ARTIGO ORIGINAL

Isolation and identification of compounds from the essential oil of *Aloysia gratissima* using column liquid chromatography (CLC)

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ABSTRACT: *Aloysia gratissima* is well known as "Mimo do Brasil" in the Brazilian folk medicine and the infusion of its branches and twigs is an important analgesic used against stomached and bladder pains. However, few investigations about specific and efficient eluent systems applied for the purification of its essential oil have been reported. Essential oil extracted from dried leaves was obtained by hydrodistillation using a Clevenger apparatus. The isolation of *cis*-pinocarveyl acetate and *trans*-pinocamphone compounds were performed by Column Liquid Chromatography (CLC) through a number of appropriate solvent systems used as mobile phase. Results have showed that isolation of *cis*-pinocarveyl acetate and *trans*-pinocamphone were possible using, respectively, ethyl acetate/hexane 1:59 (v/v) and 1:5 (v/v) as solvent systems. Therefore, the aims of this work were to provide the ideal solvent systems for the isolation of important compounds from *Aloysia gratissima* essential oil using Column Liquid Chromatography (CLC) as well as identify them from GC retention indices, relative to C_6-C_{24} n-alkanes, by comparison of their MS spectra with those reported in the literature (Adams 1995) and by computer matching with the NBS-Reve mass spectra library.
Keywords: *Aloysia gratissima*, chromatography, *cis*-pinocarveyl acetate and *trans*-pinocamphone.

RESUMO: Isolamento e identificação dos compostos do óleo essencial de *Aloysia gratissima* usando cromatografia líquida em coluna (CLC). *Aloysia gratissima* é conhecida na medicina popular como Mimo do Brasil. A infusão de ramos e folhas desta espécie é usada como anticatarral, sudorífera, analgésico, afrodisíaco, além de combater dores estomacais e na bexiga. Entretanto, são poucas as investigações reportadas sobre específicos e eficientes sistemas de eluentes utilizados na purificação de seu óleo essencial. O óleo extraído a partir das folhas secas foi obtido por hidrodestilação, usando o sistema de Clevenger. O isolamento dos compostos *cis*-acetato de pinocarvil e t*rans*-pinocanfona foi realizado por cromatografia líquida em coluna (CLC) através de um conjunto de sistemas de solventes apropriados utilizados como fase móvel. Os resultados mostraram que os isolamentos do *cis*-acetato de pinocarvil e *trans*-pinocanfona foram possíveis utilizando-se, respectivamente, os sistemas acetato de etila/hexano 1:59 (v/v) e 1:5 (v/v). Assim, os objetivos deste trabalho foram fornecer os sistemas de solventes ideais para o isolamento de compostos importantes do óleo essencial de *Aloysia gratissima* usando Cromatografia Líquida de Coluna (CLC) e identificá-los partir de índices de retenção de GC, relativos aos n-alcanos C_6-C_{24}, por comparação de seus espectros de MS com aqueles relatados na literatura (Adams 1995) e por correspondência de computador com a biblioteca de espectros de massa NBS-Reve
Palavras-chave: *Aloysia gratissima*, cromatografia, *cis*-acetato de pinocarvil, *trans*–pinocanfona.

INTRODUCTION

Essential oils are volatile compounds present in aromatic plants as secondary metabolites. They are liquid, volatile, limpid and rarely colored, soluble in organic solvents with a generally lower density than water (Chaar 2000). They play an important role in the protection of plants and also against herbivores; repelling undesirable insects or attracting some which promote the dispersion of pollens and seeds. Essential oils can be synthesized by all plant organs, i.e., buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark and are

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stored in secretor cells, cavities, canals, epidermic cells or glandular trichomes (Oussalah et al. 2007). Furthermore, it is well known that in the past, they were commonly used in folk medicine, food flavorings and fragrance. Most of chemical constituents of plant essential oils belong to terpenoid compounds, including monoterpenes, sesquiterpenes and their oxygenated derivatives. These low molecular weight (most below 300 g/mol) compounds easily diffuse across cell membranes to induce biological reactions (Soler and Dellacassa 1986). For this reason, their multiple bioactive functions have been examined and developed in recent years (Uchiyama et al. 2009; Tewary et al. 2005; Philip et al. 2016).

