



Isolation of syringaldehyde from *Mikania laevigata* medicinal extract and its influence on the fatty acid profile of mice

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RESUMO: “Extrato medicinal de *Mikania laevigata*: influência no perfil de ácidos graxos em camundongos e isolamento de siringaldeído por CLAE semipreparativa”. O gênero *Mikania* é popularmente conhecido como “guaco” e é utilizado para tratar febre, reumatismo, resfriados e afecções respiratórias. Em trabalho prévio demonstramos sinergismo entre os componentes do extrato de *M. laevigata* para produzir os efeitos farmacológicos esperados, incluindo a cumarina e seu precursor ácido *o*-cumárico como marcadores. Muitas espécies de *Mikania* são produtoras de diterpenos *ent*-caurenos que apresentam atividade antispasmódica e relaxante da musculatura lisa. Buscando a padronização do extrato medicinal de guaco (preparado segundo a farmacopéia brasileira 1ª edição), este trabalho visou determinar a presença de ácido caurenóico através de CLAE-DAD e isolou siringaldeído através de CLAE semipreparativa, sendo que o primeiro não foi encontrado no extrato e o siringaldeído é um dos seus componentes majoritários. Camundongos isogênicos Balb-C portadores de pneumonite alérgica foram tratados com este extrato, e amostras de pulmão e fígado foram analisadas por CG-DIC quanto ao seu conteúdo de ácidos graxos. A quantidade de ácido araquidônico (ARA) e de ácido docosahexaenóico (DHA) encontrada demonstrou que a composição é distinta em ambos tecidos, e apenas a concentração de DHA hepático foi alterado em função do tratamento, o qual não foi encontrado no pulmão. Não foi detectada diferença significativa na produção de ARA. Tanto o extrato aquoso, quanto a cumarina e o ácido *o*-cumárico, estimularam a síntese de DHA no fígado ($p < 0.05$).

Unitermos: *Mikania laevigata*, Asteraceae, ácido caurenóico, siringaldeído, ácido araquidônico, ácido docosahexaenóico.

ABSTRACT: The *Mikania* genus is widely known as “guaco” and is used to treat fever, rheumatism, flu and respiratory diseases. Our previous work evidenced the synergism among *M. laevigata* extract components to produce desirable effects, and included the coumarin precursor, *o*-coumaric acid as marker. Many *Mikania* species are producers of *ent*-kaurene diterpenes which presented antispasmodic and relaxant activities on smooth muscle. Seeking to standardize the *guaco* extract, which is registered in the Brazilian Pharmacopoea, this paper deals with the determination of kaurenoic acid through LC-PDA and the isolation through LC of syringaldehyde. Kaurenoic acid was not found in the extract, and syringaldehyde is one of the major compounds of pharmacopoeal extract, together with coumarin and *o*-coumaric acid. Samples from the lung and liver of Balb-C isogenic allergic pneumonitis bearing mice, treated with the same extract, were analyzed through GC-FID, and the fatty acid content was determined and analyzed. The results obtained by measuring the arachidonic acid (ARA) and docosahexaenoic acid (DHA) in the liver and lung of treated animals demonstrated that the fatty acid composition is distinct in both tissues, and that in the liver, only the DHA was altered as a result of the treatments. DHA is absent in the lung and in both organs, no significant difference in ARA production was observed. The aqueous extract, coumarin and *o*-coumaric acid stimulated DHA synthesis in the liver ($p < 0.05$).

Keywords: *Mikania laevigata*, Asteraceae, kaurenoic acid, syringaldehyde, arachidonic acid, docosahexaenoic acid.

INTRODUCTION

Asteraceae is one of the largest Angiospermae families with *ca* 1300 genera distributed in 3 subfamilies and 17 tribes. Eupatorieae includes *ca* 2400 species and the subtribe Mikaniinae contains only the *Mikania* genus, a species which is neotropical and widely known as “guaco”, and is used to treat fever, rheumatism, flu and respiratory diseases (Agra et al., 2007; Soares et al., 2006; Silva et al., 2006). Less than 10% of 430 species of *Mikania* has been investigated (Nunez et al., 2004). With morphology, organoleptic characteristics and analogous medicinal uses, *M. glomerata* and *M. laevigata* are the most widely studied. The main difference between them is the blooming season, which occurs in July for *M. glomerata* and in September for *M. laevigata*. Also, the leaves of *M. laevigata* tend to present more prominent lobes than those of *M. glomerata* (Moraes, 1997). *M. glomerata* grows in Brazil, Argentina, Paraguay and Uruguay and *M. laevigata* grows closer to the coast, from São Paulo to Rio Grande do Sul, and is also commonly cultivated.

