DOI 10.1515/JAS-2017-0004 J. APIC. SCI. VOL. 61 NO. 1 2017

Original Article

OPTIMAL CONCENTRATION OF ORGANIC SOLVENTS TO BE USED IN THE BROTH MICRODILUTION METHOD TO DETERMINE THE ANTIMICROBIAL ACTIVITY OF NATURAL PRODUCTS AGAINST *PAENIBACILLUS LARVAE*

Noelia Melina Cugnata [1,2]
Elisa Guaspari [1,2]
Maria Celeste Pellegrini [1,3]
Sandra Rosa Fuselli * [1,3]
Rosa Maria Alonso-Salces [1,2]

[1] Grupo de Investigación Microbiología Aplicada, Centro de Investigación en Abejas Sociales, Departamento de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, 7600 Mar del Plata, Argentina
[2] Consejo Nacional de Investigaciones Científicas y Técnicas, C1425FQB Buenos Aires, Argentina
[3] Comisión de Investigaciónes Científicas, 1900 La Plata, Argentina

*corresponding author: sfuselli@gmail.com
Received: 8 March 2016; accepted: 29 April 2017

Abstract

American Foulbrood (AFB) is a bacterial disease, caused by *Paenibacillus larvae*, that affects honeybees (*Apis mellifera*). Alternative strategies to control AFB are based on the treatment of the beehives with antimicrobial natural substances such as extracts, essential oils and/or pure compounds from plants, honey by-products, bacteria and moulds. The broth microdilution method is currently one of the most widely used methods to determine the minimum inhibitory concentration (MIC) of a substance. In this regard, the fact that most natural products, due to their lipophilic nature, must be dissolved in organic solvents or their aqueous mixtures is an issue of major concern because the organic solvent becomes part of the dilution in the incubation medium, and therefore, can interfere with bacterial viability depending on its nature and concentration. A systematic study was carried out to determine by the broth microdilution method the MIC and the maximum non inhibitory concentration (MNIC) against *P. larvae* of the most common organic solvents used to extract or dissolve natural products, i.e. ethanol, methanol, acetonitrile, n-butanol, dimethylsulfoxide, and acidified hydromethanolic solutions. From the MIC and MNIC for each organic solvent, recommended maximum concentrations in contact with *P. larvae* were established: DMSO 5% (v/v), acetonitrile 7.5% (v/v), ethanol 7.5% (v/v), methanol 12% (v/v), n-butanol 1% (v/v), and methanol-water-acetic acid (1.25:98.71:0.04, v/v/v).

Keywords: American foulbrood, *Paenibacillus larvae*, antimicrobial activity, broth microdilution, natural product, organic solvent

INTRODUCTION

Paenibacillus larvae is the etiological agent of American Foulbrood (AFB), a bacterial disease that affects the breeding of honeybees (*Apis mellifera*) during the larval or pupal stages (Genersch et al., 2006; De Graaf et al., 2013). The use and abuse of antibiotics to treat AFB has led to the increase in the incidence of bacterial resistance (Eguaras et al., 2005) and the contamination of the apiary products (Mutinelli, 2003). In this context, the development of alternative and effective methods for the control and prevention of AFB disease is crucial. In this sense, one of the alternative methods proposed to control AFB is the treatment of the beehive with antimicrobial natural bioactive substances, i.e. plant extracts (Flesar et al., 2010; Sabaté et

al., 2012; Boligon et al., 2013; Reyes et al., 2013; Damiani et al., 2014; Hernández-López et al., 2014); essential oils (Alippi, 1996; Fuselli et al., 2006a); pure compounds extracted from plants, bacteria or fungus (Feldlaufer et al., 1993; Lokvam et al., 2000; Hornitzky, 2003; Gallardo et al., 2004; Fuselli et al., 2006b; Flesar et al., 2010; Sabaté et al., 2012; Reyes et al., 2013; Hernández-López et al., 2014); and honeybee by-products, such as propolis (Antúnez et al., 2008; Bíliková et al., 2013) and royal jelly (Bachanová et al., 2002; Bíliková et al., 2009).

The most common methods used to determine the antimicrobial activity of a substance are the agar diffusion methods (CLSI, 2009b) and the dilution methods (CLSI, 2009a). However, the agar diffusion methods present important disadvantages. The minimum inhibitory concentration (MIC) cannot be determined, the agar diffusion data is not standardized for natural product antimicrobial susceptibility testing so the inhibition diameters cannot be interpreted, and the antimicrobial activity of natural products is underestimated due to their diffusion rates in the aqueous agar media, and furthermore, being labour-intensive and time-consuming (Dickert et al., 1981; Recio et al., 1989). Dilution methods are able to overcome some of the diffusion method limitations, the MIC values for antimicrobials are determined, and therefore quantitative conclusions can be drawn from the results. The sample is diluted in a liquid medium for the broth macro and micro-dilution methods, or in a solid medium for the agar dilution method (CLSI, 2009a), and must be dispersed homogeneously in water (Recio et al., 1989). The low solubility in water of essential oils, non-polar extracts and pure organic compounds from plants makes it difficult to use aqueous media in the liquid dilution methods, which proves to be an important issue to deal with in practice (Allegrini et al., 1973; Pellecuer et al., 1976). In fact, the solvents used to extract natural products from plants or dissolve pure natural compounds are usually organic of different polarities or their aqueous mixtures, such as ethyl acetate, ethanol, chloroform, dichloromethane, hexane, methanol and n-butanol (Lokvam et al., 2000; Gallardo et al., 2004; Flesar et al., 2010; Sabaté et al., 2012; Boligon et al., 2013; Reyes et al., 2013; Damiani et al., 2014; Hernández-López et al., 2014). Pure natural compounds also need to be dissolved in organic solvents or aqueous solutions containing organic solvents for the antimicrobial assays to be performed (Hornitzky, 2003; Reyes et al., 2013; Hernández-López et al., 2014). The influence of the organic solvent on the results of the antimicrobial activity determined by the liquid dilution methods is an issue of major concern due to the well known antiseptic properties of organic solvents. Indeed, organic solvents become part of the dilution in the incubation medium and, depending on their nature and concentration, can interfere with bacterial viability.

