Vol.56, n.2: pp. 283-292, March-April 2013
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Chemical Composition and Antibacterial Activity of *Aloysia triphylla* (L'Hérit) Britton Extracts Obtained by Pressurized CO_2 Extraction

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ABSTRACT

This study investigated the chemical composition of five different extracts of Aloysia triphylla *and their activity against* Aeromonas *sp. The extracts were obtained from the dried leaves by pressurized CO_2 extraction at 30, 50 and 70ºC, and 100, 150, and 200 bar, and analyzed by GC/FID and GC-MS. The antibacterial activity was assayed by the microdilution method. The tested microorganisms comprised seven* Aeromonas *isolates obtained from the kidney of infected silver catfish,* Rhamdia quelen*. The yield, chemical composition and antibacterial activity of the extracts were dependent on the extraction conditions. Mono and sesquiterpenoids were the major constituents of all the extracts and the highest extraction yield was obtained at 70ºC and 200 bar.* A. triphylla *presented moderate antibacterial activity against* Aeromonas *sp.*

Key words: *Aloysia triphylla*, pressurized carbon dioxide extraction, *Aeromonas* sp.

INTRODUCTION

Aloysia triphylla (L'Herit.) Britton [syn. *Aloysia citriodora* Palau, *Lippia citriodora* (Ort.) HBK, Verbenaceae] is cultivated mainly due to the lemon-like scent emitted from its leaves and is utilized for the preparation of herbal tea (Argyropoulou et al. 2007) which is known to have antispasmodic, antipyretic, sedative, and digestive activity (Carnat et al. 1999; Valentão et al. 1999; Pascual et al. 2001; Gomes et al. 2005), antimicrobial activity (Oskay et al. 2005) and antioxidant properties (Pereira et al. 2007). This plant has a long history of folk use to treat asthma, spasms, cold, fever, flatulence, colic, diarrhea, indigestion, insomnia, and anxiety (Carnat et al. 1999; Pascual et al. 2001; Gomes et al. 2005). The supply of *Aloysia triphylla* is from the Mediterranean countries such as Morocco, Portugal, Italy, and France, as well as Southeastern Asian countries. Some Latin American countries such as Chile, Columbia, Uruguay and Brazil also produce and supply the leaves of *A. triphylla* (Bandoni et al. 2008).

The conventional processes for extracting the terpenoids such as hydrodistillation and solvent extraction are usually inferior to supercritical fluid extraction (SFE), mainly regarding selectivity. It is

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well known that the SFE process makes use of mild temperatures, which avoids the degradation of thermo-sensitive compounds, and the low water content limits hydrolytic processes (Crabas et al. 2003; Wenqiang et al. 2007). This extraction method offers many important advantages over the hydrodistillation such as low energy cost and the possibility of extraction optimization by changing the processing parameters such as temperature, content of modifier (co-solvent), dynamic extraction time and fine tuning the extraction pressure (Khajeh et al. 2004). Moreover, the use of carbon dioxide brings the additional advantages of being non-flammable, fairly non-toxic, cost-effective, easily removed from the extract following decompression (Scalia et al. 1999) and, to a certain extent, solubilizes the lipophilic substances (Sahena et al. 2009).

The genus *Aeromonas* comprises a group of Gram-negative, facultative anaerobic bacteria that are pathogenic to aquatic and terrestrial animals and have also been associated with a wide spectrum of infectious diseases in humans and animals (Paniagua et al. 1999; Krzyminska et al. 2009). The widespread distribution of *Aeromonas* species in the aquatic environment combined with the stress induced by intensive culture practices predisposes fish to infections (Saavedra et al. 2004). *Aeromonas* infection is a significant concern in aquaculture as it is often associated with high mortality and morbidity rates (Clermont et al. 2008).

In this context, the aim of this work was to investigate the influence of temperature and pressure on the extraction yield and chemical composition of the extracts obtained by the pressurized fluid extraction of *A. triphylla* leaves. In order to determine the best parameters for plant material extraction in terms of activity optimization, the antibacterial activity of the extracts against *Aeromonas* sp was also evaluated.