Many authors attribute the pharmacological effect of “guaco” to the coumarin (1,2-benzopyrone), but is also clear from the pharmacological experiments that this is not the only bioactive component of the tested extracts (Aboy et al., 2002, Leite et al., 1993, Fierro et al., 1999, Soares de Moura et al., 2002, Suyenaga et al., 2002). Our previous work on experimental allergic pneumonitis also demonstrated a synergism among “guaco” medicinal extract components to produce the desirable pharmacological effect, and also included the coumarin precursor, *o*-coumaric acid, as marker (Santos et al., 2006).

Many *Mikania* species are producers of *ent*-kaurene diterpenes: *M. lindbergii*, *M. laevigata*, *M. oblongifolia*, *M. sessilifolia* (Fabbri et al., 1997), *M. glomerata* (Veneziani and Oliveira, 1999; Taleb-Contini et al., 2006), *M. hirsutissima* (Ohkoshi et al., 2004), *M. hookeriana* (Reis et al., 2003), *M. banisteriae* (Lobitz et al., 1997), *M. stipulata* (Nascimento and Oliveira, 2001), *M. officinallis*, *M. luetzelburgii*, *M. belemii* and *M. malacolepsis* (Rodrigues et al., 1996). Kaurene diterpenes presented antiespasmic and relaxant activities on smooth muscle. Kaurenoic acid (*ent*-kaur-16-en-19-oic acid) and its derivatives are potent uterine muscle relaxants, through β_2 adrenergic receptor independent mechanisms, probably antagonist of the calcium channel (Tirapelli et al., 2004). *In vitro*, PMN and lymphocyte proliferative activities are reported (Ohkoshi et al., 2004), as well as antimicrobial and antifungal activity (Yatsuda et al., 2005, Kuate et al., 2006), and non-specific cytotoxicity (Costa-Lotufo et al., 2002). Kaurenoic acid is considered a universal marker of Copaiba oil, but could not be found in every harvest (Biavatti et al., 2006).

Coumarin and kaurenoic acid were analyzed

together, through capillary gas chromatography in *M. glomerata*, extracted exhaustively with hexane (Vilegas et al., 1997) and also through exhaustive maceration in ethanol-water (7:3), these being the main compounds (Yatsuda et al., 2005).

Bronchial asthma, defined as a chronic inflammatory pathology, affects about 10% of the population in developed countries. This complex disease is characterized by recurrent episodes of several symptoms such as breathlessness, wheezing, tightness of the chest, obstruction of the airways obstruction and coughing. Essential alterations of asthma are bronchospasm (hyperreactivity), oedema and mucous hypersecretion (bronchial obstruction). Many cell types, including inflammatory cells (T lymphocytes, eosinophils, and activated mast cells) and structural cells (epithelial cells, smooth muscle cells, endothelial cells) play a key role in this disease. These few cells release more than 50 different inflammatory mediators responsible for the clinical and pathologic events in asthma. Among these mediators, autacoids, histamine, bradykinine and lipid-derived mediators such as prostaglandins (D2, F2 α , I2), TXA2 and cysteinyl leukotrienes (C4, D4, E4), contribute powerfully to the regulation of the airflow by constricting bronchial smooth muscles, increasing mucous secretion and microvascular leakage, and acting as chemoattractant factors for inflammatory cells. It is well accepted that arachidonic acid metabolites (prostanoids and isoprostanes) play a key role in the pathophysiology of diseases associated with inflammation, platelet aggregation and vasoconstriction/relaxation. Consequently, the inhibition of their production and/or the blockade of their respective receptors have greatly contributed to the understanding of their mode of action and their involvement in the pathology, and have also demonstrated clinical effectiveness in bronchial asthmas (Rolin et al., 2006).

