C in a vacuum. All extracts were stored in a domestic freezer at −20°C until analysed.

4.4. Total phenolics content (TPC)

The total phenolics content was determined in all extracts using the Folin-ciocalteau reaction. An aliquot of 0.5 mL of a methanolic solution of the dried extracts (1.5 $mg.mL^{-1}$) was added to 2.5 mL of Folin-ciocalteau reagent diluted with water (1/11). After 5 minutes 2.0 mL of 7.5% sodium carbonate was added and vigorously agitated in a vortex mixer. The mixture was incubated for 2 h in the dark at room temperature. The absorbance at 740 nm was measured and converted to the phenolics content according to a calibration curve made with gallic acid (2.0, 1.0, 0.5, 0.25, 0.125 $mg.mL^{-1}$, $r^2 = 0.9995$).

4.5. Antioxidant Activity

The antioxidant activity of the extracts and standards was determined from the radical scavenging activity determined using 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) according to Scherer and Godoy (2008). In each case an aliquot of 0.1 mL of a methanolic solution of the sample or standard at the different concentrations, was added to 3.9 mL of $DPPH^{\bullet}$ methanolic solution. The $DPPH^{\bullet}$ solution was prepared at 0.2 mM by dissolving 39.4 mg in 500 mL of methanol. The blank sample consisted of 0.1 mL of methanol added to 3.9 mL of $DPPH^{\bullet}$. The tests were carried out in triplicate. After a 90 min

incubation period at room temperature in the dark, the absorbance was measured at 517 nm. The radical scavenging activity was calculated as follows: $I\% = [(Abs_0 - Abs_1)/Abs_0] \times 100$, where Abs_0 was the absorbance of the blank and Abs_1 was the absorbance in the presence of the test compound at the different concentrations. The IC_{50} (concentration providing 50% inhibition) was calculated graphically using a calibration curve drawn in linear range by plotting the extract concentration vs the corresponding scavenging effect. The antioxidant activity was expressed as the antioxidant activity index (AAI) calculated as follows:

$$AAI = \frac{\text{final concentration of DPPH}^{\bullet}\ (\mu g.mL^{-1})}{IC_{50}\ (\mu g.mL^{-1})} \qquad \text{(Equation 1).}$$

Thus, the AAI considering the mass of $DPPH^{\bullet}$ and the mass of compound tested in the reaction, resulted in a constant for each compound, independent of the concentration of $DPPH^{\bullet}$ and sample used. In this work the plant extracts were considered to show poor antioxidant activity when AAI < 0.5, moderate antioxidant activity when the AAI was between 0.5 – 1.0, strong antioxidant activity when the AAI was between 1.0 – 2.0, and very strong when the AAI > 2.0. The assays were carried out in triplicate and all the samples and standard solutions, as well as the $DPPH^{\bullet}$ solutions, were prepared daily.

4.6. HPLC analysis of phenolic compounds

The analyses was carried out using a HP 1100 series (Agilent) liquid chromatograph equipped with degasser, quaternary pump, automatic sampler adjusted to a 20 μL volume injection and a UV-visible diode array detector (DAD) set at 280 and 320 nm. The separation was performed using a RP-C18 VYDAC^{TM} column (5 μm particle size, 250 x 4.6 mm I.D., kept at 25°C) with a gradient elution procedure using a flow rate of 0.7

$mL.min^{-1}$. The mobile phase A consisted of water with 0.1% formic acid and B was methanol with 0.1% formic acid. The gradient elution starting at 85% A and 15% B, changing to 50% A and 50% B after 10 minutes, followed by 20% A and 80% B in 25 minutes. The column equilibration time before each new injection was from 25 to 35 minutes under the initial condition. The peaks were identified from their retention times, comparing the UV-visible spectra and spike with that of a commercial standard. Quantification was carried out using a 7-point external standard curve. The concentrations of the chlorogenic and ferulic acid standard solutions were 30, 15, 7.5, 3.75, 1.875, 0.375 and 0.1875 $\mu g.mL^{-1}$, and for the *trans*-cinnamic acid 100, 50, 25, 12.5, 6.25, 1.25 and 0.625 $\mu g.mL^{-1}$. The validation parameters consisted of the linearity range, the precision and limits of detection and quantification. The peak purity was verified from the diode array spectra. The Linearity range was evaluated by plotting the peak area corresponding to each analyte, as a function of the concentration introduced. Precision involved repeatability (10 successive injections) and intermediate precision (three injections on three different days in different weeks). The limit of detection was the minimal concentration of the analyte giving a peak height that was 3 times the noise base line, and 5 times for the limit of quantification.

4.7. LC-ESI-MS-MS-TOF analysis

The FIA system (flow-injection analysis), in which the separation column was removed from the HPLC system, was used for confirmation of the phenolic compounds in *X. strumarium* extracts. The analyses was carried out using a Waters ALLIANCE 2695 liquid chromatograph equipped with degasser, quaternary pump, automatic sampler adjusted to a 5 μL volume injection. The mobile phase consisted of water with 0.8% of

NH_4OH and methanol (1/1) in isocratic elution procedure using a flow rate of 0.1 $mL.min^{-1}$. ESI(-)–MS analysis of phenolics in *X. strumarium* extracts was performed using a high-resolution and high-accuracy (5 ppm) Q-TOF micro™ (Waters/Micromass, USA). Mass spectra were achieved by electrospray ionization in negative mode. The following ion optics was used: capillary 2900V, cone 25 V, and extractor 2 V. The source block temperature was 100°C, the desolvation temperature was 300°C and the collision energy was 5V**.** Continuous mass spectra were obtained by scanning from 100 to 400 m/z. Structural analysis of single ions in the mass spectra was performed by ESI-MS/MS. The ion with the *m/z* of interest was selected and submitted to 15–30 eV collisions with argon in the collision quadrupole. The collision gas pressure was optimized to produce extensive fragmentation of the ion under investigation.

4.8. Statistical analysis

The data obtained were analyzed using ANOVA/Tukey ($P < 0.05$). The statistical package used was Statistica™ 6.0 data analysis software by Statsoft, Inc, USA.

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Table 1

Yield and total phenolic content (TPC) of the *Xanthium strumarium* extracts.

Extracts	Yield (%)	TPC (mg.g^{-1} extract)	TPC (g.100 g^{-1} leaves)
1A	12.3 ± 0.5^{d}	64.51 ± 1.0^{d}	0.80 ± 0.01^{e}
2A	14.1 ± 1.0^{cd}	70.07 ± 1.6^{c}	0.99 ± 0.02^{d}
3A	19.3 ± 0.8^{b}	69.38 ± 1.3^{cd}	1.34 ± 0.03^{b}
1B	14.3 ± 0.3^{cd}	93.68 ± 2.1^{a}	1.34 ± 0.02^{b}
2B	14.9 ± 1.3^{c}	78.23 ± 0.9^{b}	1.17 ± 0.01^{c}
3B	26.4 ± 1.3^{a}	81.35 ± 1.3^{b}	2.15 ± 0.04^{a}
1C	6.5 ± 0.4^{ef}	21.98 ± 3.6^{f}	0.14 ± 0.02^{f}
2C	4.6 ± 0.6^{f}	27.19 ± 1.0^{e}	0.13 ± 0.00^{f}
3C	6.9 ± 0.3^{e}	23.19 ± 0.3^{ef}	0.16 ± 0.00^{f}
1D	6.2 ± 0.4^{ef}	13.30 ± 0.8^{g}	0.08 ± 0.00^{g}
2D	5.3 ± 0.2^{ef}	18.85 ± 2.4^{f}	0.10 ± 0.01^{g}

1: maceration; 2: dynamic maceration; 3: soxhlet; A: 80% ethanol; B: 80% methanol; C: ethyl acetate; D: chloroform/dichloromethane (1:1). The results are the mean of triplicate assays. Values with different superscripts in the same column were significantly different ($P < 0.05$).

Table 2

Antioxidant activity index (AAI) of *Xanthium strumarium* extracts and pure compounds.

Extract/pure compound	r^2	IC_{50} (mean ± SD)	AAI (mean ± SD)
1A	0.9992 – 1.0000	47.83 ± 1.40	1.61 ± 0.05^{g}
2A	0.9985 – 0.9997	53.01 ± 1.20	1.45 ± 0.03^{g}
3A	0.9974 – 0.9989	53.34 ± 1.52	1.44 ± 0.04^{g}
1B	0.9963 – 0.9995	44.94 ± 1.06	1.71 ± 0.04^{f}
2B	0.9984 – 0.9990	45.05 ± 1.15	1.70 ± 0.03^{f}
3B	0.9983 – 0.9986	43.53 ± 1.61	1.77 ± 0.07^{f}
1C	0.9869 – 0.9988	346.35 ± 16.50	0.22 ± 0.01^{h}
2C	0.9899 – 0.9971	369.83 ± 13.58	0.21 ± 0.01^{hi}
3C	0.9809 – 0.9993	423.97 ± 22.27	0.18 ± 0.01^{hi}
1D	0.9978 – 0.9999	657.10 ± 24.01	0.12 ± 0.00^{i}
2D	0.9984 – 0.9997	674.61 ± 28.57	0.11 ± 0.00^{i}
Gallic acid	0.9992 – 1.0000	2.83 ± 0.07	27.1 ± 0.68^{a}
Protochatechuic acid	0.9988 – 1.0000	3.82 ± 0.15	20.2 ± 0.77^{b}
Quercetin	0.9939 – 0.9998	4.88 ± 0.56	15.9 ± 1.76^{c}
Chlorogenic acid	0.9986 – 0.9995	7.44 ± 0.12	10.3 ± 0.17^{d}
Caffeic acid	0.9966 – 0.9983	8.21 ± 0.31	9.4 ± 0.35^{d}
BHA	0.9969 – 0.9991	8.23 ± 0.41	9.3 ± 0.46^{d}
Rutin	0.9996 – 0.9998	12.09 ± 0.70	6.4 ± 0.37^{e}
Ferulic acid	0.9992 – 0.9998	14.45 ± 0.66	5.3 ± 0.25^{e}
trans-cinnamic acid	-	-	-

1: maceration; 2: dynamic maceration; 3: soxhlet; A: 80% ethanol; B: 80% methanol; C: ethyl acetate; D: chloroform/dichloromethane (1:1). Values with different superscripts in the same column were significantly different ($P < 0.05$); r^2: linearity coefficient; IC_{50}: concentration providing 50% inhibition ($\mu g.mL^{-1}$).

Table 3

HPLC-DAD analyses of chlorogenic, ferulic and *trans*-cinnamic acids in the *Xanthium strumarium* extracts.

Validation parameters	Chlorogenic acid	Ferulic acid	*trans*-Cinnamic acid
Linearity (r^2)	0.9992	0.9999	0.9980
Repeatability (RSD %)	1.53	1.07	1.03
Intermediate precision (RSD %)	2.10	1.52	1.49
Limit of detection ($\mu g.mL^{-1}$)	0.07	0.03	0.07
Limit of quantification ($\mu g.mL^{-1}$)	0.12	0.06	0.13
Extract ($mg.g^{-1}$ of dry extract)			
1A	18.18 ± 0.72	23.12 ± 0.89	75.03 ± 0.27
2A	17.13 ± 0.40	22.92 ± 0.62	76.31 ± 0.59
3A	16.23 ± 0.35	26.55 ± 0.22	80.80 ± 0.62
1B	28.28 ± 0.42	39.01 ± 0.41	67.13 ± 0.71
2B	24.97 ± 0.75	30.47 ± 0.58	71.45 ± 0.31
3B	23.95 ± 1.09	41.85 ± 0.69	52.48 ± 0.04
1C	0.12 ± 0.00	0.74 ± 0.06	29.33 ± 0.18
2C	0.03 ± 0.00	0.12 ± 0.01	24.11 ± 0.27
3C	0.08 ± 0.01	0.26 ± 0.01	36.45 ± 0.34
1D	0.05 ± 0.00	0.11 ± 0.00	23.25 ± 0.36
2D	0.03 ± 0.00	0.06 ± 0.00	22.53 ± 0.07

RSD: relative standard deviation; 1: maceration; 2: dynamic maceration; 3: soxhlet; A: 80% ethanol; B: 80% methanol; C: ethyl acetate; D: chloroform/dichloromethane (1:1).