MATERIALS AND METHODS

The leaves of *Aloysia triphylla* were purchased from the local market of Santa Maria (RS, Brazil) and identified by Dr. Gilberto Dolejal Zanetti (Department of Industrial Pharmacy, UFSM). A voucher specimen (SMDB No. 11169) was deposited in the herbarium of the Department of Biology, UFSM. Leaves were dried at 40ºC in a ventilated drying oven and subsequently powdered in a knife mill, homogenized and the particles classified in a sieve (Taylor series), excluding the particles smaller than 200 mesh, then stored in light-protected plastic bags at - 4^{o}C in a domestic freezer.

The experiments were performed in a laboratory scale unit (Mazutti et al. 2006). Basically, it consisted of of a CO_2 (99.9% purity, purchased from White Martins) reservoir, two thermostatic baths, a syringe pump (ISCO 260D), a 0.1dm^3 jacketed extraction vessel, an absolute pressure transducer (Smar, LD301) equipped with a portable programmer (Smar, HT 201) with a precision of $\pm$ 0.12 bar, a collector vessel with a glass tube and a cold trap. About 25 g of dried and powdered leaves of *A. triphylla* were loaded into the extraction vessel. The CO_2 was pumped into the bed at a constant flow rate of 2 g min^{-1}, which was supported by two 300 mesh wire disks at both ends, and was kept in contact with the herbaceous matrix for at least one hour to allow the system to stabilize. Afterwards, the extract was collected by opening the micrometering valve and the CO_2 mass flow was recorded by the pump recordings. Then the extract was weighed, the glass tube was re-connected to the equipment, and this procedure was repeated until no significant mass was extracted. The extraction was completed within approximately 120 min, isothermally at desired pressure and temperature. An experimental factorial design using three temperatures (30, 50 and 70ºC) and three pressure levels (100, 150 and 200 bar) was established so as to assess the influence of process variables on the extraction yield, extract composition and antimicrobial activity.

GC-FID and TIC analysis was performed on a Varian gas chromatograph (Model CP-3800) equipped with mass detector Saturno using a capillary column fused silica VF-5 MS (Varian) (30 m x 0.25 mm x 0.25 µm). The conditions of analysis included: injector 1177 (MS), 250ºC, split 1:20 (FID and MS); injetor 1093 (FID), 250ºC. The flow of the carrier gas (He) was 1 mL/min (both FID and MS). The column oven temperature was initially kept at 50ºC for 4 min and then heated up to 280°C (increasing rate 4ºC/min). Injection volume was 1 µL and the detector temperatures were 200ºC (MS) and 310ºC (FID). The GC-MS analysis was performed with eletronionization (70 eV).

The constituents of the extracts were identified based on the retention index (RI) determined by

using a calibration curve of a homologous series of n-alkanes (C_8-C_{32}) injected under the same chromatographic conditions of the samples and fragmentation models of mass spectra. Both data were compared with the literature (Adams, 2001) and with the equipment's library (NIST, 1998). The quantitative data were obtained from the FID eletronic integration peak areas.

The tested microorganisms comprised seven *Aeromonas* sp. isolates obtained from the kidney of infected silver catfish, *R. quelen*. The isolates were phenotypically identified by the conventional methods to genus according to Quinn et al. (1994).

Inoculum suspensions were prepared as described in CLSI M7-A6 protocol (NCCLS, 2003) by diluting the scraped cell mass in 0.85% NaCl solution adjusted up to 0.5 in the McFarland scale and confirmed by spectrophotometrical reading at 580 nm. The cell suspensions were finally diluted to 5 x 10^4 UFC mL^{-1} to be used in the activity assays in microdilution plates, where each hole contained 200 µL Mueller-Hinton broth and different concentrations of the test extracts. Mueller-Hinton broth, methanol and bacterial inoculum were used as negative control. For positive control, Mueller-Hinton broth and bacterial inoculum were added. The plates were incubated at 37ºC for 24 h.

The different extracts were dissolved in methanol at the ratio of 1:2. The final extracts concentrations in the plates were: 25000; 12500; 6250; 3125; 1562.5; 781.25; 390.62; 195.31µg mL^{-1}. The tests were performed in duplicate. After the incubation period, all the plates containing Mueller-Hinton broth were replicated and incubated at 37ºC for 24 h to evaluate the Minimum Bactericidal Concentration (MBC). Minimum Inhibitory Concentrations (MIC) were not determined due the difficulty of solubilizing the extract in the broth.