Linolenic acid (Omega-3) is the precursor for the long chain polyunsaturated fatty acid docosahexaenoic (DHA) and linoleic (Omega-6) acid is the precursor of arachidonic acid (ARA). Omega-3 plays a protective role in the development of inflammatory and cardiovascular diseases, by competitively inhibiting the biosynthesis of leukotrienes and other pro-inflammatory eicosanoids (Wong, 2005).

Seeking to standardize guaco extract, which is officially registered in the Brazilian Pharmacopoea I (Brandão et al., 2006), this paper deals with the determination of kaurenoic acid through LC-PDA and the isolation through semi-preparative LC of syringaldehyde. Samples from lung and liver of Balb-C isogenic allergic pneumonitis bearing mice, treated with the same extract, were analyzed through GC-FID, and the fatty acid content was determined and analyzed, leading to some conclusions about the mechanism of action of “guaco” in asthma.

MATERIAL AND METHODS

Plant material

Leaves of cultivated specimens obtained from vegetative propagation of authentic *M. laevigata* Sch ex Baker (identified by Pedro M. Magalhães, Universidade Estadual de Campinas, Campinas, São Paulo, Brazil) were collected in Itajaí (Santa Catarina, Brazil). Voucher specimens of *M. laevigata* are deposited at the Herbarium Barbosa Rodrigues [HBR Biavatti 30 (30/08/01)] Itajaí, Santa Catarina, Brazil

Extract and samples

The leaves were air dried (40° C, forced ventilation), powdered and sieved. Only particles between 0.85 - 1.4 mm were used to prepare the extracts. Hydroalcoholic extracts were prepared by percolation with ethanol-water 1:2 (v/v) and concentrated to obtain a 1:2 (w/v) extract. For the LC analytical injections of the hydroalcoholic extract, the extract was diluted in ultra pure water 1:5.

For the semi preparative injections, the hydroalcoholic extract was reduced to 1:1 (w/v) in a rotary evaporator, then partitioned with *n*-butanol, and injections of 2.5 mg were performed. A lyophilized aqueous extract (1%, prepared by infusion of the leaves) was used to *in vivo* tests.

LC analysis

The HPLC system consisted of a Waters 600 pump and 2996 PDA detector, a manual injector Rheodyne 7725i, *in line* Degasser AF and software Millennium Empower. The analytical injections (loop 20 µL) were carried out on a RPC18 Nova-Pak Waters (150 x 3.9 mm, 4 µM), and the semi preparative injections (loop 500 µL) on a RPC18 Nova-Pak Waters (300 x 7.8 mm, 6 µM). The mobile phase for the analytical conditions was MeOH-H₂O 0.01% acetic acid (30:70 v/v), flow rate 1.5 mL/min, the analysis was monitored at 200 nm, and the column oven fit to 30 °C. The mobile phase for the semi-preparative analysis was MeOH 2% formic acid-H₂O 2% formic acid (30:70 v/v), flow rate 6 mL/min, the analysis was monitored at 225 nm, and the column oven fit to 30 °C.

All the solvents were HPLC grade, and were degassed using an ultrasonic bath before use. The water was purified using a Milli-Q system (Millipore). All the solutions were filtered through 0.45 µM membranes (Schleicher & Schuell, Germany).

The kaurenoic acid was kindly supplied by Ângela Malheiros, UNIVALI.

NMR analysis

A Bruker AVANCE DRX 400 was used to record mono and bidimensional spectra.

Syringaldehyde or *4-hydroxy-3,5-dimethoxybenzaldehyde*: ¹H NMR (400 MHz, CDCl₃, TMS) δ 9.83 s (1H), 7.16 s (2H) and 3.98 s (6H). ¹³C NMR (100 MHz, CDCl₃, TMS) δ 190.6, 147.5, 141.1, 128.6 and 106.9.

Pharmacological analysis

To determine the influence of *M. laevigata* aqueous and hydroalcoholic extracts, coumarin and *o*-coumaric acid on the fatty acid profile of mice, a previously developed model of allergic pneumonitis was used (Landgraf et al., 2004).