FIGURE 1.

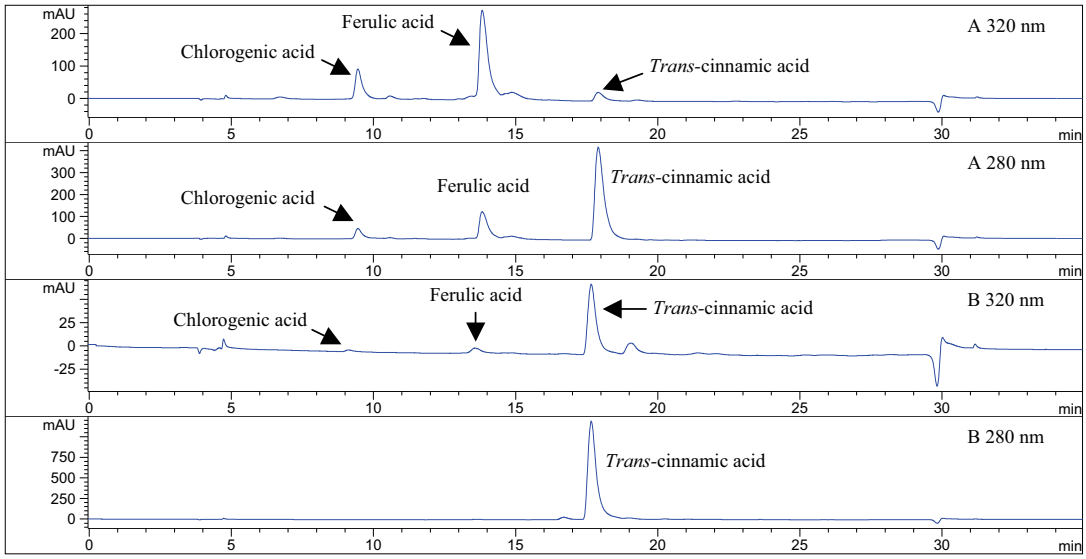

Fig.1. HPLC-DAD chromatograms of the dried *Xanthium strumarium* extracts. A – maceration with methanol (0.8 mg.mL^{-1}); B – maceration with dichloromethane/chloroform (1:1) (5 mg.mL^{-1}). The chromatography conditions were described in the text.

Capítulo 4

Antimicrobial activity and analysis of carboxyatractyloside (CAT) by electrospray ionization mass spectrometry of X*anthium strumarium* L.

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Antimicrobial activity and analysis of carboxyatractyloside (CAT) by electrospray ionization mass spectrometry of *Xanthium strumarium* L.

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ABSTRACT: The aim of this work was to evaluate the antimicrobial activity of extracts of *Xanthium strumarium* L. leaves against *Staphylococcus aureus, Escherichia coli, Salmonella thyphimurium*, *Pseudomonas aeruginosa e Clostridium perfringens*, as well as to investigate the carboxyatractyloside in different parts of the plant. *S. aureus* and *C. perfringens* were more sensible to non-polar fractions than to polar fractions, since that to the other stains no difference between extracts was observed. All extracts exhibit strong antimicrobial activity against the species studied. The results show that carboxyatractyloside is indeed present in the seeds, inside the fruits and in the cotiledonary stage (two leaves stage) but not in adult leaves and in the shell of burr. Therefore for medicinal use, only the adult leaves should be used.

Key words: cocklebur, antimicrobial activity, medicinal plants.

RESUMO: Atividade antimicrobiana e análise de carboxiatractilosideo por espectrometria de massas com ionização por electrospray de *xanthium strumarium* L. O objetivo do presente trabalho foi avaliar a atividade antimicrobiana de vários extratos de folhas adultas de *Xanthium strumarium* L. (carrapicho) sobre os microrganismos *Staphylococcus aureus, Escherichia coli, Salmonella thyphimurium*, *Pseudomonas aeruginosa e Clostridium perfringens*, bem como verificar a presença do composto tóxico carboxiatractilosideo em diferentes partes da planta através de injeção direta em um espectrômetro de massas com ionização por electrospray. As bactérias *S. aureus* e *Clostridium perfringens* foram mais sensíveis às frações não polares do que as polares, sendo que para as outras bactérias não foi verificada diferença entre os extratos. Todos os extratos apresentaram uma forte ação antimicrobiana sobre os microrganismos avaliados. O carboxiatractilosideo foi encontrado nos cotilédones e nas sementes, entretanto, não foi encontrado nas folhas em estádio adulto e na carapaça espinhosa que envolve a semente. Portanto, para o uso medicinal dessa planta somente as folhas na fase adulta podem ser utilizadas.

Palavras-chave: carrapicho, atividade antimicrobiana, plantas medicinais.

INTRODUCTION

Cocklebur (*Xanthium strumarium* L.) is an herbaceous annual plant with a worldwide distribution. The plant is erect, up to 2.5 m tall, with blotched purple stems. The leaves are dark green on the upper surface, similar in shape to grape leaves, 15 cm in diameter and roughly textured with minute bristles. The flowers are inconspicuous, both male and female, occurring in leaf axils towards the end of the branches. The flowers develop into hard, woody, spiny burrs (fruits), 1.2 cm to 2 cm long with numerous hooked spines. *X. strumarium* is extremely competitive with other agronomic crops. It has long been regarded as one of the worst weeds in soybean plantations (Bozsa & Oliver, 1993).

Some investigations of the biological properties of *X. strumarium* L. have been reported, such as anti-ulcerogenic (Favier et al. 2005), antitrypanosomal (Talakal et al. 1995), anti-helmintic (Sharma et al. 2003), anti-inflammatory (Kim et al. 2005; Yadava & Jharbade, 2007), diuretic action (Nieves et al. 1999), anti-leishmanial and antifungal activities (Lavault et al. 2005) and hypoglycemic action (Hsu et al. 2000), inhibition on the proliferation of cultured human tumor cells (Kim et al. 2003), and has a significant depressant action of the central nervous system (Mandal et al. 2001).

Previous studies have reported that *X. strumarium* induced intoxication and could be lethal to cattle (Colodel et al. 2000), sheep (Loretti et al. 1999, pigs (Stuart et al. 1981) and humans (Turgut et al. 2005). The results have shown that consumption of the fruits (burs) and the cotyledonary stage (two-leaf stage) induces hepatic necrosis and myocardial injury in humans. The toxic principle in the poison of *X. strumarium* was isolated and identified as carboxyatractyloside (CAT) (Cole et al. 1980), a highly selective inhibitor of oxidative phosphorylation (Scott et al. 1993).

The chemical composition of *X. strumarium* include phenolic compounds, like as chlorogenic and ferulic acids, thiazinediones (Han et al. 2006; Qin et al. 2006), triterpenoid saponin (Yadava & Jharbade, 2007), carboxyatractyloside (CAT) (Cole et al. 1980), xanthanolide sesquiterpene lactones (8-epi-xanthatin and 8-epi-xanthatin epoxide) (Kim et al. 2003), several xanthanolides (Riscala et al. 1994), beta-sitosterol (Bisht et al. 1977) and strumasterol (C-24 epimer of stigamsterol) (Bisht et al. 1978), monoterpene and sesquiterpene hydrocarbons (Taher et al. 1985), caffeic acid, 1,3,5-tri-O-caffeoyl quinic acid, 1,5-di-O-caffeoyl quinic acid (Sheu et al. 2003).

However, there are no reports about the antimicrobial activities of the extracts of *X. strumarium* against human pathogenic microorganisms, such as *Escherichia coli* and about the presence of the toxic compound in different parts of the plant. Therefore, the aim of this work was to investigate the antimicrobial activities of several extracts of *X. strumarium* adult leaves against *Staphylococcus aureus, Escherichia coli, Salmonella thyphimurium*, *Pseudomonas aeruginosa* and *Clostridium perfringens*, as well as to investigate the presence of carboxyatractyloside in different parts of the plant.

MATERIAL AND METHOD

Reagents and standards

Chromatographic grade methanol (Tedia, Fairfield, OH, USA), ammonium hydroxide (Merck, Darmstadt, Germany), nutrient agar (Merck, Germany), dimethyl sulfoxide (DMSO) (Sigma, USA), triphenyl tetrazolium chloride (TTC) (Merck, Germany), culture medium (Mueller-Hinton 2.1%, Merck, Germany) were used. The antibiotics amoxicillin, enrofloxacin, erythromycin, lincomycin, tetracycline, oxytetracycline, penicillin G,

spiramycin, sulfadiazine and sulfanilamide were purchased from Sigma (USA). The carboxyatractyloside were from Calbiochem (Darmstadt, Germany).

Plant material

X. strumarium (common namne: cocklebur, Family: *Asteraceae*) used in this work was cultivated on the experimental farm of the Faculty of Agricultural Engineering (FEAGRI) of the Campinas State University (UNICAMP, Campinas, São Paulo, Brazil). A voucher specimen was deposited at the Campinas State University Herbarium denominated as number 134865, and identified by Dr. Washington M. F. Neto (curator). The leaves were separated and dried in a tray drier with air circulation at 45 °C (Marconi, model 035, Piracicaba, Brazil), packed in dark plastic bags and stored in a domestic freezer at −20 °C until extracted. Before being submitted to extraction the material was triturated in a domestic food processor (Wallita, model Master, São Paulo, SP) and the particles from 24 - 48 mesh selected using a magnetic agitator (Bertel, Model 1868, Caieiras, SP).

Extracts

The extractions were carried out using 3 different extraction methods and 4 different solvents in triplicate. The methods were maceration (1), dynamic maceration (2) and soxhlet (3). 80% Ethanol (A), 80% methanol (B), ethyl acetate (C) and dichloromethane/chloroform (1:1) (D) were the solvents used. Maceration was carried out with 20 g plus 100 mL of the different solvents giving the extracts 1A, 1B, 1C and 1D. After 7 days with periodic agitation, the extracts were filtered through paper and the residue extracted again with 100 mL of the respective solvents for 10 min under agitation. Both fractions were then blended and evaporated to dryness at 38°C in a vacuum. Dynamic maceration was carried out with 20 g plus 100 mL of the different solvents giving the extracts 2A, 2B, 2C and 2D. After 3 h of agitation, the extracts were filtered through paper

and the residue extracted again with 100 mL of the respective solvents for 1 h under agitation. Both fractions were then blended and evaporated to dryness at 38°C in a vacuum. The Soxhlet method was carried out in a soxhlet apparatus with 15 g for 5 h of extraction with the solvents A, B and C, these extracts were denominated as 3A, 3B and 3C. The extracts were dried to dryness at 38°C in a vacuum. All extracts were stored in a domestic freezer at −20°C until analyzed.

Antimicrobial activity.