Data are reported as the mean values ± standard deviations (σ). The homogeneity of variances among the groups was tested with the Levene test. As the data did not present homogeneous variances, groups were compared by Kruskal-Wallis test, followed by Mann-Whitney post hoc tests. All the tests were performed with Statistica 5.1 (1997; StatSoft Inc., Tulsa, OK, USA). The minimum significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

The semisolid extracts obtained by the pressurized CO_2 extraction were light or dark yellow in color, similar to the extracts of *Ocimum basilicum* L. obtained by the supercritical carbon dioxide extraction (Lachowicz et al. 1997). Figure 1 shows the extraction kinetics for all the experimental conditions. As commonly found for SFE of vegetable matrices, extraction kinetic curves were characterized by a linear portion followed by the decreasing and nearly zero extraction rates (Fig. 1). The cumulative yield of the extracts increased up to 100-120 min, except at 70°C and 100 bar. As seen from Table 1, the yield at this experimental condition was significantly lower than those obtained under the other experimental conditions. Here, the extraction yield is defined as the weight percentage of the extract in relation to the initial load of the raw material in the extractor (Table 1).

According to Table 1 and Figure 1, the highest extraction yield was obtained at 70°C and 200 bar, thus corroborating the positive influence of higher system pressures.

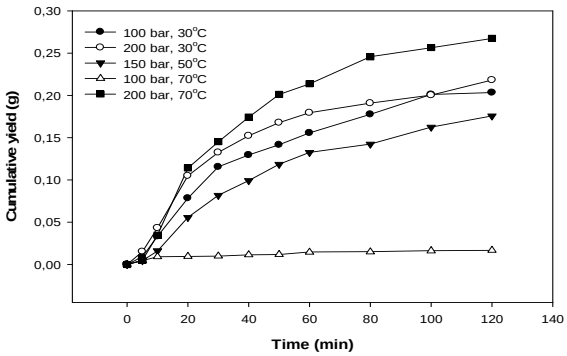

Figure 1 - Relationship between cumulative yields of the extracts obtained from leaves of *Aloysia triphylla* by supercritical fluid at different temperatures and pressures.

Table 1 - Extraction yield and antimicrobial activity against *Aeromonas* sp. of the supercritical fluid extracts obtained from leaves of *Aloysia triphylla*.

Temperature (°C)	Pressure (bar)	Yield* ± σ (wt%)	Minimum bactericidal concentration (μg mL^{-1})
30	100	0.8 ± 0.007^{A}	1339.0^{A}
70	100	0.07 ± 0.002^{B}	4464.0^{B}
50	150	0.73 ± 0.04^{A}	3125.0^{C}
30	200	0.91 ± 0.05^{A}	1674.0^{AC}
70	200	1.13 ± 0.03^{C}	1562.0^{AC}

Means identified by different capital letters in columns were significantly different ($p < 0.05$) as determined by Kruskal-Wallis and Mann-Whitney tests. * mean ± σ, n=2

These cause an enhancement of solvent power, and system temperature, affecting the vapor pressure of the solute to be extracted (Khajeh et al. 2004). This result was in agreement with the best extraction yield obtained by the supercritical CO_2 extraction of *O. basilicum* L., in which the highest yield was obtained at the highest temperature (50°C) and pressure (250 bar) (Mazutti et al. 2006). In the present study, at the highest temperature chosen, 70°C, the pressure was really determinant for the extraction yield. Hence, the extract obtained at 100 bar showed the smallest yield, while the extract obtained at 200 bar presented the highest cumulative yield.

It is well known that supercritical fluid extraction recovery and selectivity depends greatly on the balance between the fluid density and solute vapor pressure. For instance, the extracts obtained at 30°C and 100 bar (Table 2), 30°C and 200 bar (Table 3), 50°C and 150 bar (Table 4), and 70°C and 200 bar (Table 5) contained mainly monoterpenoids (C_{10} compounds), which are lipophilic compounds with low molecular weight, followed by sesquiterpenoids (C_{15} compounds).