Aloysia is a genus of sweet aromatic shrubs disseminated mainly in tropical and subtropical regions probably originated in South American (Fester et al. 1961; Amorin 1988; Zygadlo et al.1995; Bassols and Gurni 1996; Silva et al. 2006; Ricciardi et al. 2000). In the Brazilian folk medicine, *Aloysia gratissima* **(**Gillies & Hook.) Tronc. is well known as "Mimo do Brasil" and the infusion of its branches and twigs is used as analgesic, aphrodisiac, against stomached and bladder pains (Troncoso 1974; Botta 1979; Alice et al. 1995; Bailac et al. 1999). The essential oil from aerial parts of *Aloysia gratissima* has been investigated and demonstrated a high chemical variability in its composition, as well as important bioactivities (Soler et al. 1986; Sartoratto and Augusto 2003; Franco et al. 2007; Tovati et al. 2009; Santos et al. 2013; Santos et al.,2015). According to Santos et al. (2013), the essential oil composition from *Aloysia gratissima* leaves have been identified and showed as major compounds *trans*-pinocarveyl acetate (17.6%), *trans*-pinocamphone (16.3%) and guaiol (11.5%), representing 45.4% of identified compounds. Authors also have reported the efficiency of this essential oil against *P. aeruginosa* and *S. pneumonia* bacteria.

Some bioactivities have been reported for virucidal (Garcia et al. 2003), nematicidal (Duschatzky et al. 2004), and fungicidal against *Ascophaera apis* (Dellacasa et al. 2003). The organic extract has demonstrated antibacterial and antiedematogenic effects (Vandresen et al. 2010), while the aqueous extract has exerted antidepressant-like and neuroprotective activities (Zeni et al. 2011). Recently, both antibacterial and antifungal activities against several microorganisms, mainly *B. cereus and T. mentagrophytes*, respectively, have been reported (Santos et al. 2015).

Thus, the isolation of two compounds previously identified as *trans*-pinocarveyl acetate isomer and *trans*-pinocamphone have been reported in this work. Column Liquid Chromatography (CLC) was applied for the isolation of these constituents present in the raw essential oil. Furthermore, a simple and appropriate system of eluents, which allowed their isolation, was also suggested. The isolated compounds identification was made using a combination of Gas Chromatography (GC-FID) and Gas Chromatography - Mass Spectrometry (GC-MS) techniques. Considering the importance of this Brazilian plant, and also of its isolated constituents, we hope that this work can contribute to new researches regarding the bioactivities of these constituents, since we have proposed here a new and simple method its isolation.

MATERIAL AND METHODS

Plant Material and Oil Extraction

Leaves of *Aloysia gratissima* were collected in São Carlos – Brazil situate, 22º 01' 03" S and 47º 53' 27" W at 854 m. Plant identification (No. 006697) was determined by Maria Inês Salgueiro Lima and has been deposited at the herbarium of Federal University of São Carlos (UFSCar). Leaves were dried in a room with controlled humidity for ten days (50% of relative humidity) at 30ºC until reaching constant weight and then subjected to hydrodistillation using a Clevenger apparatus for 4.5 h. The yield of the oil was found to be 2.3% (v/w) and it was stored at -18 ºC until analysis.

Physical Properties

Density

Relative density was estimated according to the equation (d = m/v). Essential oil volume was fixed at 1.0 ml (constant volume) and its mass was determined using an analytical balance at 29 °C. The ratio between oil mass and volume was estimated by the method described in Adolfo Lutz Institute (Zenebon et al.2008)

Refraction Index

Refraction index of the raw essential oil was determined at 22 °C using an Abbe-type refractometer (Enciclopédia Técnica Universal 1958).