The minimal inhibitory concentration (MIC) tests were carried out according to the NCCLS (National Committee of Laboratory Standards, 2003) (*31*) using a tissue culture test plate (96 wells). *Staphylococcus aureus* (ATCC 6538)*, Escherichia coli* (isolated from swine)*, Salmonella thyphimurium* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 13388) and *Clostridium perfringens* (ATCC 1324) were obtained from the CBMAI (Brazilian Collection of Environmental and Industrial Microorganisms, CPQBA/UNICAMP, Brazil). The microorganisms were grown overnight at 36 °C in nutrient agar plates in aerobiosis, except for *Clostridium perfringens*, cultivated in thyoglicolate broth in anaerobiosis. The inoculum for the assays was prepared by diluting scraped cell mass in 0.85% NaCl solution, adjusted to the McFarland scale 0.5. Cell suspensions were finally diluted to 10^4 CFU.mL^{-1} for use in the assays. The extracts and the antibiotics were diluted in DMSO and stored at −20 °C until used. The final concentrations of the extracts were 1.8, 1.5, 1.2, 1.0, 0.8, 0.6, 0.4 and 0.2 mg.mL^{-1}, while serial dilutions of the antibiotics were prepared between 0.25 and 0.00195 mg.mL^{-1}. The inoculum was added to all the wells and the plates were incubated at 36 °C for 24 h. To verify the sterility of the extracts and culture medium (Mueller-Hinton 2.1%), as well as the viability of the microorganism, a control test was made with all the test plates. Antimicrobial activity was

detected by adding 0.05 mL of an aqueous 0.5% triphenyl tetrazolium chloride (TTC) solution. MIC was defined as the lowest concentration of oil or extract that visually inhibited the growth, as indicated by the TCC staining (dead cells are not stained by TTC).

Analysis of the carboxyatractyloside (CAT)

The adult and cotiledonary stage dried leaves, seeds and shell of the burr were extracted with methanol 50% (100 mg to 5 mL) for 20 minutes in ultrasom and filtered in membrane 0.5 μm (FHLP13, Millipore). Samples of plant extracts were analyzed by direct infusion ESI-MS by means of a syringe pump (Harvard Apparatus) at a flow rate of 10 μL min^{-1}. ESI-MS fingerprints and ESI-MS/MS in the negative ion mode were acquired using a hybrid high-resolution and high-accuracy (5 ppm) Micromass Q-TOF mass spectrometer. Capillary and cone voltages were set to -3000 V and -50 V respectively, with a desolvation temperature of 100°C. One milliliter of each sample was added to 10 mL of a solution containing 70% (v/v) chromatographic grade methanol and 30% (v/v) deionized water and 5 μL of ammonium hydroxide per milliliter. Fingerprint mass spectra were acquired in the *m/z* range between 650 and 920.

RESULT AND DISCUSSION

Table 1 shows the antimicrobial activity of the *X. strumarium* extracts and antibiotics. No difference between the *X. strumarium* extracts were observed except for *Staphylococcus aureus* and *Clostridium perfringens*, that were affected by the solvent used, but not by the extraction method.

TABLE 1. Minimal inhibitory concentration (MIC, $mg.mL^{-1}$) of *Xanthium strumarium* extracts and antibiotics.

Minimal inhibitory concentration (MIC, mg/mL)					
Extract/antibiotic	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. thyphimurium*	C. perfringens
1A	0,4 - 0,6	0,4 - 0,6	0,4 - 0,6	0,6 - 0,8	0,6 - 0,8
1B	0,4 - 0,6	0,4 - 0,6	0,4 - 0,6	0,6 - 0,8	0,6 - 0,8
1C	0,2 - 0,4	0,4 - 0,6	0,4 - 0,6	0,6 - 0,8	0,4 - 0,6
1D	0,2 - 0,4	0,4 - 0,6	0,4 - 0,6	0,6 - 0,8	0,4 - 0,6
2A	0,4 - 0,6	0,4 - 0,6	0,4 - 0,6	0,6 - 0,8	0,6 - 0,8
2B	0,4 - 0,6	0,4 - 0,6	0,4 - 0,6	0,6 - 0,8	0,6 - 0,8
2C	0,2 - 0,4	0,4 - 0,6	0,4 - 0,6	0,6 - 0,8	0,4 - 0,6
2D	0,2 - 0,4	0,4 - 0,6	0,4 - 0,6	0,6 - 0,8	0,4 - 0,6
3A	0,4 - 0,6	0,4 - 0,6	0,4 - 0,6	0,6 - 0,8	0,6 - 0,8
3B	0,4 - 0,6	0,4 - 0,6	0,4 - 0,6	0,6 - 0,8	0,6 - 0,8
3C	0,2 - 0,4	0,4 - 0,6	0,4 - 0,6	0,6 - 0,8	0,4 - 0,6
Penicilin G	*	0,008-0,015	0,062-0,125	0,062-0,125	0,062-0,125
Enrofloxacin	*	*	*	0,004-0,008	*
Erythromycin	*	*	0,031-0,062	0,031-0,062	0,031-0,062
Sulfadiazine	0,062-0,125	0,004-0,008	0,062-0,125	0,062-0,125	0,062-0,125
Oxytetracycline	*	0,008-0,015	0,062-0,125	0,062-0,125	0,062-0,125
Lincomycin	*	0,031-0,062	0,031-0,062	0,031-0,062	0,031-0,062
Spiramycin	0,002-0,004	0,004-0,008	0,062-0,125	0,062-0,125	0,062-0,125
Amoxicillin	*	0,031-0,062	0,062-0,125	0,062-0,125	0,031-0,062
Tetracycline	*	*	0,031-0,062	0,031-0,062	0,008-0,015
Sulfanilamide	0,062-0,125	0,004-0,008	0,062-0,125	0,062-0,125	0,062-0,125

1: maceration; 2: dynamic maceration; 3: soxhlet; A: 80% ethanol; B: 80% methanol; C: ethyl acetate; D: chloroform/dichloromethane (1:1). * < 0.0019 $mg.mL^{-1}$.

The extracting solvents ethyl acetate (C) and dichloromethane/chloroform (1:1) (D) were more effective than 80% ethanol (A) and 80% methanol (B). Since the *Staphylococcus aureus* and *Clostridium perfringens* are gram-positive bacteria, maybe in the non-polar fraction have concentrated some active compound with cell wall mechanism, but more study is needed. *S. thyphimurium* and *C. perfringens* were more resistant to the methanolic and ethanolic *X. strumarium* extracts than *E. coli* and *P. aeruginosa*. Xanthatin was found in small quantities in *X. strumarium* essential oil (Scherer et al. 2007). This compound was isolated from the extracts of *X. spinosum L.* and was active against *Colletotrichum gloesporoides, Trichothecium roseum, Bacillus cereus* and *Staphylococcus aureus* (Ginesta-Peris et al., 1994). Previous study reported that cinnamic acid was

effective against *E. coli*, *P. aeruginosa*, *S. aureus* and *Salmonella sp.* with MIC values of 1.0 $mg.mL^{-1}$ for all strains (Chang et al. 2001). Cinnamic acid was found in the *X. strumarium* extracts between 22 to 80 $mg.g^{-1}$ of dry extract (Scherer et al. 2008). So, maybe the compounds xanthatin and cinnamic acid could have some contribution for the antimicrobial property of *X. strumarium*.

Staphylococcus aureus was more susceptible to the antibiotics tested followed by *E. coli*, the MIC values for *Staphylococcus aureus* were below to 0.0019 $mg.mL^{-1}$ for penicillin G, enrofloxacin, erythromycin, oxytetracycline, lincomycin, amoxicillin and tetracycline. The antibiotic enrofloxacin was more effective against all tested strains, followed by tetracycline and erythromycin, respectively (Table 1). On the other hand, sulfanilamide and sulfadiazine showed the higher MIC values for *Staphylococcus aureus*,

Duarte et al. (2005) proposed a classification for plant materials, based on the MIC results as follows, strong inhibitors when MIC below 0.5 $mg.mL^{-1}$ moderate inhibitors when MIC between 0.6 and 1.5 $mg.mL^{-1}$ and weak inhibitors when MIC above 1.6 $mg.mL^{-1}$. Thus, considering these classifications, all the *X. strumarium* extracts showed potential antimicrobial activity against the microorganisms evaluated (Table 1).

Figure 1A shows the ESI-MS fingerprint of *X. strumarium* seed. Note the ion of *m/z* 769 that correspond to the deprotonated molecule of carboxyatractyloside. Its ESI-MS/MS (Figure 1B) confirms its structure since the ion is found to dissociate nearly exclusively to HSO_4^- of *m/z* 97. Via ESI-MS/MS monitoring, we found the carboxyatractyloside in extracts from the seed and cotiledonary stage, but not in adult leaves and in the shell of burr.

Carboxyatractyloside is toxic and was originally isolated from the Mediterranean thistle *Atractylis gummifera*, a highly selective inhibitor of the cytosolic side-specific

mitochondrial ADP/ATP carrier (Huber et al. 1999). As it was mentioned, previous reports reported that *X. strumarium* induces liver damage and can be deathful, thus, care should be taken for the medicinal use of *X. strumarium* and only the leaves in adult stage are recommended, since that showed strong antimicrobial activity and the toxic compound was not found.

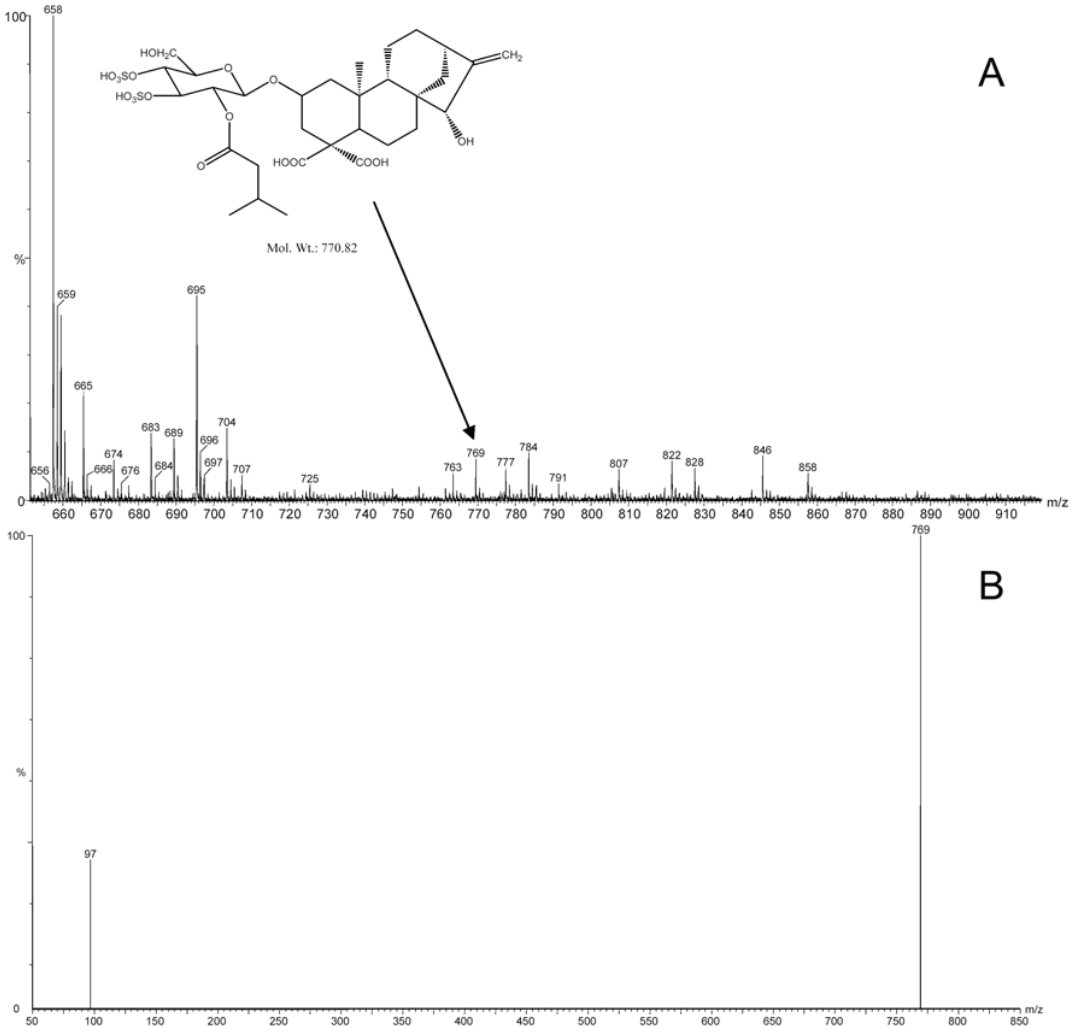

Figure 1. (A) ESI-MS of the *Xanthium strumarium* seed extract and (B) ESI-MS/MS of deprotonated carboxyatractyloside.