Animals

Male BALB/c mice weighing 28-32 g, 6-8 weeks old, from our own animal facilities, were housed in a room with a 12 h light-dark cycle (19-25 °C) and water and food *ad libitum*. Animal care and research protocols were used, in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Committee for Animal Research (UNIVALI).

Induction of the allergic pneumonitis

The mice were sensitized on days 0 and 7, by intraperitoneal injection of a mixture containing 50 µg of ovalbumin and 1 mg of Al₂O₃ in saline (a total volume of 0.6 mL). At 14 and 21 days after the first immunization, the animals were challenged by exposure to an ovalbumin aerosol (50:50 grade II and III, Sigma) generated by a nebulizer (INALAR, SC, Brazil) delivering particles of 0.5 - 10 µM in diameter at approximately 0.75 cc/min for 20 min. The concentration of ovalbumin in the nebulizer was 2.5% w/v. The control group consisted of animals immunized as described and challenged with saline solution.

Treatments

By gavages, the animals received the following treatment once a day (twice a day on days 14, 15 and 16) for 21 consecutive days: Group 1 (n = 7): negative control (saline). Group 2 (n = 6): positive control (saline). Group 3 (n = 8): aqueous lyophilized extract of *M. laevigata* (63.92 g/kg). Group 4 (n = 8): hydroalcoholic extract 1:2 of *M. laevigata* (35.9 µg/kg). Group 5 (n = 8): *o*-coumaric acid (0.42 mg/kg). Group 6 (n = 9): coumarin (1.10 mg/kg). Group 7 (n = 6): coumarin + *o*-coumaric (1.52 mg/kg). Doses were selected according to the traditional doses of extracts popularly indicated, and extrapolated to the amount of markers in the extracts (Santos et al., 2006).