Ethanol Solubility

Successive volumes of 0.1 ml hydroalcoholic solution were added in a set of four aliquots containing 50.0 µl of the raw essential oil. Each aliquot represents a specific concentration of hydroalcoholic solution (30, 50, 70, and 90%) with final volume of 2.0 ml (Enciclopédia Técnica Universal 1958).

Column Liquid Chromatography (CLC)

A piece of signalized glass wool was introduced in a glass column that was packed with 5.0 g of silica gel Aldrich (100–200 mesh) previously

activated at 140 ºC for 4 h (cooled in a desiccator), which was conditioned with 25 ml of *n*-hexane. After the introduction of the sample, 0.20 g of raw essential oil dissolved in 500 µl chloroform, the column was eluded with 19 ml of ethyl acetate/hexane (1:59 v/v). Thirteen fractions were collected (TL1 to TL13). In sequence, 17 ml of the eluent ethyl acetate/hexane (1:5 v/v) was used, and seven news fractions also were obtained (TL14 to TL20). The fractions were keeping at room temperature for solvent evaporation and submitted to analysis of GC/FID and GC/MS.

Gas Chromatography (CG/FID)

The GC analysis was carried out using a Hewlett-Packard 5890 instrument equipped with a flame ionization detector (FID) and a HP-5 column, (25 m x 0.22 mm, film thickness 0.30 µm). The operating conditions were as follows: injector and detector temperatures were 300 ºC; hydrogen was used as carrier gas with flow rate of 1ml/min; column temperature was kept at 80 ºC for 2 min, heated to 150 ºC with 7 ºC/min rate and then heated to 300 ºC with 12 ºC/min rate and kept constant at 300 ºC for 20 min; split ratio, 1:50.

Gas Chromatography/Mass Spectrometry (GC/MS)

GC/MS analysis was performed using a Hewlett-Packard 5970 with HP-1 column (50 m x 0.22 mm, film thickness 0.25 µm). The operating conditions were as follows: injector and detector temperatures were 300 ºC; column temperature was kept at 80 ºC for 2 min, heated to 150 ºC with 7 ºC/min rate and then heated to 300 ºC with 12 ºC/min rate and kept constant at 300 ºC for 20 min. Split ratio, 1:50. Scan time: 45 min. Acquisition mass range: 32 – 420 Daltons. Helium was used as carrier gas with a flow rate of 1 ml/min. MS were taken at 70 eV. The identification of the isolated constituents of the oil compounds was established from their GC retention indices, relative to C_6-C_{24} *n*-alkanes, by comparison of their MS spectra with those reported in the literature and by computer matching with the NBS-Reve mass spectra library (Adams 1995).

RESULTS AND DISCUSSION

Essential Oil Extraction

The extraction made by the Clevenger system offer, in addition, different results in what regards the time of extraction (Figure 1). As can be seen, time is an important variable in the process to obtain oil. Indeed, it was observed an increase of 157% in the yield performance of oil gain after 3.5 hours of extraction if compared to the 1 hour period (Table 1).

Physical Properties

Essential oil physical parameters are shown in Table 1. Comparing the values in literature, there is a similarity regarding density, refraction index, performance, color and appearance. It was not possible to compare the analyses of solubility in ethanol because this information has not been reported (Ricciardi et al. 2000).