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Capítulo 5

Composição e atividades antioxidante e antimicrobiana dos óleos essenciais de cravo, citronela e palmarosa.

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COMPOSIÇÃO E ATIVIDADE ANTIOXIDANTE E ANTIMICROBIANA DOS ÓLEOS ESSENCIAIS DE CRAVO-DA-ÍNDIA, CITRONELA E PALMAROSA

(Atividade antioxidante e antimicrobiana)

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Resumo

Foram avaliadas a ação antioxidante, ação antimicrobiana e a composição dos óleos essenciais de cravo-da-índia (*Caryophillus aromaticus L.*), citronela (*Cymbopogon winterianus*) e palmarosa (*Cymbopogon martinii*). A ação antioxidante foi avaliada pelo método de DPPH (2,2-difenil-1-picril-hidrazila), e a ação antimicrobiana determinada pelo método da microdiluição definindo-se a concentração inibitória mínima para os microrganismos *Staphylococcus aureus, Escherichia coli, Salmonella thyphimurium*, *Pseudomonas aeruginosa* e *Clostridium perfringens*. A composição química dos óleos foi determinada por CG-DIC e a identificação dos compostos voláteis por CG-EM, em ambos os casos, utilizando uma coluna capilar DB-5. O óleo de cravo-da-índia apresentou uma forte atividade antioxidante e ação antimicrobiana moderada a forte, sendo o eugenol o componente majoritário do óleo de cravo-da-índia (83,7%). Por outro lado, as amostras de citronela e palmarosa apresentaram fraca ação antioxidante, porém a ação antimicrobiana foi moderada a forte.

Palavras-chave. radicais livres, cravo-da-índia, óleos essenciais, antioxidantes.

Abstract

COMPOSITION AND ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF CLOVE, CITRONELLA AND PALMAROSA ESSENTIAL OILS. Antioxidant and antimicrobial activity, as well as the chemical composition of essential oils of clove (*Caryophillus aromaticus L.*), citronella (*Cymbopogon winterianus*) and palmarosa (*Cymbopogon martinii*) were studied. Antioxidant activity was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl) method and the antimicrobial activity determinate by the microdillution method. The minimal inhibition concentration was defined against *Staphylococcus aureus, Escherichia coli, Salmonella thyphimurium*, *Pseudomonas aeruginosa* e *Clostridium perfringens*. The chemical composition was performed by GC-FID and volatile compounds were identified by GC-MS, using a DB-5 fused silica capillary column. Clove essential oil showed very strong antioxidant activity and moderate to strong antimicrobial activity for all tested strains, eugenol was the majority compound (83.7%) in the clove essential oil. On the other hand, citronella and palmarosa samples presented very low antioxidant activity, but showed moderate to strong antimicrobial activity for all tested strains.

Keywords: clove, free radicals, antioxidants, essential oils

1. Introdução

Óleos essenciais são produtos aromáticos de metabolismo secundário de plantas, normalmente formados por células especiais ou grupos de células, sendo encontrados em diversas partes do vegetal, como folhas e talos. São comumente concentrados em uma região do vegetal, como nas folhas, casca ou frutos, e freqüentemente apresentam composição diferente (CONNER, 2003). A aplicação de óleos essenciais como ingredientes funcionais em formulações alimentícias, cosméticas ou ainda em formulações sanitizantes, tem despertado grande interesse neste setor industrial devido à grande aceitação dos consumidores por produtos naturais, bem como pelos danos à saúde propiciados pelos aditivos sintéticos.

A literatura atual apresenta inúmeros relatos sobre atividade biológica de extratos vegetais, como ação antifúngica (ALANÍS-GARZA et al, 2007; KORUKLUOGLU, SAHAN e YIGIT, 2008), ação antibacteriana (ZHANG et al, 2007; AI-TURKI, EI-ZINEY e ABDEL-SALAM, 2008), atividade anti-tumoral (JIMÉNEZ-MEDINA et al, 2006; KAILEH et al, 2007), ação antiinflamatória e analgésica (BOSE et al, 2007; DÍAZ-VICIEDO et al, 2008) atividade antioxidante (FERRERES et al, 2007; STARZYŃSKA-JANISZEWSKA, STODOLAK e JAMRÓZ, 2008), dentre outras.

O cravo-da-índia (*Caryophillus aromaticus L.*) é uma planta amplamente utilizada para as mais diversas aplicações, como, por exemplo, anestesia para peixes durante o transporte ou para minimizar o estresse antes do abate (ROUBACH et al, 2005), ou ainda como anestésico tópico (ALQAREER, ALYAHYA e ANDERSSON, 2006), tratamento de candidíase vaginal (AHMAD et al, 2005), além de ser muito utilizado na culinária. A literatura apresenta algumas publicações atribuindo atividades

biológicas ao óleo de cravo-da-índia, tais como antimicrobiana (NUNEZ et al, 2001; VELLUTI et al, 2004; VIUDA-MARTOS et al, 2007), antioxidante (BAMDAD, KADIVAR e KERAMAT, 2006; JIROVETZ et al, 2006; YANISHLIEVA, MARINOVA e POKORNY, 2006) e anestésica (ALTUN, HUNT e USTA, 2006; SEOL et al, 2007).

SUHR e NIELSEN (2003) estudaram o efeito de óleos essenciais e extratos sobre a deterioração de pão de centeio por fungos. Os resultados mostraram que extratos de capim-limão, canela, cravo, mostarda e tomilho apresentaram um efeito positivo na preservação do pão. MYTLE et al. (2006) relataram que a aplicação de óleo de cravo na proporção de 1 e 2% reduziu a contagem de *Listeria monocytogenes* em salsicha alemã.

As plantas citronela (*Cymbopogon winterianus*) e palmarosa (*Cymbopogon martinii*) têm sido estudadas em relação a sua atividade repelente de insetos (MAKHAIK, NAIK e TEWARY, 2005; KUMAR, SRIVASTAVA e DUBEY, 2007) e ação antifúngica (DUARTE et al, 2005), entretanto, não há relatos sobre a atividade antioxidante. Em outro estudo prévio, MENDONÇA et al. (2005) relataram que o óleo essencial de citronela mostrou elevada atividade contra larvas do mosquito *Aedes Aegypti*.

Compostos com propriedades biológicas, produzidos por diversas plantas, podem ser utilizados para síntese de novos medicamentos, ou mesmo, serem utilizados como substitutos de princípios ativos sintéticos, como os antibióticos**,** no intuito de reduzir a resistência microbiana (AHMAD e BEG, 2001). Sendo assim, o objetivo do presente trabalho foi investigar as atividades antioxidante e antimicrobiana dos óleos essenciais de cravo-da-índia, citronela e palmarosa, bem como caracterizar os constituintes voláteis das amostras.

2. Material e Métodos

Os óleos essenciais de cravo-da-índia (*Caryophillus aromaticus L.*), citronela (*Cymbopogon winterianus*) e palmarosa (*Cymbopogon martinii*) foram adquiridos comercialmente da Dierberger Óleos Essenciais S.A. (Brasil) em embalagens de 5 Kg (1 embalagem de cada óleo) de onde foram retiradas alíquotas para as análises. Os antibióticos, os compostos fenólicos, BHA (butil-hidroxianisol) e o DPPH (2,2-difenil-1-picril-hidrazila) foram adquiridos da Sigma-Aldrich (EUA).

A análise dos constituintes químicos das amostras foi realizada em um cromatógrafo a gás (Varian 3800) acoplado a um detector de ionização em chama (DIC), em triplicata. Os compostos voláteis foram separados em uma coluna capilar DB-5 (30 m x 0,25 mm d.i. x 0,25 μm, J&W Scientific, Folson Califórnia, USA). A programação de temperatura iniciou a 60 °C, aumentando até 240 °C por uma rampa de temperatura de 3 °C/min, permanecendo por 7 min. Utilizou-se o gás hélio para arraste, com uma vazão constante de 1 mL/min. As temperaturas do injetor e do detector foram mantidas em 230 e 250 °C, respectivamente. As amostras foram diluídas em hexano (1mg/mL) e o volume injetado foi de 1,0 μL com o injetor no modo *splitless*. As percentagens relativas dos compostos foram calculadas a partir das áreas médias obtidas dos cromatogramas. A série homóloga de alcanos (C6-C19) foi analisada nas mesmas condições cromatográficas e, a partir dos tempos de retenção destes, foram calculados os Índices de Kovats (IK) dos compostos voláteis.

A identificação dos constituintes químicos das amostras foi realizada em um cromatógrafo a gás (Shimadzu 17A) acoplado a um espectrômetro de massas (EM) (Shimadzu QP-5000) nas mesmas condições cromatográficas descritas acima, exceto que a temperatura da interface (CG-EM) foi mantida em 240 °C. O detector de massas

foi operado no modo de ionização por elétrons (+70 eV), utilizando o modo de varredura, realizada de 35 a 400 *m/z* e a voltagem do detector foi de 1,6 kV. A identificação dos compostos foi realizada primeiramente pela comparação da similaridade dos espectros de massa obtidos com os da literatura (NIST 98; ADAMS, 1995), e posteriormente realizou-se a comparação do Índice Kovats com a literatura (IK) (ADAMS, 1995). Alguns compostos foram considerados positivamente identificados, quando comparados através dos espectros de massas e tempos de retenção dos analitos e dos padrões.

A atividade antioxidante foi determinada pelo método DPPH, segundo SCHERER e GODOY (2008). Foram utilizados 3,9 mL de solução de DPPH (0,2 mM) em metanol e 0,1 mL de amostra ou padrões diluídos em metanol em diferentes concentrações. No branco foram utilizados 3,9 mL de DPPH (0,2 mM) em metanol e 0,1 mL de metanol. Após a adição dos extratos, os tubos foram agitados vigorosamente e mantidos em repouso por 90 minutos no escuro. Após esse período, a atividade antioxidante foi medida em um espectrofotômetro (λ = 517 nm), previamente calibrado com metanol puro. Todas as análises foram realizadas em triplicata. O índice DPPH foi calculado através da equação 1: I (%) = [(Abs_0 – Abs_1)/Abs_0] x 100 (1), onde Abs_0 é a absorbância do branco e Abs_1 é a absorbância da amostra. O IC_{50} (quantidade suficiente para 50% de inibição) foi calculado através da equação da reta obtida da curva de calibração (concentração versus o índice DPPH correspondente). A ação antioxidante dos extratos foi expressa pelo Índice de Atividade Antioxidante (IAA), onde tanto a massa de DPPH quanto a massa do extrato utilizada no ensaio foram consideradas para gerar a constante. O IAA é calculado pela equação 2: IAA = massa de DPPH (μg/mL)/IC_{50} (μg/mL) (2). No presente trabalho, considerou-se uma ação antioxidante

fraca quando o IAA < 0,5, ação moderada quando o AAI estiver entre 0,5 e 1,0, ação antioxidante forte quando o IAA for de 1,0 a 2,0, e ação muito forte para um valor de IAA > 2,0. Todas as soluções das amostras e padrões, bem como as soluções de DPPH, foram preparadas diariamente antes da utilização.