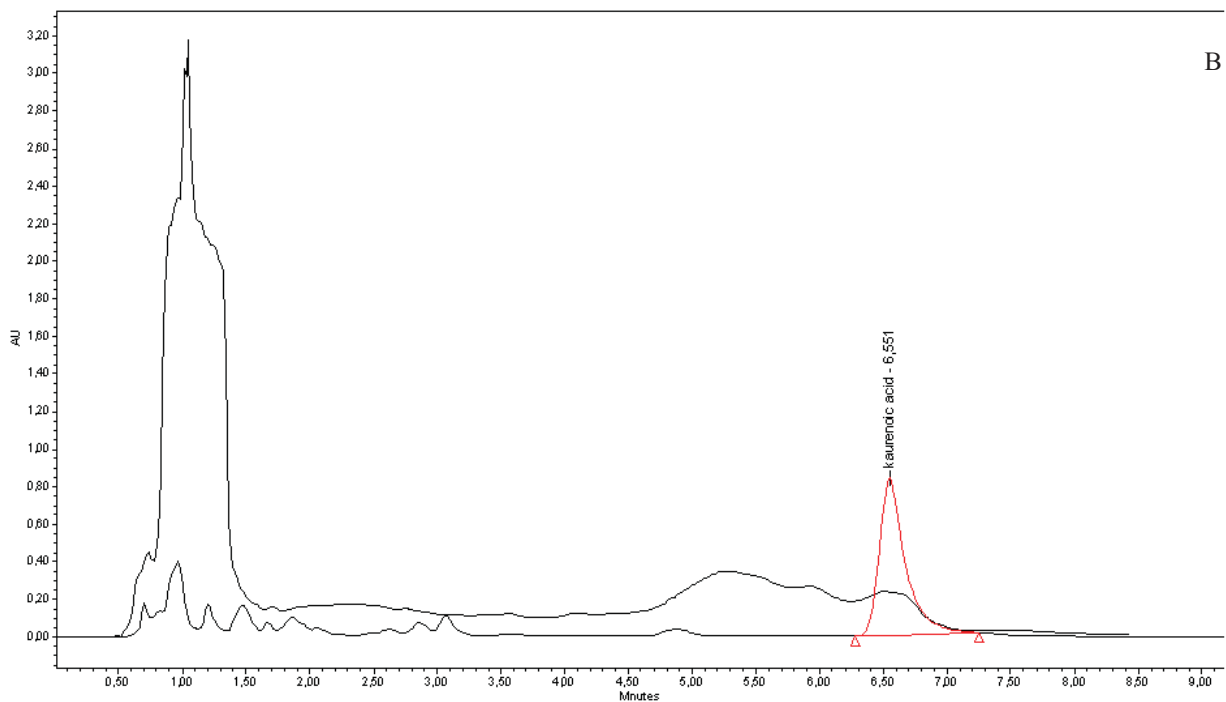
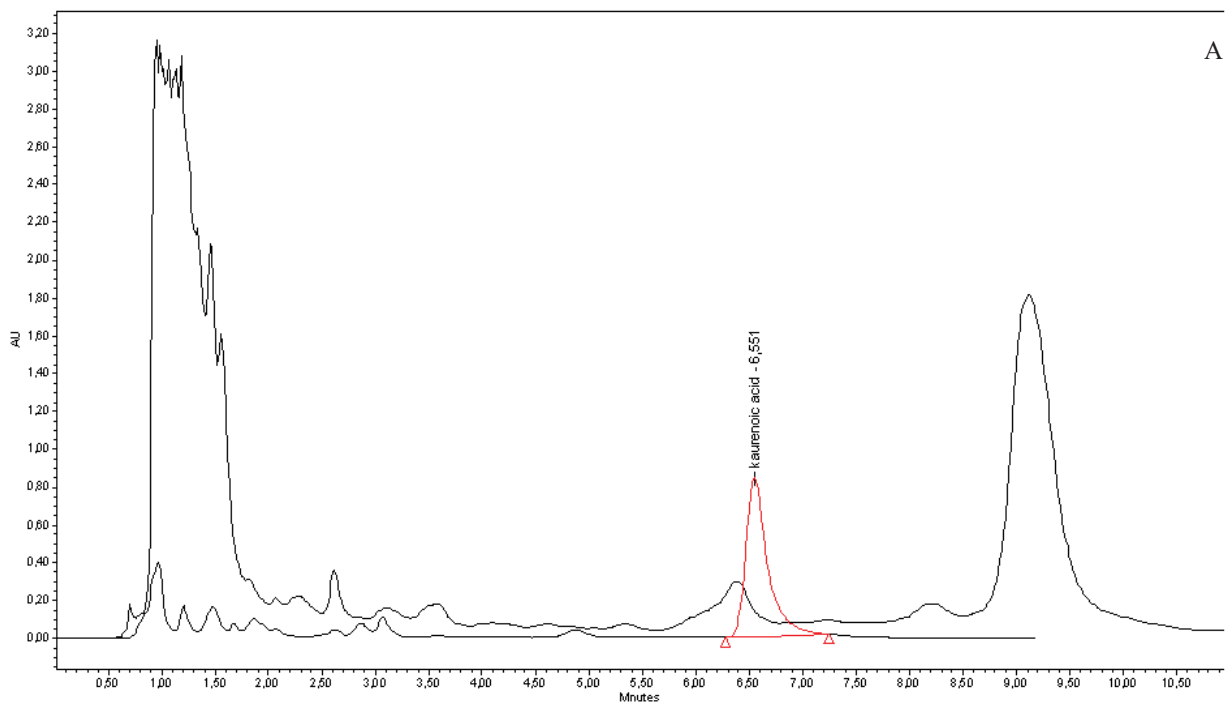


Figure 1. A - Hydroalcoholic extract of *M. laevigata* and kaurenoic acid (standard). B - Dichlorometane extract of *M. laevigata* and kaurenoic acid (standard). For chromatographic conditions, see experimental.