Isolation and Identification of Secondary Metabolites

Figure 2 shows the chromatogram for *Aloysia gratissima* raw essential oil and many chromatographic peaks can be observed. These results reveal a great amount of compounds with different polarities and intermediate volatility since de main mechanism of separation of the chromatographic column is by dielectric constant. Another possibility is to infer the presence of

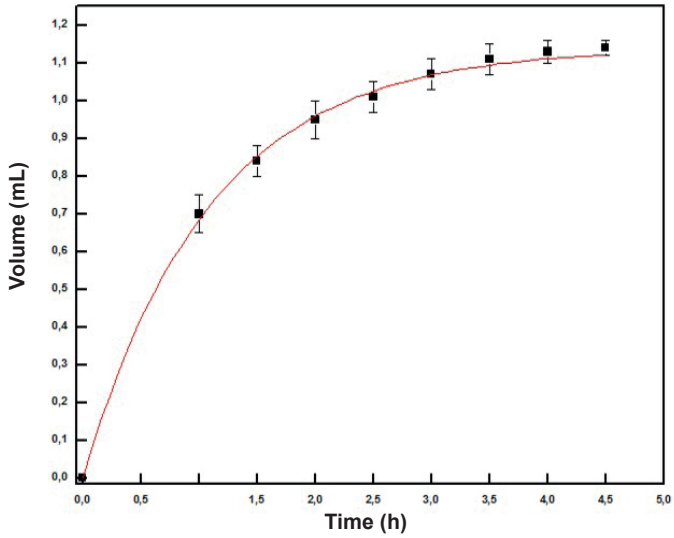

FIGURA 1. Extraction time and essential oil performance obtained through the Clevenger system for dried leaves.

TABLE 1. Physical parameters of the essential oil from *Aloysia gratissima.*

Physical Parameters	Experimental Parameters	Literature Parameters
Ethanol Solubility	1:10	———
Density (g/ml)	0.922 $^{29°C}$	0.927 $^{20°C}$
Index of Refraction	1.493 $^{22°C}$	1.486 $^{22°C}$
Yield (%v/w)	2.3	0.8
Color	yellow	yellow

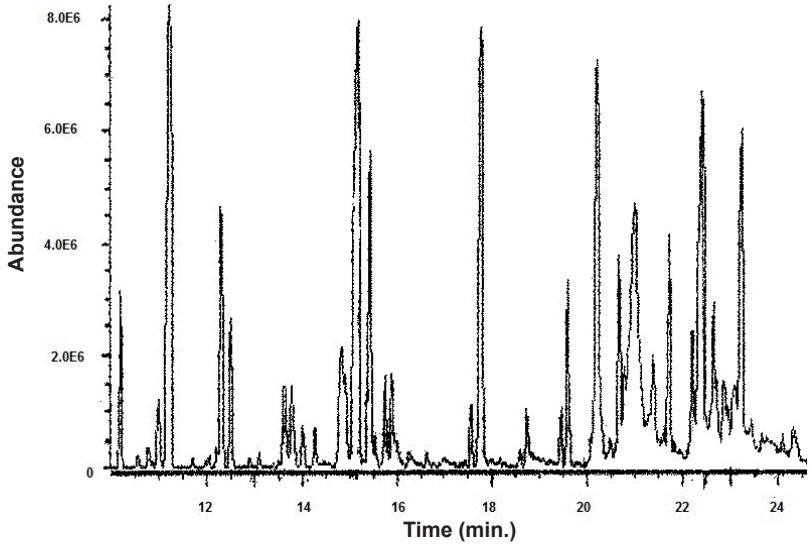

FIGURE 2. Chromatogram of raw essential oil from *Aloysia gratissima* analysed by GC/MS.

an ample band ebullition points based in the temperature program of the chromatograph oven.

The analytical study of the oil essential leaves was based on the isolation and identification of oxygenated monoterpene, *cis*-pinocarveyl acetate and *trans*-pinocamphone, present in raw essential oil. The results obtained through the fractioning of *Aloysia gratissima* essential oil are presented in Figure 3. The fractions of initial sample (oil + chloroform) were done by column liquid chromatography have been codified as TL1 to TL20 and the best mixtures of testes solvents were ethyl acetate / hexane (1:59, v/v) and (1:5 v/v).

In this work only the fractions TL13 and TL 17 were subjected of the study. The chromatograms Figures 4 and 5 illustrate the effect of the purification process, which reveal high efficiency and capability for concentration of the targeted compounds.