Table 2 - Chemical composition of major compounds present in the supercritical fluid extracts from leaves of *A. triphylla* obtained at 30°C and 100 bar.

Compound	RT	RC	RTIR	%
Limonene	9.532	1036	1029	0.7
Tagetone	10.045	1052	1053	1.5
Linalool	11.577	1098	1097	1.2
Z-Citral	16.566	1244	1238	13.6
E-Citral	17.590	1363	1362	19.3
Z-Citral dimethoxy	19.106	1320	1318	0.8
Citronellyl acetate	20.055	1349	1353	2.5
Neryl acetate	20.463	1362	1362	0.9
Geranyl acetate	21.105	1381	1381	3.1
Total of monoterpenoids				43.6
E-β-Caryophyllene	22.462	1424	1427	5.6
α-Curcumene	24.363	1485	1481	11.0
γ-Cadinene	25.383	1519	1514	1.2
α-cadinene	26.086	1543	1539	0.8
Z-Muurol-5-en-4-β-ol	26.502	1557	1552	1.2
Caryophyllene oxide	27.470	1590	1581	14.3
9-epi-*E*-Caryophyllene-14-ol	29.732	1671	1670	0.7
Caryophyllene acetate	30.338	1692	1701	1.9
Acorenone B	30.553	1700	1698	1.5
2*E*, 6*E* Farnesyl acetate	34.152	1837	1847	2.1
8*S*-13-Cedranediol	35.247	1881	1880	2.1
Total of sesquiterpenoids				42.4
n-Octanol	10.236	1058	1068	0.7
Methyl butyl 2-methyl butanoate	11.773	1104	1100	0.7
Cinerolone	22.188	1415	1426	1.0
Total of constituents with no terpenoid structure				2.4
Total percentage of identified components				88.4

RT= retention time; RC= retention index calculated; RTIR= retention index reference; % = relative percentage

Table 3 - Chemical composition of major compounds in the supercritical fluid extracts from leaves of *A. triphylla* obtained at 30°C and 200 bar.

Compound	RT	RC	RTIR	%
Limonene	9.336	1031	1029	86.0
Total of monoterpenoids				86.0
Total percentage of identified components				86.0

RT= retention time; RC= retention index calculated; RTIR= retention index reference; % = relative percentage

Table 4 - Chemical composition of major compounds present in the supercritical fluid extracts from leaves of *A. triphylla* obtained at 50°C and 150 bar.

Compound	RT	RC	RTIR	%
Z-Citral	16.545	1244	1238	12.6
E-Citral	17.571	1274	1282	16.3
Geranyl acetate	21.091	1381	1380	2.7
Total of monoterpenoids				29.1
β-Caryophyllene	22.436	1424	1418	6.5
α-Caryophyllene	23.580	1460	1451	1.8
Aromadendrene	23.709	1464	1460	0.9
Germacrene D	24.163	1479	1474	2.4
α-Curcumene	24.340	1484	1481	1.2
τ-Elemene	24.810	1500	1482	1.8
β-Himachalene	25.179	1512	1494	1.1
τ-Cadinene	25.357	1518	1514	0.8
β-Cadinene	25.483	1522	1519	0.9
α-Muurolene	26.060	1542	1537	1.6
Isoaromadendrene epoxide	27.248	1582	1579	1.0
Total of sesquiterpenoids				26.0
Squalene	54.399	2929	2914	11.4
Total of triterpenoids				11.4
Octadecyne	34.133	1836	1828	3.0
2-Methyl-7-octadecyne	35.255	1881	1863	1.4
Linoleic acid, ethyl ester	41.976	2169	2166	2.1
Heptacosanol	57.088	2938	2948	9.5
Total of constituents with no terpenoid structure				16.0
Total percentage of identified components				82.5

RT= retention time; RC= retention index calculated; RTIR= retention index reference; % = relative percentage