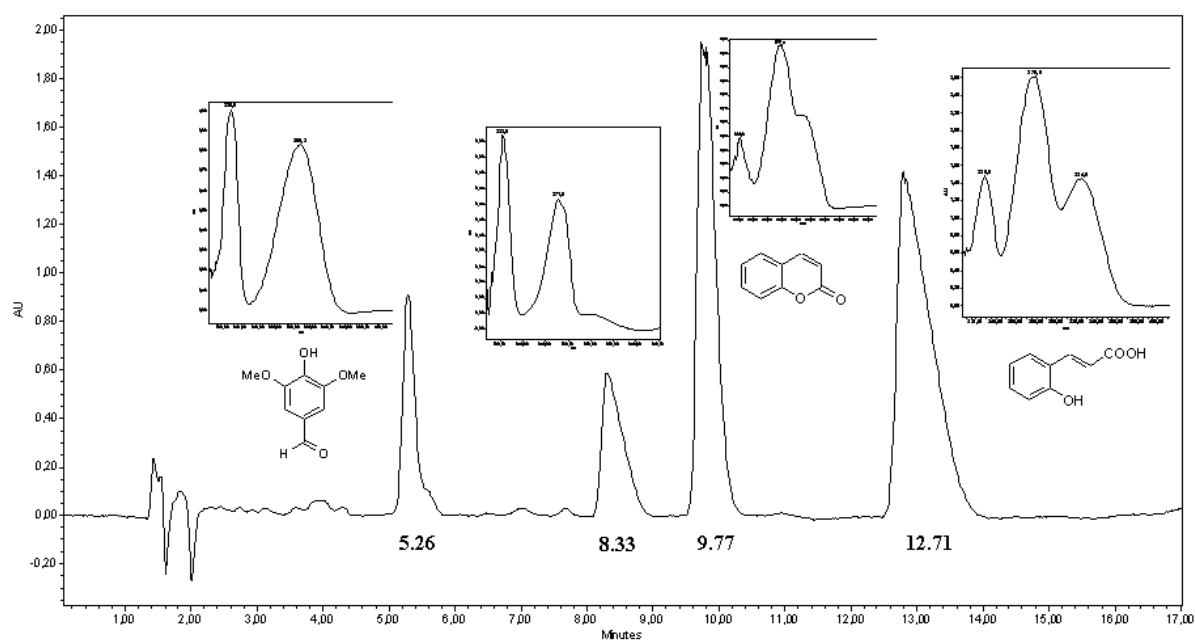


Figure 2. Semi-preparative analysis of *guaco* medicinal extract. For chromatographic conditions, see experimental.

Table 1. Influence of the treatments (aqueous, hydroalcoholic extracts and markers) on the fatty acid profile in mice lung and liver. Mean followed by different letters differ from the Duncan test by 5% of error probability.

Tissue	Treatment	Palmitic	Estearic	Saturated	Oleic	Linoleic	ARA	DHA	Unsaturated
Lung	Negative Control	32.92	15.20 ^d	32.92	24.46 ^a	20.55 ^a	5.92	.	5.92
	Positive Control	34.40	24.04 ^{bc}	34.40	18.49 ^{bc}	14.90 ^b	8.16	.	8.16
	Aqueous extract	34.20	35.96 ^a	34.20	12.86 ^d	9.82 ^d	7.14	.	7.14
	Hydroalcoholic ext	34.38	27.48 ^b	34.38	17.36 ^c	13.08 ^{cd}	7.69	.	7.69
	<i>O</i> -coumaric acid	34.04	20.20 ^c	34.04	21.56 ^{ab}	17.82 ^{ab}	6.37	.	6.37
	Coumarin	33.33	29.98 ^b	33.33	15.39 ^{cd}	13.51 ^c	7.76	.	7.76
	Coumarin + acid	33.28	28.87 ^b	33.28	17.13 ^c	13.25 ^{cd}	7.45	.	7.45
Liver	Negative Control	26.33	20.57	46.89	10.92	18.60	15.53	8.05 ^{ab}	45.05
	Positive Control	26.61	20.75	47.37	11.90	19.19	14.55	6.99 ^b	45.64
	Aqueous extract	26.23	20.01	46.24	10.15	18.81	16.23	8.56 ^a	45.19
	Hydroalcoholic ext	26.05	19.57	45.62	11.15	19.47	15.72	8.04 ^{ab}	46.34
	<i>O</i> -coumaric acid	26.01	21.15	47.16	9.45	18.12	16.98	8.28 ^a	44.55
	Coumarin	25.41	19.68	45.10	9.64	18.75	17.60	8.91 ^a	45.99
	Coumarin + acid	26.20	22.94	49.14	9.40	17.02	16.45	7.98 ^{ab}	42.87