Chromatogram of the TL13 fraction (Figure 4) showed two distinct peaks. The highest peak was identified as *cis*-pinocarveyl acetate, which represents the isomer of the major constituent of the essential oil collected at Lavras city state of Minas Gerais – Brazil, with retention time of 18.39 min. (Santos et al. 2013). Another observed short peak was identificated as *trans*-pinocamphone, also present in essential oil from leaves, as the second majority compound of the plant collected at Lavras, with retention time of 15.83 min.

TL17 fraction showed the same compounds previously identificated in its chromatogram, with solvents ration of 1:5 (v/v). On the other hand, the highest peak was identified as *trans*-pinocamphone, with retention time of 17.25 min and the shorter peak the compound *cis*-pinocarveyl acetate, with retention time of 19.59 min. Figures 6 and 7 shows the mass spectrum of the isolated compounds from raw essential oil.

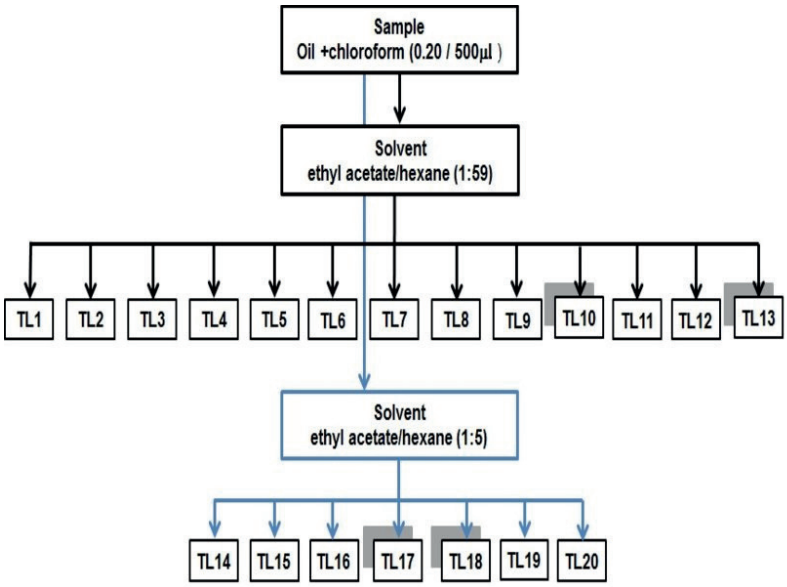

FIGURE 3. Fractions of essential oil from *Aloysia gratissima* leaves by CLC.

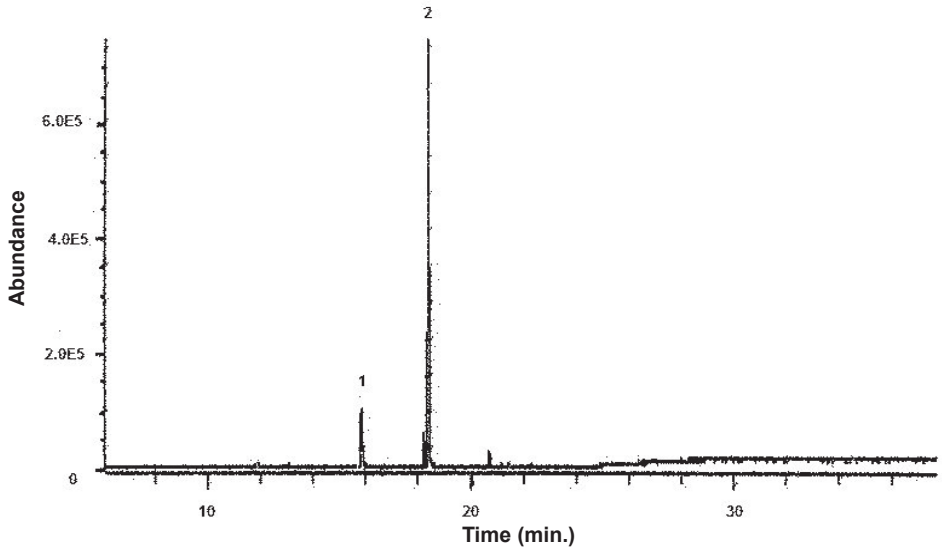

FIGURE 4. Chromatogram of the TL13 fraction from *Aloysia gratissima* analysed by GC/MS.