Both groups are the main constituents of the most essential oils and are easily extracted by hydrodistillation, as they are generally volatile. In the extracts obtained at 100 bar and 30°C (Table 2), 150 bar and 50°C (Table 4), and 200 bar and 70°C (Table 5), the monoterpenoid citral were the major component, while in the extract obtained at 200 bar and 30°C (Table 3), only one constituent, the monoterpenoid limonene was detected. Limonene has been extensively investigated due its application in the flavor and fragrance industries (Erasto and Viljoen 2008) as well as its medicinal potential (Bakkali et al. 2008; Yoon et al. 2010). However, the extract obtained at 70°C and 100 bar (Table 6) showed the presence of high amount of caryophyllene oxide, a compound with sesquiterpenoid structure which has been considered to be an artifact produced during the distillation as a consequence of the relatively high temperatures involved (Silva et al. 2010). Sköld et al. (2006) also described the oxidation of caryophyllene when exposed to air, yielding the corresponding oxide. However, their findings did not explain the fact that only one extract contained large amounts of caryophyllene oxide, since the entire plant material was processed in the same way prior to extraction. There was no report in the literature about this compound as an artifact during the supercritical fluid extraction process. However, caryophyllene, which has been considered its precursor, was detected in three of the five extracts obtained (see Tables 2, 4, and 5).

As regards the number of constituents detected by GC, the extract obtained at 70°C and 200 bar was the richest one, with 29 different compounds (Table 5), followed by the extracts obtained at 50°C and 150 bar (27 compounds, Table 4), and at 30°C and 100 bar (23 compounds, Table 2).

However, for the extracts obtained at 30°C and 200 bar and 70°C and 100 bar, only one constituent was detected, limonene and caryophyllene oxide, respectively (Tables 3 and 6).

Table 5 – Chemical composition of major compounds present in the supercritical fluid extracts from leaves of *A. triphylla* obtained at 70°C and 200 bar.

Compound	RT	RC	RTIR	%	
Limonene	9.336	1031	1029	2.0	
p-Mentha-triene	9.675	1071	1085	0.5	
Z-Citral	16.572	1244	1238	15.3	
E-Citral	17.605	1275	1282	17.8	
α-Cubebene	21.024	1349	1345	0.8	
Geranyl acetate	21.121	1382	1381		2.5
Total of monoterpenoids					38.9
α-Caryophyllene	23.586	1457	1466		1.2
(-)-allo Aromadendrene	23.713	1464	1460		1.0
Germacrene D	24.189	1479	1474		3.0
α-Curcumene	24.367	1483	1481		2.0
τ-Elemene	24.827	1500	1482		2.8
β-Himachalene	24.954	1504	1494		5.5
γ- Cadinene	25.370	1518	1514		6.1
(-) *β*-Cadinene	25.497	1523	1519		2.2
α-Muurolene	26.063	1542	1537		1.2
Caryophyllene oxide	26.607	1561	1581		5.3
Hexahydro farnesyl acetone	34.351	1845	1833		2.4
Total of sesquiterpenoids					32.7
3-Octadecyne	34.143	1837	1828		9.7
(9,12) – Hexadecadienoic acid methyl ester	35.246	1880	1894		3.0
Total of constituents with no terpenoid structure					12.7
Total percentage of identified components					84.3

RT= retention time; RC= retention index calculated; RTIR= retention index reference; % = relative percentage

Table 6 - Chemical composition of major compounds present in the supercritical fluid extracts from leaves of *A. triphylla* obtained at 70°C and 100 bar.

Compound	**RT**	**RC**	**RTIR**	**%**
Caryophyllene oxide	27.446	1590	1585	88.0
Total of sesquiterpenoids				88.0
Total percentage of identified components				88.0

RT= retention time; RC= retention index calculated; RTIR= retention index reference; % = relative percentage

The chemical composition varied according to the experimental conditions, but some similarities could be observed. A predominance of compounds with terpenoid structure was observed in all the extracts. Monoterpenoids were the major components in almost all the extracts and were absent only in the extract obtained at 70°C and 100 bar (Table 6). Only the extract obtained at 50°C and 150 bar contained squalene, a precursor of triterpenoids and steroids in the biosynthetic pathway of plants (Table 4).