Para a determinação da atividade antimicrobiana, as amostras e os padrões antibióticos foram diluídos em Dimetilsulfóxido (DMSO) e armazenados -20 °C até o momento das análises. A determinação da concentração inibitória mínima (CIM) foi realizada em placas de cultura com 96 poços, segundo as normas do NCCLS (National Committee of Laboratory Standards, 2003). Os microrganismos utilizados foram *Staphylococcus aureus* (ATCC 6538)*, Escherichia coli* (isolada de suínos)*, Salmonella thyphimurium* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 13388) e *Clostridium perfringens* (ATCC 1324). A concentração final de células, ajustada em um espectrofotômetro pela escala de McFarland 0,5, foi na ordem de 10^4 UFC/mL. Em cada poço foi adicionado meio de cultura (Mueller-Hinton 2,1%), amostra ou o antibiótico, e o inóculo. As concentrações finais avaliadas dos extratos foram de 1,8, 1,5, 1,2, 1,0, 0,8, 0,6, 0,4 e 0,2 mg/mL, enquanto que para os antibióticos foram de 250 a 1,95 μg/mL, diluídos em série. Foram incluídos nos testes, controles para verificar a esterilidade do meio, do extrato e da viabilidade do microrganismo. Após a adição do inóculo, as placas foram incubadas a 36 °C por 24 h, e posteriormente adicionado 50 μL de cloreto de trifenil tetrazolium (CTT) (0,5% em solução aquosa). Após mais 4 h de incubação, a CIM foi determinada como a menor concentração capaz de inibir o crescimento visível de células, conferido pelo CTT (células mortas não são coradas).

A análise estatística foi realizada utilizando o software Statistica 6.0. As diferenças foram consideradas significativas quando $P < 0,05$ (ANOVA/teste de Tukey).

3. Resultados e Discussão

Na composição volátil do óleo essencial de cravo-da-índia, três compostos foram considerados majoritários, destacando-se o eugenol, com 83,75% da área total, seguido pelo β-cariofileno, com 10,98%, e com 1,26%, o α-humuleno. Outros compostos também foram detectados em quantidades diminutas (Tabela 1). Os resultados presentes foram similares aos encontrados por outros pesquisadores (PINO et al, 2001; TOMAINO et al, 2005; JIROVETZ et al, 2006). No óleo essencial de citronela os compostos majoritários foram o β-citronelal (45%), geraniol (20,71%) e β-citronelol (14,49%), além de outros compostos com área superior a 1% (Tabela 1). No óleo essencial de palmarosa, o geraniol constituiu mais de 80%, sendo o acetato de geranila o segundo composto em maior percentagem (12%) (Tabela 1), concordando com RAO et al. (2005) onde foram encontrados teores de 78 – 85% de geraniol e 3,0 - 12% de acetato de geranila em diferentes amostras de óleo essencial de palmarosa. Em estudo prévio, foi encontrado no óleo essencial de citronela o linalol (27,4%), citronelol (10,9%), geraniol (8,5%), cis-calameneno (4,3%), β-elemeno (3,9%) dentre outros compostos (MALELE et al, 2007). DUARTE et al. (2005) avaliaram a composição dos óleos essenciais de citronela e palmarosa, e diferentemente dos resultados obtidos neste estudo, os autores encontraram para o óleo de citronela 36% de β-citronelal, 18% de β-citronelol e 11% de geraniol, enquanto que no óleo de palmarosa, o geraniol apresentou 63% da área total e o acetato de geranila 28%.

A composição e a concentração dos componentes dos óleos essenciais, de plantas de mesma espécie, podem variar devido a fatores ecológicos e as condições edafoclimáticas (YIN, 1991). Assim, estas diferenças químicas existentes entre os óleos essenciais podem refletir diretamente nas propriedades funcionais, como a atividade antimicrobiana e antioxidante. Em outro estudo prévio, os componentes do óleo essencial de citronela foram afetados pela época de coleta e espaçamento entre as plantas, sendo que os constituintes citronelol e citronelal foram significativamente mais elevados em plantas cultivadas com espaçamento intermediário e colhidas após seis meses do plantio, e com 30 cm de altura, por outro lado, o teor de geraniol foi superior em plantas colhidas quatro meses após o plantio, porém não houve influência do espaçamento e de altura de corte sobre o seu teor (MARCO et al, 2007). Esses resultados concordam com BLANK et al. (2007), que reportaram que os componentes do óleo essencial de citronela foram alterados conforme a estação do ano e o horário em que a planta foi coletada.

Na determinação do IC_{50} foram elaboradas curvas de calibração para cada composto, pois o IC_{50} deve ser calculado em um intervalo de linearidade do índice DPPH. Sendo assim, através da equação da reta calculou-se o IC_{50} de cada amostra e, posteriormente o índice de atividade antioxidante (IAA), cujos valores estão apresentados na Tabela 2. Os compostos fenólicos empregados neste estudo serviram como parâmetro de equivalência de atividade antioxidante aos óleos essenciais testados. O ácido gálico apresentou o maior valor de IAA seguido pelo ácido protocatecuico e quercetina ($P < 0,05$). Não houve diferença significativa no valor de IAA, entre o ácido clorogênico, óleo essencial de cravo-da-índia, ácido caféico e BHA, entretanto apresentaram IAA significativamente maior do que a rutina e o ácido ferúlico

(Tabela 2). O óleo essencial de cravo-da-índia apresentou uma ação antioxidante muito forte devido ao elevado teor de eugenol em sua composição, pois segundo SCHERER e GODOY (2008) o eugenol apresenta um valor de IAA entre 10 e 11. Por outro lado, o óleo de citronela apresentou um valor de IC_{50} muito alto e conseqüentemente valor de IAA muito baixo indicando fraca ação antioxidante (IAA < 0,5) e o óleo de palmarosa não apresentou habilidade de reduzir o DPPH, mesmo na maior concentração final avaliada (800 μg/mL).

Estruturalmente, os compostos fenólicos consistem em um anel aromático ligado a um ou mais grupos hidroxila, ocorrendo desde simples fenólicos até moléculas altamente polimerizadas (BRAVO, 1998). A estrutura dos compostos fenólicos é um fator determinante para a sua atividade anti-radical livre e quelante de metais, assim conhecido como relação estrutura-atividade (BALASUNDRAM, SUNDRAM e SAMMAN, 2006). A ação antioxidante dos compostos fenólicos ácidos aumenta com o grau de hidroxilação, como no caso do ácido gálico (trihidroxilado), que apresentou maior IAA do que o ácido protocatecuico (dihidroxilado) (Tabela 2). A substituição de um grupo hidroxila no anel aromático por um grupo metoxil reduziu a atividade antioxidante do composto ácido ferúlico em relação ao ácido caféico (Tabela 2), fato também reportado por RICE-EVANS, MILLER e PAGANGA (1996). Isso pode ser explicado pela redução da capacidade de doação de átomos de hidrogênio da molécula.

A relação estrutura-atividade em flavonóides é geralmente mais complicada do que nos ácidos hidroxibenzóicos e hidroxicinâmicos, devido à maior complexidade das moléculas. Van ACKER et al. (1996) relataram que o grau de hidroxilação, bem como a posição dos grupos hidroxilas influenciam na atividade antioxidante dos flavonóides. Além disso, uma dupla ligação combinada com um radical –OH no anel central aumenta

a ação antioxidante. Por esse motivo, a quercetina apresentou um valor de IAA significativamente maior em relação à rutina, onde o radical –OH do anel central foi substituído por um açúcar (rutinose).

Na Tabela 3 estão apresentados os valores de CIM das amostras e dos antibióticos. O óleo de cravo-da-índia apresentou melhor atividade antimicrobiana nos microrganismos *S. aureus*, *E. coli* e *P. aeruginosa*, sendo que os microrganismos *S. thyphimurium e C. perfringens* foram os mais resistentes. Os microrganismos *E. coli* e *P. aeruginosa* foram mais sensíveis ao óleo de citronela do que os microrganismos *S. aureus, S. thyphimurium* e *C. perfringens*. Por outro lado, o óleo de palmarosa apresentou maior eficácia contra os microrganismos *S. aureus, E. coli* e *S. thyphimurium* (Tabela 3). Os resultados do presente trabalho foram similares aos encontrados por OUSSALAH et al. (2007), onde o óleo essencial de citronela (Vietnam) apresentou valores de CIM de 0,8, 0,4 e 0,05 mg/mL, para *E. coli, S. thyphimurium* e *S. aureus*, respectivamente, enquanto que os óleo essenciais de palmarosa (Índia) e cravo-da-índia (Madagascar) apresentaram valores de CIM de 0,2, 0,2 e 0,1 mg/mL e 0,1, 0,1, e 0,05 mg/mL, para *E. coli, S. thyphimurium* e *S. aureus*, respectivamente. A diferença na atividade antimicrobiana, como citado anteriormente, pode ser atribuída às diferenças na composição dos óleos essenciais, pois no óleo de cravo, cujos valores de CIM foram bem menores do que os valores encontrados no presente trabalho, o teor de eugenol e acetato de eugenila foram de 78 e 14%, sendo que no presente trabalho o teor de eugenol foi de 85% e não foi encontrado acetato de eugenila.

De maneira geral, os microrganismos testados apresentaram menor resistência ao antibiótico enrofloxacina, sendo a *S. thyphimurium* a bactéria mais resistente a esse antibiótico (Talela 3).

DUARTE et al. (2005) estabeleceram com base no trabalho de ALIGIANNIS et al. (2001), uma classificação para atividade antimicrobiana de materiais vegetais, de acordo com os resultados de CIM, sendo considerada como forte, moderada e fraca atividade antimicrobiana, óleos ou extratos que apresentem CIM até 0,5 mg/mL, entre 0,6 e 1,5 mg/mL, e acima de 1,6 mg/mL, respectivamente. Sendo assim, todos os óleos essenciais testados nesse trabalho apresentaram ação antimicrobiana de moderada a forte.

As ações antimicrobiana e antioxidante do óleo de cravo-da-índia podem ser atribuídas ao eugenol, pois o mesmo representa mais de 80% da composição deste óleo. O geraniol encontrado no óleo de citronela e no óleo de palmarosa tem sido relatado como um composto de elevada atividade antimicrobiana (PATTNAIK et al, 1997; Van ZYL et al, 2006; DUARTE et al, 2007; JIROVETZ et al, 2007), assim como os compostos citronelal e citronelol encontrados no óleo de citronela (SATO, KRIST e BUCHSAUER, 2006; KORDALI, KOTAN e CAKIR, 2007).

Conclusões

O óleo de cravo-da-índia apresentou atividade antioxidante muito forte e ação antimicrobiana moderada a forte sobre os microrganismos avaliados. Os óleos essenciais de citronela e palmarosa apresentaram fraca ação antioxidante, porém moderada a forte ação antimicrobiana. Desta forma, considerando as propriedades estudadas das amostras de óleos essenciais, pode se propor que, em algumas circunstâncias, como na forma microencapsulada, estas essências podem atuar de forma eficiente como antimicrobianos, assim tornando-se uma alternativa aos antimicrobianos sintéticos, como no caso dos promotores de crescimento adicionados a ração de animais.

Adicionalmente, o óleo de cravo-da-índia pode ser utilizado também quando há a necessidade de um antimicrobiano e antioxidante, simultaneamente.

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Tabela 1: Composição química dos óleos essenciais de cravo, citronela e palmarosa, determinadas por CG-DIC.