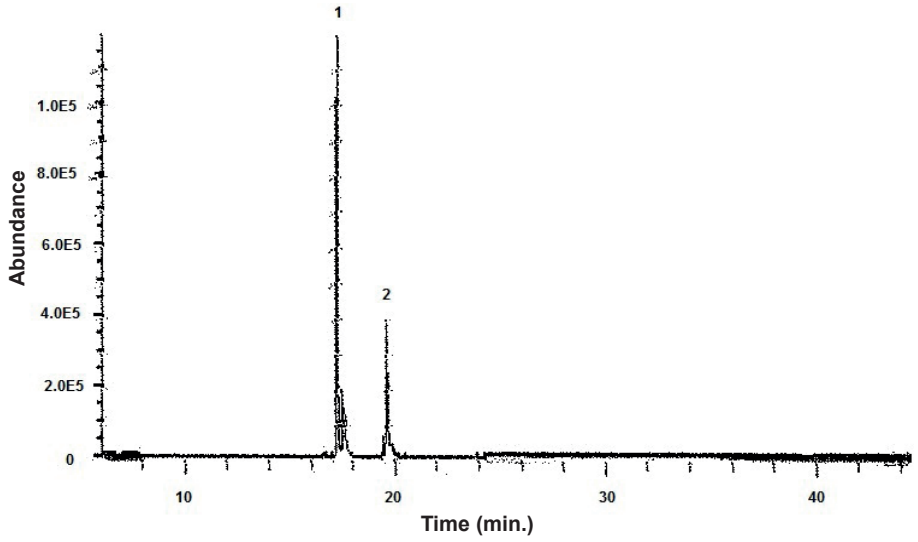

FIGURE 5. Chromatogram of the TL17 fraction from *Aloysia gratissima* analysed by GC/MS.