It was also possible to obtain the compounds with a non-terpenoidic structure and a high molecular weight, which seemed to be influenced by the temperature and pressure since different structures were obtained at different extraction conditions (Tables 2, 4 and 5). The same method of extraction also presented variations in the extraction of compounds of high molecular weight from *Tanacetum cinerariifolium* when using different temperatures and pressures (Marongiu et al. 2009). In a general sense, this work showed that it was possible to select the most favorable conditions for the extraction of different terpene groups present in the leaves of *A. triphylla*. The qualitative and quantitative differences observed indicated that the main constituents of the extract could then be

chosen by adjusting the operating parameters such as pressure and temperature.

Three of the extracts from the leaves of *A. triphylla* (those obtained at 100 bar and 30°C and at 200 bar and 30°C and 70°C) showed activity against *Aeromonas* sp (Table 1). Antibacterial activity against another genus of Gram-negative bacterium (*Escherichia coli*) was previously described for the ethanolic extract of *A. triphylla* (Oskay et al. 2005). The essential oil of this species also presented antibacterial activity against four Gram-negative clinical isolates from the urinary tract infections (Rojas et al. 2010).

The MBC values of the obtained extracts did not differ statistically among themselves, nevertheless differences appeared with regard to the extracts obtained at 50°C and 150 bar and also at 70°C and 100 bar (Table 1), which showed a weaker antibacterial activity. Probably this activity was due to the synergistic action of several components because most of them were present in small percentages. The present study did not analyze the antibacterial activity of the isolated substances from the extracts. However, considering the chemical class of the major components of each extract, it was observed that the extract with the weakest antibacterial activity was the only one, which did not have the monoterpenoids in its composition. This result, obtained for the extract produced at 70°C and 100 bar, indicated that the monoterpenoids were probably important for the antimicrobial activity of *A. triphylla* leaves extracts against *Aeromonas* sp. The caryophyllene oxide, which was identified as the major compound of this extract (Table 6), lacked the antimicrobial activity against yeasts, Gram-positive and Gram-negative bacteria at concentrations up to 1716 μg/mL (Silva et al. 2010). On the other hand, monoterpenoids are recognized as food preservatives due their antimicrobial activity (Singh et al. 2010).

The tolerance of Gram-negative bacteria such as *Pseudomonas aeruginosa* for monoterpene constituents in consequence of their outer membrane has been reported in the literature (Mann et al. 2000). This could be explained by the presence of a hydrophilic membrane in the outer portion of Gram-negative bacterial cell wall that blocks the penetration of hydrophobic substances (Ozturk and Ercisli 2006), such as terpene constituents that are part of the chemical composition of essential oils (Ceylan and Fung 2004) and were present in the extracts obtained by the supercritical fluid extraction as described in this work. This obstacle imposed by the Gram-negative bacteria could have reduced the antimicrobial activity of the substances reported for this purpose such as limonene (Ozturk and Ercisli 2006) and citral (Kim et al. 1995; Fisher and Phillips 2006). Although Gram-negative bacteria are usually more resistant to plant-origin antimicrobials, *Aeromonas hydrophila* has been described as the most sensitive one (Tajkarimi et al. 2010).

The essential oil of *A. triphylla* showed antibacterial activity against different strains (*Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae*) observing inhibition zones with diameters between 7 and 19 mm and MIC values between 10 and 60 mg/mL, without activity against *Pseudomonas aeruginosa* (Rojas et al. 2010). There was no activity of the oil of *A. triphylla* obtained by hydrodistillation of the aerial parts of the plant against *P. aeruginosa*. The antimicrobial activity against genito-urinary Gram-positive pathogens such as *S. aureus* (MIC $\geq$ 18.75 mm) and *E. faecalis* (MIC $\geq$ 17.75 mm) has also been observed for the essential oil of *A. triphylla* (Rojas et al. 2010).