Composto	IK*	IK**	CRA (%)	CIT (%)	PAL (%)
Campfeno	954	952	-	-	-
β-Mirceno	994	994	-	-	0,08
Limoneno	1032	1031	-	3,37	-
Eucaliptol	1036	1035	-	-	-
cis-Ocimeno	1041	1040	-	-	0,15
trans-Ocimeno	1051	1050	-	-	1,52
NI	1101	1100	-	0,54	1,55
NI	1149	1148	-	2,17	-
β-Citronelal	1159	1156	-	45,00	-
β-Citronelol	1232	1231	-	14,49	-
Chavicol	1255	1256	0,33	-	-
Geraniol	1259	1260	-	20,71	81,22
Geranial	1273	1273	-	0,67	0,53
Acetato de citronelila	1357	1355	-	1,46	-
Eugenol	1365	1363	83,75	-	-
α-Copaeno	1378	1377	0,24	-	-
Acetato de geranila	1386	1385	-	1,40	12,80
β-Elemeno	1393	1392	-	1,50	-
β-Cariofileno	1420	1420	10,98	-	0,86
α-Humuleno	1455	1454	1,26	-	-
γ-Muroleno	1478	1477	0,21	0,19	-
germacreno D	1482	1481	-	0,57	-
α-Muroleno	1500	1499	-	0,38	-
γ-Cadineno	1515	1513	-	0,46	-
Δ-Cadineno	1525	1524	0,40	1,27	-
Elemol	1551	1550	-	1,24	-
Butanoato de geranila	1563	1562	-	-	0,15
Germacreno D-4-ol	1577	1575	-	0,25	-
Total			97,17	95,67	98,86

IK: Índice de Kovats calculado; *CG-EM, **CG-DIC; NI: não identificado; CIT: óleo de citronela; PAL: óleo de palmarosa; CRA: óleo de cravo.

Tabela 2: Índice de atividade antioxidante (IAA) dos óleos essenciais de cravo, citronela, palmarosa e dos compostos fenólicos.

	r^2	IC_{50} (μg/mL)	IAA
Ácido gálico	0,9992 – 1,0000	2,83 ± 0,07	27,1 ± 0,7[a]
Ácido protocatecuico	0,9988 – 1,0000	3,8 ± 0,15	20,2 ± 0,8[b]
Quercetina	0,9939 – 0,9998	4,9 ± 0,56	15,9 ± 1,7[c]
Ácido clorogênico	0,9986 – 0,9995	7,4 ± 0,12	10,3 ± 0,1[d]
Óleo de cravo	0,9986 – 0,9999	7,8 ± 0,65	9,9 ± 0,8[d]
Ácido cafeico	0,9966 – 0,9983	8,2 ± 0,31	9,4 ± 0,3[d]
BHA	0,9969 – 0,9991	8,2 ± 0,41	9,3 ± 0,4[d]
Rutina	0,9996 – 0,9998	12,1 ± 0,70	6,4 ± 0,3[e]
Ácido ferúlico	0,9992 – 0,9998	14,4 ± 0,66	5,3 ± 0,2[e]
Óleo de citronela	0,9947 – 0,9991	743 ± 18	0,1 ± 0,0[f]
Óleo de palmarosa	-	-	-

Os valores apresentados correspondem à média e desvio padrão de triplicatas. Letras diferentes correspondem diferenças significativas ($p < 0,05$).

Tabela 3: Concentração Inibitória Mínima (CIM – mg/mL) dos óleos essenciais de cravo, citronela e palmarosa e dos antibióticos.

Concentração Inibitória Mínima (CIM – mg/mL)					
	S. aureus	*E. coli*	*P. aeruginosa*	*S. thyphimurium*	*C. perfringens*
Óleo de citronela	0,600-0,800	0,400-0,600	0,400-0,600	0,600-0,800	0,600-0,800
Óleo de palmarosa	0,400-0,600	0,400-0,600	0,600-0,800	0,400-0,600	0,600-0,800
Òleo de cravo	0,400-0,600	0,400-0,600	0,400-0,600	0,600-0,800	0,600-0,800
Amoxicilina	*	0,031-0,062	0,062-0,125	0,062-0,125	0,031-0,062
Enrofloxacina	*	*	*	0,004-0,008	*
Eritromicina	*	*	0,031-0,062	0,031-0,062	0,031-0,062
Lincomicina	*	0,031-0,062	0,031-0,062	0,031-0,062	0,031-0,062
Oxitetraciclina	*	0,008-0,015	0,062-0,125	0,062-0,125	0,062-0,125
Penicilina G	*	0,008-0,015	0,062-0,125	0,062-0,125	0,062-0,125
Sulfadiazina	0,062-0,125	0,004-0,008	0,062-0,125	0,062-0,125	0,062-0,125
Sulfanilamida	0,062-0,125	0,004-0,008	0,062-0,125	0,062-0,125	0,062-0,125
Tetraciclina	*	*	0,031-0,062	0,031-0,062	0,008-0,015

* < 0,0019 mg/mL

Capítulo 6

Utilização de eucaliptol e eugenol microencapsulados como promotores de crescimento em frangos de corte.

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Utilização de eucaliptol e eugenol microencapsulados como promotores de crescimento em frangos de corte no período de 1 a 21 dias.

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RESUMO - O objetivo do presente trabalho foi avaliar diferentes níveis de eucaliptol e eugenol microencapsulados e previamente analisados quanto ao teor de pureza, atividade antioxidante e atividade antimicrobiana como alternativa aos promotores de crescimento em frangos de corte no período de 1 a 21 dias. Foram utilizados 392 pintos machos (Ross) distribuídos em 7 tratamentos: T1 - controle, T2 - avilamicina (10 mg/kg), T3 - eucaliptol (500 mg/kg), T4 - eucaliptol (1000 mg/kg), T5 - eugenol (500 mg/kg), T6 - eugenol (1000 mg/kg) e T7 - eucaliptol e eugenol (500 mg/kg de cada). O delineamento foi inteiramente casualizado com 7 tratamentos, 8 repetições e 7 aves por unidade experimental. Os resultados mostraram que a associação dos dois compostos afetou negativamente o desempenho das aves, entretanto, a utilização de eucaliptol (500 mg/kg) ou eugenol (500 mg/kg) melhorou o desempenho das aves em relação ao controle, e não foram verificadas diferenças significativas ($P < 0,05$) com 10 mg/kg de avilamicina, portanto, é possível substituir a avilamicina pelos extratos vegetais testados sem comprometer o desempenho das aves na fase inicial.

Palavras-chave: fitoterápicos, frangos de corte, promotores de crescimento, desempenho.

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The use of eucalyptol and eugenol microencapsulated as growth promoters in broilers in the period 1 to 21 days of age

ABSTRACT – The aim of this work was to evaluate different levels of eucalyptol and eugenol microencapsulated and previously tested of purity, antioxidant activity and antimicrobial activity as alternative growth promoters in broilers from 1 to 21 days of age. In this experiment were used 392 male broilers (Ross), distributed in 7 treatments as follow: T1 - control, T2 - avilamycin (10 mg/kg), T3 - eucalyptol (500 mg/kg), T4 - eucalyptol (1000 mg/kg), T5 - eugenol (500 mg/kg), T6 - eugenol (1000 mg/kg) and T7 - eucalyptol and eugenol (500 mg/kg of each one). The experimental design was entirely randomized, with 7 treatments with 8 replicates of 7 chicks each. The results showed that the association of the two compounds had negative effect in the broilers performance, however, when the eucalyptol (500 mg/kg) or eugenol (500 mg/kg) was used, the broilers performance were better than control group, and no significant difference ($P < 0,05$) to 10 mg/kg of avilamicyn was observed. So, the avilamycin can be substituted by the tested plant extracts without to lose the performance of birds in the growing phase.

Key words: phytotherapics, broilers, growth promoters, performance.

Introdução

Algumas cepas enterotoxigênicas de *Escherichia coli*, assim como outras bactérias, são responsáveis por grandes perdas econômicas na suinocultura e na avicultura em várias partes do mundo (White et al., 2003). Tanto em aves como em suínos, a infecção causada por *E. coli* é chamada de colibacilose. Em aves, o trato respiratório é a principal entrada do microrganismo onde forma colônias na traquéia causando quadros como peritonite, pneumonia, doença respiratória crônica complicada (DRCC) entre outros quadros clínicos (Ferreira & Knöbl, 2000).

Devido à alta incidência de problemas relacionados com microrganismos nos aviários, o uso de promotores de crescimento tem sido uma medida muito utilizada para a redução dos prejuízos e melhora do desempenho dos animais (Freed et al., 1993). O efeito benéfico pode ser mais bem expresso em animais jovens, devido sua proteção imunológica ser deficiente (Rutz & Lima, 2001). Entretanto, o uso de antibióticos tem sido questionado, tanto sobre a quantidade de resíduos que estes podem deixar nos produtos em que consumimos (Vassalo et al., 1997), bem como ao surgimento de bactérias resistentes a estes medicamentos. Blanco et al. (1997) e Cardoso et al. (2002) encontraram uma alta resistência microbiana de amostras de *E. coli* isoladas de frango frente aos principais antibióticos utilizados, como sulfadiazina-trimetropina, tetraciclina, ampicilina e outros. A alta resistência microbiana tem se mostrado um problema preocupante para a suinocultura e avicultura mundial (Baccaro et al., 2002). Ela é transmitida por diversos mecanismos em uma mesma população ou em populações diferentes, como da microbiota animal para a humana e vice-versa (Nijsten et al., 1993; Casewell et al., 2003).

Devido a opiniões públicas, políticas e o conceito científico de que a resistência de animais pode ser transmitida para os humanos, a União Européia vem banindo

gradativamente o uso de antibióticos como promotores de crescimento, como recentemente ocorreu com a avoparcina e espiramicina (Casewell et al., 2003). No Brasil, muitos antibióticos já foram banidos, como a avoparcina pelo ofício Circular DFPA Nº 047/98, penicilina, tetraciclinas e sulfonamidas sistêmicas pela Portaria 193 de 12/05/1998, o cloranfenicol e os nitrofuranos pela Instrução Normativa 09, de 27/06/2003, o olaquindox pela Instrução Normativa 11, de 24/11/2004 e o carbadox pela Instrução Normativa 35, de 14/11/2005 (Brasil, Ministério da Agricultura, Pecuária de Abastecimento). Portanto, o estudo de substitutos aos antibióticos como promotores de crescimento é uma necessidade atual e mundial. Por essa razão, o presente experimento teve o objetivo de avaliar o desempenho de frangos de corte alimentados com dietas contendo o antibiótico avilamicina e os fitoterápicos eucaliptol e eugenol microencapsulados, no período de 1 a 21 dias de idade.

Material e Métodos

As frações de eucaliptol e eugenol para a produção das rações foram adquiridas comercialmente da Dierberger Óleos Essenciais S.A. (Brasil) em embalagens de 5 Kg (1 embalagem de cada óleo) de onde foram retiradas alíquotas para as análises e para a elaboração das microcápsulas utilizadas na ração. Os antibióticos avilamicina, halquinol, lincomicina, tiamulina e tilosina foram fornecidos pela POLINUTRI (São Paulo, SP, Brasil). Os compostos fenólicos, BHA (butil-hidroxianisol) e o DPPH (2,2-difenil-1-picril-hidrazila) foram adquiridos da Sigma-Aldrich (EUA).

A atividade antioxidante das frações foi determinada pelo método DPPH (2,2-difenil-1-picrilhidrazila) utilizando o índice de atividade antioxidante (IAA) segundo Scherer & Godoy (2008). Para a determinação da atividade antimicrobiana, as frações e os padrões de antibióticos foram diluídos em Dimetilsulfóxido (DMSO) e armazenados -20 °C até o

momento das análises. A determinação da concentração inibitória mínima (CIM) foi realizada em placas de cultura com 96 poços, segundo as normas do NCCLS (National Committee of Laboratory Standards, 2003). Os microrganismos utilizados foram *Staphylococcus aureus* (ATCC 6538)*, Escherichia coli* (isolada de suínos)*, Salmonella thyphimurium* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 13388) e *Clostridium perfringens* (ATCC 1324). A concentração final de células, ajustada em um espectrofotômetro pela escala de McFarland 0,5, foi na ordem de 104 UFC/mL. Em cada poço foi adicionado meio de cultura (Mueller-Hinton 2,1%), fração ou o antibiótico, e o inóculo. As concentrações finais avaliadas das frações foram de 1,8; 1,5; 1,2; 1,0; 0,8; 0,6; 0,4 e 0,2 mg/mL, enquanto que para os antibióticos foram de 250 a 1,95 μg/mL, diluídos em série. Foram incluídos nos testes, controles para verificar a esterilidade do meio, do extrato e da viabilidade do microrganismo. Após a adição do inóculo, as placas foram incubadas a 36 °C por 24 h, e posteriormente adicionado 50 mL de cloreto de trifenil tetrazolium (CTT) (0,5% em solução aquosa). Após mais 4 h de incubação, a CIM foi determinada como a menor concentração capaz de inibir o crescimento visível de células, conferido pelo CTT (células mortas não são coradas). A análise de pureza das frações foi realizada por cromatografia a gás acoplada a espectrometria de massas (Shimadzu 17A, QP-5000) para identificação e, por cromatografia a gás acoplado a um detector de ionização em chama (Varian 3800) para a quantificação segundo Scherer et al. (2008).