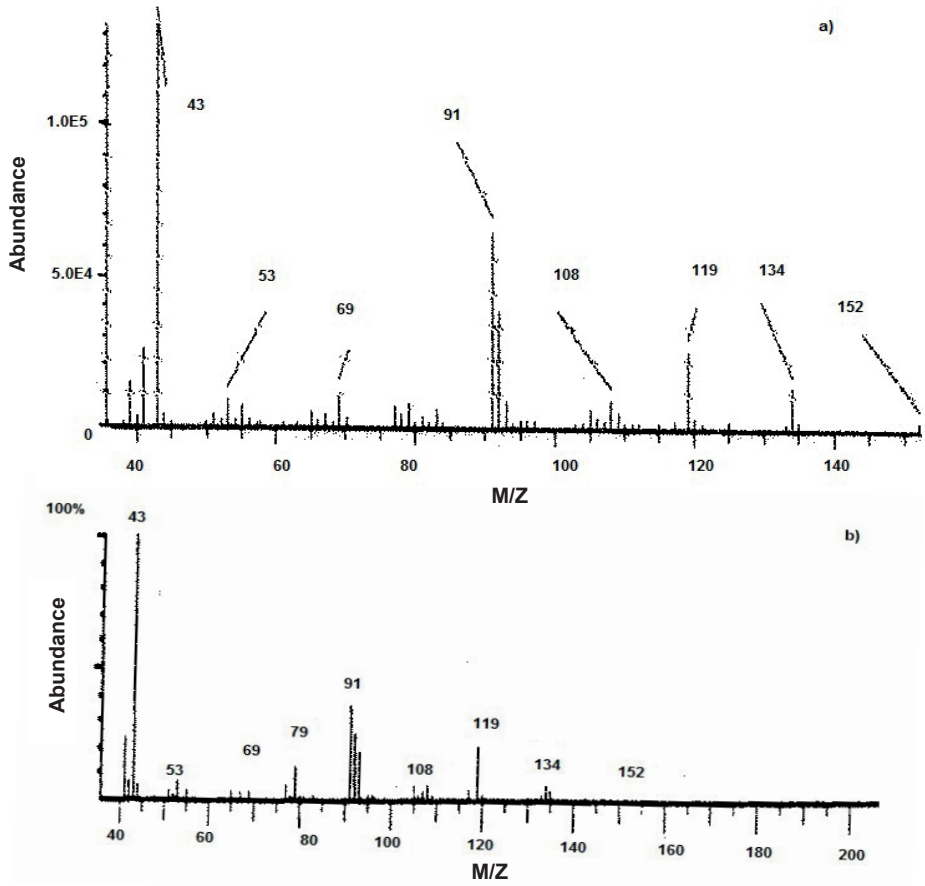

FIGURE 6. Mass spectrum of the *cis*-pinocarveyl acetate: (a) experimental, (b) theoretical (Adams 1995).

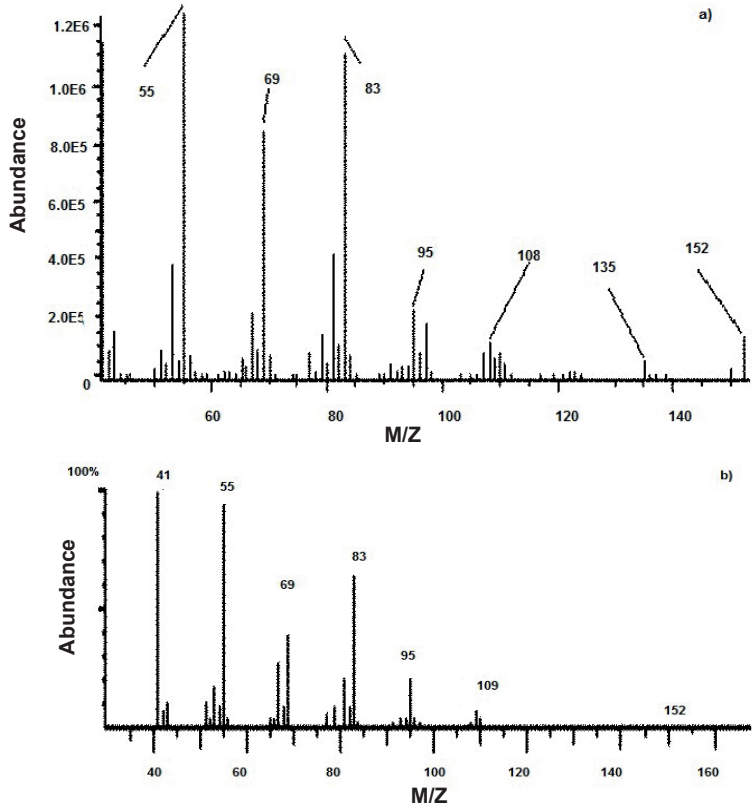

FIGURE 7. Mass spectrum of the *trans*-pinocamphone: (a) experimental, (b) theoretical (Adams 1995).

CONCLUSION

This work provided the purification of oil essential from *Aloysia gratissima* using silica and systems of solvents, with appropriate dielectric constant for further biologic test. Physical properties of essential oil as well as ethanol solubility were reported, which were not previously reported in literature. Generally, the dielectric constant of the solvent provides the measure of a solvent's polarity. Then, using system of solvents ethyl acetate/ hexane 1:59 (v/v) and 1:5 (v/v) it was possible to obtain different concentrations of the two targets components (*cis*-pinocarveyl acetate and *trans*-pinocamphone) and also their isolation from raw essential oil. Even so, it is still necessary research to prove the folk culture importance as start point to diseases treatments.

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