Biochemical analysis

The right lung (200 mg) and liver (300 mg) were removed and the total lipids were extracted using the Bligh and Dyer (1959) method. To determine the fatty acids in the lipids, they were converted to methyl esters by adding KOH 0.1 M in MeOH and kept at 60 °C/1 h. After cooling, H₂SO₄ 1 M in MeOH was added

and it was warmed again to 60 °C/1 h, cooled, and extract with *n*-hexane and filtered samples injected (1 µL) on GC-FID, using a Shimadzu GC-17A, equipped with automatic injector AOC20I. Helium was used as the carrier gas (17 mL/min), and nitrogen, hydrogen and synthetic air with 30, 30 and 300 mL/min. The temperature program was an isotherm 140 °C (5 min) followed by 4 °C/min until 240 °C, kept 10 min. Injector

and detector temperatures were 240 °C and 260 °C, respectively, and the capillary column used was SP2340 [100% poly (bis-cyanopropyl-siloxane) - 60 m x 0.25 mm id x 0.2 µM], from Supelco.

The fatty acids were identified by comparing the retention time with the standards (Supelco 37 Components FAME Mix) and the percent of every component in the sample was determined in relation to the total area of identified peaks.

Statistical analysis

The Duncan's statistical test ($p < 0.05$) was used to contrast the averages of the treatments.

RESULTS AND DISCUSSION

The chromatographic profile of the hydroalcoholic extract 1:2 can be seen in Figure 1A. Compared with the standard kaurenoic acid, any corresponding peak can be recognized in the extract, by the detector employed. The PDA detector is not effective for detecting compounds presenting few chromophores. To check whether the absence of kaurenoic acid was due to the extraction procedure, an extract with dichloromethane was prepared and injected under the same conditions (Figure 1B). Any free kaurenoic acid is present in the medicinal extract of "guaco". This diterpene and others are reported as antimicrobial, but Barbosa et al. (1994) using a "guaco" extract produced using the same method, did not find any antimicrobial activity. On the other hand, hexane fraction (with kaurenoic acid as a major compound) of ethanol extract of *M. laevigata* and *M. glomerata*, were active against *Streptococcus mutans* (Yatsuda et al., 2005). The glycosilated form of kaurenoic acid was found in other species of *Mikania* (Ohkoshi et al., 2004).

From the semipreparative analysis of the *n*-butanol fraction of the medicinal extract, four well-defined peaks were found (F1-F4, Figure 2). The two last correspond to coumarin and *o*-coumaric acid. The two first peaks present a phenol profile, F1 being identified as syringaldehyde through NMR mono and bidimensional analysis and also by direct comparison with the respective literature data (SBDS database). The F2 peak was not identified, but according to the UV spectrum, a furofuran lignan derivative can be expected (Cuenca et al., 1991).

As a general rule, syringaldehyde and vanillin are the predominant compounds in aged alcoholic beverages due to the lignin hydrolysis, which is the major chemical process that occurs during aging in wooden barrels. This is the process from which several phenolic compounds are extracted and affect the final organoleptic characteristic of the beverage (Aquino et al., 2006). Is not commonly isolated from the leaves, and is isolated from *Mikania* (Sharp et al., 2001) for the

first time here. Little research has been conducted in order to verify its bioactivity, and only weak antioxidant activity has previously been found (Bortolomeazzi et al., 2007).

The results obtained by measuring the ARA and DHA in the liver and lung of treated animals demonstrated that the fatty acid composition is distinct in both tissues, while in the liver, only the DHA was altered as a result of the treatments. DHA is absent in the lung and in both organs, no significant difference in ARA production was observed (Table 1). The aqueous extract, coumarin and *o*-coumaric acid stimulated DHA synthesis in the liver ($p < 0.05$). The presence of inflammation enhanced the stearic acid concentration in the lungs, perhaps to reinforce the energy supply in the cells or to stabilize the membranes due to the increased temperature, or to reduce the unsaturated proportion to decrease the possibility of oxidation due to the enhanced oxidation in the lung environment. It seems that effects of the coumarin are prevalent in comparison with the *o*-coumaric acid, since it did not alter the fatty acid profile.

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