A microencapsulação das frações foi realizada utilizando maltodextrina (20 DE), capsul (amido modificado), fração e água, na proporção de 1,5; 1,0; 1,5 e 3,5 g, respectivamente. A mistura foi homogeneizada em ultra turrax e submetida à secagem a 180 ºC em um equipamento SPRAY DRYER modelo Lab Plant SD-05 (L.P. Technology,

UK), utilizando um atomizador de duplo fluido (1,5 mm de diâmetro). Para assegurar a qualidade das microcápsulas, a quantidade de recheio foi determinada por hidrodestilação em triplicata (5 g com 200 mL de água destilada em sistema Clevenger) sendo posteriormente analisada por cromatografia a gás como descrito acima.

O experimento com as aves foi conduzido nas instalações do Setor de Avicultura do Departamento de Nutrição e Produção Animal, Faculdade de Medicina Veterinária e Zootecnia da USP de Pirassununga. Foram utilizados 392 pintos machos "Ross" de um dia de idade alojados em baterias metálicas (gaiolas) com lâmpadas de 100 W para o aquecimento, comedouro tipo calha e bebedouro tipo nipple. O delineamento foi inteiramente casualizado com 7 tratamentos, 8 repetições e 7 aves por unidade experimental. Os tratamentos utilizados foram: T1 – controle (ração basal), T2 – ração basal com 10 mg/kg de avilamicina, T3 - ração basal com 500 mg/kg de eucaliptol, T4 - ração basal com 1000 mg/kg de eucaliptol, T5 - ração basal com 500 mg/kg de eugenol, T6 - ração basal com 1000 mg/kg de eugenol, e T7 - ração basal com 500 mg/kg de eucaliptol e 500 mg/kg de eugenol. As rações (Tabela 1) e a água foram fornecidas à vontade no período de 1 a 21 dias. O desempenho das aves foi avaliado através das variáveis: peso inicial, consumo de ração (CR), ganho de peso (GP), conversão alimentar (CA), taxa de mortalidade e peso final dos frangos. Todas as aves foram pesadas com 1, 10 e 21 dias de idade.

A análise estatística foi realizada utilizando o software Statistica 6.0. As diferenças foram consideradas significativas quando $P < 0,05$ (ANOVA/teste de Tukey).

Resultados e Discussão

A atividade antioxidante (IAA) dos compostos está apresentada na Tabela 2. A fração eugenol, que apresentou 99,9% de pureza segundo as análises cromatográficas, demonstrou

elevada atividade antioxidante, com valor de IAA significativamente maior ($P < 0,05$) que a rutina e o ácido ferúlico, porém significativamente menor ($P < 0,05$) que o ácido gálico e o ácido protocatecuico, além disso, não houve diferença significativa entre o eugenol, ácido clorogênico, ácido caféico e BHA. Por outro lado, o eucaliptol, que apresentou 99,95% de pureza, não apresentou habilidade de reduzir o DPPH, mesmo na maior concentração final avaliada (0,8 mg/mL). Estudos prévios demonstraram que o BHA e o BHT, principais antioxidantes utilizados em alimentos e rações, se acumulam no organismo podendo causar danos ao fígado e carcenogênese (Ito et al., 1986; Whysner et al., 1986). Considerando o presente resultado, o eugenol poderia substituir o BHA em rações para animais, entretanto, mais estudos são necessários, pois não foram realizados ensaios para monitorar oxidação lipídica na ração.

Segundo Zuanon et al. (1998) a ação antimicrobiana dos extratos vegetais sobre a microbiota intestinal pode controlar ou inibir o crescimento dos patógenos e, conseqüentemente, proporcionar crescimento dos microrganismos benéficos, favorecendo o desempenho animal. Por essa razão foi avaliada a ação antimicrobiana das frações eucaliptol e eugenol em comparação com os principais antibióticos utilizados como promotores de crescimento, para desta forma, estabelecer uma concentração apropriada para os experimentos 'in vivo'. Na Tabela 3 estão apresentados os valores de concentração mínima inibitória (CIM) das frações e dos antibióticos. Não houve diferença na ação antimicrobiana entre o eucaliptol e o eugenol para os microrganismos *E. coli*, *P. aeruginosa* e *S. thyphimurium*. Entretanto, os microrganismos *S. aureus* e *C. perfringens* foram mais sensíveis ao eugenol do que ao eucaliptol. Duarte et al. (2005) estabeleceram com base no trabalho de Aligiannis et al. (2001), uma classificação para atividade antimicrobiana de materiais vegetais de acordo com os resultados de CIM, sendo considerada como forte,

moderada e fraca atividade antimicrobiana, óleos ou extratos que apresentem CIM até 0,5 mg/mL, entre 0,6 e 1,5 mg/mL, e acima de 1,6 mg/mL, respectivamente. Considerando essa classificação, o eucaliptol e o eugenol apresentaram ação antimicrobiana moderada a forte considerando todas as cepas testadas, viabilizando a utilização das frações no experimento 'in vivo'. O *S. aureus* foi o microrganismo mais sensível aos antibióticos testados (Tabela 3). A avilamicina foi mais eficaz contra o microrganismo *S. aureus*, entretanto, os antibióticos halquinol e tilosina foram os mais eficazes contra a *E. coli*.

Considerando os resultados da atividade antimicrobiana (Tabela 3), nota-se que os valores médios de CIM para a avilamicina, tilosina e halquinol são cerca de 5, 20 e 80 vezes maior, respectivamente, que os valores médios de CIM das frações, considerando o microrganismo *E. coli*. Os valores freqüentemente utilizados na ração animal dos antibióticos avilamicina, tilosina e halquinol são de 10, 50 e 20 mg/kg. Comparando a avilamicina com as frações, teoricamente seriam necessários 50 mg/kg das frações para equivalência, entretanto, considerando que podem haver diferenças entre ensaios 'in vitro' e 'in vivo', os valores das frações foram superestimados sendo escolhidas as concentrações de 500 e 1000 mg/kg das frações para os experimentos com os frangos de corte.

As análises dos teores de eucaliptol e eugenol nas microcápsulas revelaram teores de recheio de 20,4 ± 0,2% e 20,8 ± 0,9%, respectivamente. Houve uma perda, tanto de eugenol quanto de eucaliptol durante o processo de secagem no spray, provavelmente devido à volatilidade dos compostos. A fração extraída das microcápsulas foi injetada no cromatógrafo a gás para verificar se houve alguma alteração dos compostos durante o processo de microencapsulação, o que poderia provocar alterações nas propriedades biológicas. Os resultados mostraram que não houve alteração significativa, sendo de 99,5 e

99,1% os teores de pureza do eucaliptol e do eugenol, respectivamente. Nesse caso específico, o produto microencapsulado foi formado por apenas um composto (eucaliptol ou eugenol), entretanto, quando se realiza a microencapsulação de óleos essenciais, que são misturas complexas de compostos com diferentes temperaturas de volatilização, torna-se imprescindível a análise por cromatografia a gás, antes e depois da microencapsulação, pois pode haver alterações na composição desses óleos, refletindo em alterações na atividade biológica do produto final.

Os resultados do desempenho das aves estão apresentados na Tabela 4. Durante o experimento foram observadas apenas 3 mortes, sendo uma no tratamento com eugenol 1000 mg/kg e uma com eucaliptol 1000 mg/kg com 7 dias de idade, e a terceira morte ocorreu aos 19 dias de idade no tratamento com eugenol 500 mg/kg. Não houve diferença significativa entre os pesos iniciais das aves entre todos os tratamentos (Tabela 4). O tratamento T7 (500 mg/kg de eucaliptol e 500 mg/kg de eugenol) apresentou menor consumo de ração, menor ganho de peso, maior conversão alimentar e menor peso aos 10 dias ($P < 0,05$). Os tratamentos com 10 mg/kg de avilamicina, 500 mg/kg de eucaliptol, 1000 mg/kg de eucaliptol, 500 mg/kg de eugenol apresentaram melhores índices de conversão alimentar, sendo que o valor do tratamento com 500 mg/kg de eugenol foi significativamente menor ($P < 0,05$) que o do controle no período de 1 – 10 dias (Tabela 4).

No período de 1 a 21 dias de crescimento, não foi observada nenhuma diferença significativa entre os tratamentos controle (T1) e 10 mg/kg de avilamicina (T2) (Tabela 4). Esse fato talvez possa ser explicado devido às boas condições de criação, as quais não permitiram a manifestação de desafios que pudessem criar condições para se observar melhor os efeitos benéficos significativos dos promotores de crescimento testados. O tratamento em que foram utilizado 500 mg/kg de eucaliptol e 500 mg/kg de eugenol juntos,

apresentou os piores índices de desempenho das aves, sendo significativamente inferior a todos os tratamentos, seguido pelo tratamento com 1000 mg/kg de eugenol, pois este apresentou maior conversão alimentar ($P < 0,05$) e menor peso final ($P < 0,05$) em relação ao tratamento com avilamicina. Por outro lado, os tratamentos T3 (500 mg/kg de eucaliptol) e T5 (500 mg/kg de eugenol) não apresentaram nenhuma diferença significativa em relação ao tratamento com 10 mg/kg de avilamicina, considerando todas as variáveis estudadas, além disso, os tratamentos T3 e T5 apresentaram valores significativamente ($P < 0,05$) melhores de conversão alimentar e ganho de peso, respectivamente, em relação ao controle (Tabela 4).

Toledo et al. (2007) avaliaram um fitoterápico de óleos essenciais à base de orégano, canela, eucalipto, artemísia e trevo como promotor de crescimento em frangos de corte, comparando os resultados com avilamicina e com um controle. Os resultados mostraram que não houve diferença significativa no consumo de ração, peso corporal e na conversão alimentar entre todos os tratamentos no período de 1 a 21 dias. Em outro estudo realizado por Botsoglou et al. (2002), foram avaliados dois níveis de óleo essencial de orégano adicionados à dieta de frangos de corte (50 e 100 mg/kg), sendo que não foi observado uma melhora no desempenho dos animais, em comparação aos alimentados com a dieta controle. Esses resultados concordam com Freitas et al. (2001) que utilizaram diferentes concentrações de alho na dieta de frangos de corte alojados em baterias metálicas como substitutos aos promotores de crescimento. Os autores concluem que os dados de desempenho dos frangos de corte não permitiram observar efeitos significativos do uso do alho, antibióticos bacitracina de zinco e lincomicina, entretanto, relatam que talvez o uso destes promotores de crescimento na alimentação de aves criadas em cama e em condições

comerciais, em que o nível de microorganismos é maior, possa resultar em maiores benefícios.