Although it was not possible to assign the activity against *Aeromonas* sp to a component in particular, oxygenated monoterpens such as citral which were present as a major component in three of the obtained extracts, showed inhibitory effect against Gram-negative and Gram-positive (Kim et al. 1997) microorganisms and antibacterial activity (Kim et al. 1995; Fisher and Philips 2006; Tajkarimi et al. 2010). Some monoterpenoids such as citral have been tested as preservatives in fish-based food products in order to reduce the contamination and to increase the useful life of these foods (Svodoba and Greenaway 2003; Burt 2004). Limonene also showed antimicrobial activity against the food-borne pathogens, fungi and resistant strains of Gram-positive and Gram-negative bacteria (Singh et al. 2010; Tajkarimi et al. 2010). Citral and limonene have been recognized by the regulatory agencies in the US and EU as safe for the use as food additive (Tajkarimi et al. 2010).

There are no reports regarding the antimicrobial activity of the non-terpene compounds found (Tables 2, 4 and 5). This is probably because the plants that present the terpenoids as secondary metabolites are generally superior in antimicrobial

activity. However, the strength of this activity depends on external factors (Geiyd et al. 2005), including the agronomic practices and methodology of extraction, which would alter the chemical composition of the extracts and consequently influence their antimicrobial activity (Delaquis et al. 2002).

In general, the antimicrobial effect of essential oil is attributed to the C_{10} and C_{15} carbon terpenes with specific structural characteristics (Dorman and Deans 2000). Some of these terpene structures alone are bioactive and can interfere with the physical parameters of microorganisms such as absorption and bioavailability (Svodoba et al. 2006). The interaction between these terpenoid structures and microbes, which ultimately induces the antimicrobial activity is not well understood. The inhibitory effect of such molecules as citral and limonene (Svodoba and Greenway 2003) is generally explained by their interaction with the bacterial cell structural components (Belletti et al., 2004) like the phospholipid bilayer of the cell membrane, causing increase of permeability and leakage of intracellular constituents that are of vital importance for the bacteria (Singh et al. 2002). Some monoterpene alcohols can lead to inhibition of oxygen consumption, respiratory electron flow and oxidative phosphorylation of the microorganism (Reichling et al. 2009). Terpenes can also transpose the bacterial membrane, causing cell swelling, dissipation of pH gradients and electric potential (Sikkema et al. 1994).

However, when extracts are applied in a concentrated form as in the present study, the delivery of these hydrophobic compounds to bacterial cells may be reduced. An uneven distribution and accessibility of terpenes to bacterial cells may be one of the reasons for the relatively low bactericidal activity (Inouye et al. 2001), where the insolubility of the constituents in the culture medium may change the effectiveness of the extract besides chemical composition of plant material. Romeo et al. (2008) showed the antimicrobial efficacy of essential oil of *A. triphylla* in an experiment using a liquid medium. However, other essential oils in the same medium were less effective. Besides the differences in the chemical composition of these oils, the dependence of the bacterial metabolic activity from the growth medium, solid or liquid, might influence the microbial growth and survival (Skandamis et al. 2000).

Antimicrobial susceptibility tests with plant extracts using the microdilution plate may have drawbacks such as precipitation of some components in the extract and adherence of some microorganisms to the bottom of the hole making it difficult to analyze (Ostrosky et al. 2008). However, liquid medium was considered the best medium for the evaluation of antimicrobial activity using the natural compounds (Skandamis et al. 2000). Methanol was used to facilitate the dissolution of the extracts in a liquid culture medium and assess its minimum bactericidal activity (data not shown). In the present work, methanol showed no antimicrobial activity against *Aeromonas*. The lack of antimicrobial activity of methanol had also been found in Gram-negative bacteria (Pinto et al. 2001). Celiktas et al (2007) found low antimicrobial activity of methanol for the genus *Staphylococcus* and this activity was considered insignificant when compared to the essential oil tested.

CONCLUSION

The results obtained in the present work scientifically support the use of preparations of *A. triphylla*, traditionally used to treat the bacterial diseases. However, the antibacterial activity was influenced by the extraction conditions.

ACKNOWLEDGEMENTS

The authors thank Dr. Gilberto Dolejal Zanetti for the identification of the species. This work was supported by PRONEX/FAPERGS-CNPq and CAPES. B. Baldisserotto, A. P. C. Vargas, E. M. M. Flores and J. V. Oliveira were recipient of CNPq fellowships. T.V. Parodi, received PhD fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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Received: December 01, 2011;
Revised: June 27, 2012;
Accepted: December 26, 2012.