Conclusões

As frações eucaliptol e eugenol adquiridas comercialmente apresentaram elevado teor de pureza, e o processo de microencapsulação não causou alterações qualitativas nestes compostos. O eugenol apresentou ação antioxidante muito forte sendo comparado ao antioxidante sintético BHT, por outro lado, o eucaliptol não apresentou atividade antioxidante. Ambas as frações apresentaram ação antimicrobiana forte a moderada contra os microrganismos testados, viabilizando a sua utilização na ração como agentes antimicrobianos. Nos experimentos 'in vivo', a utilização conjunta de eucaliptol e eugenol (500 mg/kg de cada) promoveu um decréscimo significativo no desempenho das aves comparado ao controle, entretanto, os tratamentos com 500 mg/kg de eucaliptol ou 500 mg/kg de eugenol não apresentaram nenhuma diferença em relação ao tratamento com 10 mg/kg de avilamicina, e além disso, apresentaram algumas vantagens significativas em relação ao grupo controle. Finalizando, 10 mg/kg de avilamicina podem ser substituídos por 500 mg/kg de eugenol ou eucaliptol sem perdas no desempenho zootécnico das aves no período de 1 a 21 dias, além disso, o eugenol talvez possa substituir o BHA, e dessa forma, tornar a ração isenta de aditivos sintéticos.

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Tabela 1 - Composição percentual e análise calculada das rações utilizadas nos experimentos.

	T1	T2	T3	T4	T5	T6	T7
Milho	52	52	52	52	52	52	52
Farelo de Soja	40,1	40,1	40,1	40,1	40,1	40,1	40,1
Óleo de soja	3,52	3,52	3,52	3,52	3,52	3,52	3,52
Sal	0,35	0,35	0,35	0,35	0,35	0,35	0,35
Calcário	1,24	1,24	1,24	1,24	1,24	1,24	1,24
Fosfato bicálcico	1,6	1,6	1,6	1,6	1,6	1,6	1,6
Metionina	0,24	0,24	0,24	0,24	0,24	0,24	0,24
Premix [1]	0,3	0,3	0,3	0,3	0,3	0,3	0,3
Eucaliptol *	-	-	0,245	0,49	-	-	0,245
Eugenol *	-	-	-	-	0,24	0,48	0,24
Avilamicina	-	0,001	-	-	-	-	-
Caulim	0,65	0,649	0,405	0,16	0,41	0,17	0,165

[1] Quantidade em 100 Kg de ração: Vit. A: 1057500 UI; vit. D_3: 255375 UI; vit. K: 180 mg; vit. B_2, 450 mg; vit. B_{12}: 1200 mcg; vit. E: 787 mg; niacina: 3000 mg; ácido pantotênico: 1173 mg; Vit B_1: 201 mg; Vit B_6: 249 mg; ferro: 5589 mg; cobre: 1101 mg; zinco: 5578 mg, manganês: 7203,6 mg; iodo: 84 mg; selênio: 43,5 mg; antioxidante (BHT): 378 mg. Composição calculada: Energia metabolizável (kcal/kg): 2950; Proteína (%): 22,5; Metionina (%): 0,35; Metionina + cistina (%): 0,71; Cálcio (%): 0,95; Fósforo disponível (%): 0,45. * microcápsulas. T1 – controle, T2 – ração basal com 10 mg/kg de avilamicina, T3 - ração basal com 500 mg/kg de eucaliptol, T4 - ração basal com 1000 mg/kg de eucaliptol, T5 - ração basal com 500 mg/kg de eugenol, T6 - ração basal com 1000 mg/kg de eugenol, e T7 - ração basal com 500 mg/kg de eucaliptol e 500 mg/kg de eugenol.

Tabela 2 - Índice de atividade antioxidante (IAA) das frações eucaliptol e eugenol e dos compostos de referência.

	r^2	IC_{50} (μg/mL)	IAA
Ácido gálico	0,9992 – 1,0000	2,83 ± 0,07	27,15 ± 0,68[a]
Quercetina	0,9939 – 0,9998	4,88 ± 0,56	15,92 ± 1,76[c]
Eugenol	0,9991 – 0,9998	6,99 ± 0,32	11,02 ± 0,49[d]
Ácido clorogênico	0,9986 – 0,9995	7,44 ± 0,12	10,34 ± 0,17[d]
Ácido cafeico	0,9966 – 0,9983	8,21 ± 0,31	9,37 ± 0,35[d]
BHA	0,9969 – 0,9991	8,23 ± 0,41	9,36 ± 0,46[d]
Rutina	0,9996 – 0,9998	12,09 ± 0,70	6,38 ± 0,37[e]
Ácido ferúlico	0,9992 – 0,9998	14,45 ± 0,66	5,33 ± 0,25[e]
Eucaliptol	-	-	-

Letras diferentes correspondem diferenças significativas ($P < 0,05$).

Tabela 3 - Concentração Inibitória Mínima (CIM – mg/mL) das frações eucaliptol e eugenol, e dos antibióticos utilizados como padrões.

	Concentração Inibitória Mínima (CIM – mg/mL)				
	S. aureus	*E. coli*	*P. aeruginosa*	*S. typhimurium*	*C. perfringens*
Eucaliptol	0,600-0,800	0,400-0,600	0,400-0,600	0,600-0,800	0,800-1,000
Eugenol	0,400-0,600	0,400-0,600	0,400-0,600	0,600-0,800	0,600-0,800
Avilamicina	0,004-0,008	0,062-0,125	0,062-0,125	0,062-0,125	0,062-0,125
Halquinol	0,004-0,008	0,004-0,008	0,031-0,062	0,015-0,031	0,015-0,031
Lincomicina	*	0,031-0,062	0,031-0,062	0,031-0,062	0,031-0,062
Tiamulina	*	0,031-0,062	0,062-0,125	0,062-0,125	0,062-0,125
Tilosina	*	0,015-0,031	0,062-0,125	0,062-0,125	0,062-0,125

* < 0,0019 mg/mL.

Tabela 4 - Valores médios para, consumo de ração, ganho de peso e conversão alimentar de frangos de corte no período de 1 a 21 dias de idade

Tratamento	Peso inicial	Consumo de ração (g)	Ganho de peso (g)	Conversão alimentar (g/g)	Peso final
			1 a 10 dias		
T1	45 ± 1 [a]	228 ± 4 [a]	198 ± 4 [ab]	1,18 ± 0,05 [b]	243 ± 4 [a]
T2	45 ± 1 [a]	233 ± 9 [a]	206 ± 8 [a]	1,10 ± 0,03 [bc]	252 ± 8 [a]
T3	45 ± 1 [a]	224 ± 9 [a]	205 ± 7 [a]	1,08 ± 0,09 [bc]	251 ± 8 [a]
T4	45 ± 1 [a]	222 ± 12 [a]	201 ± 9 [a]	1,12 ± 0,08 [bc]	246 ± 9 [a]
T5	45 ± 2 [a]	229 ± 7 [a]	201 ± 8 [a]	0,99 ± 0,08 [c]	246 ± 8 [a]
T6	45 ± 1 [a]	222 ± 9 [a]	186 ± 3 [ab]	1,19 ± 0,05 [b]	232 ± 5 [b]
T7	44 ± 1 [a]	200 ± 13 [b]	142 ± 11 [c]	1,6 ± 0,14 [a]	187 ± 11 [c]
			1 a 21 dias		
T1	45 ± 1 [a]	1164 ± 38 [ab]	985 ± 30 [b]	1,18 ± 0,02 [c]	1031 ± 31 [bc]
T2	45 ± 1 [a]	1161 ± 30 [ab]	1001 ± 18 [ab]	1,16 ± 0,01 [cd]	1046 ± 19 [ab]
T3	45 ± 1 [a]	1146 ± 21 [b]	1014 ± 12 [ab]	1,13 ± 0,02 [d]	1060 ± 12 [ab]
T4	45 ± 1 [a]	1170 ± 37 [ab]	1020 ± 25 [ab]	1,15 ± 0,02 [d]	1066 ± 25 [ab]
T5	45 ± 2 [a]	1221 ± 55 [a]	1039 ± 40 [a]	1,17 ± 0,01 [c]	1085 ± 41 [a]
T6	45 ± 1 [a]	1182 ± 25 [ab]	971 ± 22 [b]	1,22 ± 0,02 [b]	1016 ± 22 [c]
T7	44 ± 1 [a]	977 ± 81 [c]	721 ± 64 [c]	1,36 ± 0,03 [a]	766 ± 65 [d]

Letras diferentes na mesma coluna correspondem diferenças significativas ($P < 0,05$). T1 – controle, T2 – ração basal com 10 mg/kg de avilamicina, T3 - ração basal com 500 mg/kg de eucaliptol, T4 - ração basal com 1000 mg/kg de eucaliptol, T5 - ração basal com 500 mg/kg de eugenol, T6 - ração basal com 1000 mg/kg de eugenol, e T7 - ração basal com 500 mg/kg de eucaliptol e 500 mg/kg de eugenol.

CONCLUSÕES GERAIS

- O índice de atividade antioxidante (IAA) proposto mostrou-se eficiente para comparar a potência antioxidante entre extratos vegetais, bem como com compostos de referência.
- Os compostos de referência que apresentaram maior ação antioxidante foram: ácido gálico > ácido protocatecuico > quercetina > eugenol = ácido clorogênico = ácido caféico = BHA > rutina > resveratrol > ácido ferúlico.
- As frações voláteis óleo essencial e extratos supercríticos de *Xanthium strumarium* apresentaram uma composição muito semelhante, entretanto, foi verificada uma diferença quantitativa dos compostos entre os extratos. Além disso, o rendimento obtido pela extração supercrítica foi até 20 vezes maior em relação à hidrodestilação.
- As frações voláteis da planta *X. strumarium* apresentaram uma fraca ação antioxidante, porém apresentaram uma forte ação antimicrobiana. Por outro lado, as frações fixas apresentaram uma forte ação antioxidante e forte ação antimicrobiana, sendo que os extratos polares (metanólicos e etanólicos) apresentaram maior ação antioxidante e maiores teores de compostos fenólicos totais em relação aos extratos apolares (acetato de etila de diclorometano/clorofórmio).
- Foi encontrado um alto grau correlação (r^2 = 0,97) entre o teor de compostos fenólicos e a atividade antioxidante dos extratos de *X. strumarium*.
- O composto tóxico carboxiatractilosideo não foi encontrado nas folhas de *X. strumarium* em estádio adulto, entretanto, foi encontrado nos cotilédones e nas sementes, sendo assim, o uso medicinal dessa planta deve ser restrito apenas as folhas na fase adulta. A presença de carboxiatractilosideo pode inviabilizar a

utilização de extratos de *X. strumarium* em formulações alimentícias, tanto para consumo humano como para consumo animal.

- Os óleos essenciais de cravo, citronela e palmarosa, bem como as frações eugenol e eucaliptol, apresentaram uma forte atividade antimicrobiana. Por outro lado, apenas o óleo de cravo e o eugenol apresentaram uma forte ação antioxidante, pois as amostras de eucaliptol e os óleos essenciais de citronela e palmarosa apresentaram uma ação antioxidante fraca.
- No óleo essencial de cravo, o eugenol foi encontrado como composto majoritário, com 83% do total, enquanto que nos óleos essenciais de citronela e palmarosa, os compostos majoritários foram o β-citronelal (45%) e o geraniol (81%), respectivamente. No óleo essencial de *X. strumarium* o composto β-guaieno foi encontrado na proporção de 79%, enquanto que nos extratos supercríticos foi encontrado menos de 1% de β-guaieno. Nos extratos supercríticos de *X. strumarium* foram encontrados dois compostos majoritários, xanthinin e um composto não identificado.
- Nos extratos fixos de *X. strumarium* foram identificados 3 compostos majoritários: ácido ferúlico, ácido clorogênico e ácido (E)-cinâmico, sendo que os extratos metanólicos e etanólicos apresentaram maiores teores desses compostos do que nos extratos obtidos com acetato de etila e diclorometano/clorofórmio.
- Os compostos eugenol e eucaliptol foram estáveis ao processo de microencapsulação utilizado.
- O uso conjunto de eugenol e eucaliptol (500 ppm de cada) afetou negativamente o desempenho das aves.

- A utilização de 500 ppm de eugenol ou de eucaliptol na ração de frangos de corte pode substituir 10 ppm de avilamicina sem comprometer o desempenho da aves no período de 1 a 21 dias.