To the authors´ knowledge, there is little information in literature regarding the solvent of plant extracts or the solvent used to prepare the solutions of pure natural compounds when they are submitted to the dilution methods for testing their antimicrobial activity against *P. larvae*. It is essential to have the information regarding the final concentration of the organic solvent in the medium once the bacterium is put in contact with the extract or the pure compound, in order to assure that the antimicrobial activity observed is only due to the extract or the compound tested and not to the organic solvent. This paper presents a systematic study to determine the MIC and the maximum non inhibitory concentration (MNIC) against *P. larvae* of organic solvents and their aqueous mixtures by the broth microdilution method, in order to establish the maximum content of the organic solvent in the sample solution needed to perform this dilution method properly. The solvents most commonly used to extract bioactive natural products from plants or to dissolve pure natural compounds are studied.

MATERIAL AND METHODS

Chemicals and materials

Mueller-Hinton broth, agar, brain-heart infusion, yeast extract, glucose and peptone were obtained from Britania S.A. (Ciudad autónoma

de Buenos Aires, Argentina). Sodium chloride (99.99 %) from Alun (San Martin, Buenos Aires, Argentina) and K_2HPO_4 (98 %) from Cicarelli (San Lorenzo, Santa Fe, Argentina) were analytical grade; sodium pyruvate (≥ 99 %) from Biopack (Zárate, Buenos Aires, Argentina) was extra pure. Distilled water was sterilized in autoclave FAC (Buenos Aires, Argentina). Sterilized polystyrene 96-well culture plates were supplied by Deltalab (Barcelona, Spain). Acetonitrile, ethanol and methanol (HPLC grade), glacial acetic acid (ACS reagent), n-butanol (FCC reagent), and resazurin sodium salt (for cell culture) were supplied by Sigma-Aldrich (Saint Louis, MO, USA). Dimethylsulfoxide (analytical grade) was from Biopack (Zárate, Buenos Aires, Argentina).

***Paenibacillus larvae* strains and growth conditions**

P. larvae strains were isolated from honey combs of beehives exhibiting clinical symptoms of American foulbrood, located in the provinces of Buenos Aires, Córdoba and Entre Ríos in Argentina, and the provinces of Reggio Emilia and Módena in Italy. Strains BAI, BAII and BAIII were from Balcarce-Buenos Aires (37°50´47´´S, 58°15´19´´W); strain BAIV from Necochea-Buenos Aires (38°33´44´´ S, 58°44´43´´ W); strain CI from Río Cuarto-Córdoba (33° 08´00´´ S, 64°21´00´´ W); strain ERI from Concordia-Entre Ríos (31°23´32´´S, 58°01´01´´W); strain IT.I from Módena (44°39´00´´ N, 10°56´00´´ E); and strain IT.II from Reggio Emilia (44°42´00´´ N, 10°38´00´´ E). The strains were identified using previously described methods (Alippi, 1991, 1992; De Graaf et al., 2013).

P. larvae growth was detected using resazurin sodium salt. Bacterial strains were grown and maintained on 2 % (w/v) MYPGP-agar plates (Mueller-Hinton broth 1 % (w/v), yeast extract 1.5 % (w/v), K2HPO4 0.3 % (w/v), glucose 0.2 % (w/v), sodium pyruvate 0.1 % (w/v), and agar 2 % (w/v)), and incubated at 37 °C and 10% (v/v) CO2 for 48 h (Dingman and Stahly, 1983; Nordström and Fries, 1995). The bacterial inoculum was prepared in sterile peptone water (peptone 0.1 % (w/v) and sodium chloride 0.85 % (w/v)) to a final optical density at 600nm (OD600nm) of 0.1 using a UV-VIS spectrophotometer Spectrum SP-1103 (Spectrum Instruments Company Ltd., Shanghai, China) (Nordström and Fries, 1995). Brain-heart infusion (3.7 %, w/v) was used as a growth media for the bacterial strains when the broth microdilution assay was being performed.

Preparation of solvent solutions

The solvents studied throughout the present work were classified according to their physicochemical parameters (Tab. S1, in the supplementary information): *(i)* miscible solvents with the culture media which included slightly volatile solvents such as dimethylsulfoxide (DMSO) and volatile solvents such as acetonitrile, ethanol, methanol, and acidified hydroalcoholic mixtures such as methanol-water-acetic acid mixture (30:69:1, v/v/v); and *(ii)* non miscible solvents with the culture media, such as n-butanol.

The following aqueous solutions of each solvent were prepared (Tab. 1): DMSO 18.75% (v/v), 22.5% (v/v), 27% (v/v) and 30% (v/v); acetonitrile 18.75% (v/v), 22.5% (v/v), 30% (v/v), 36% (v/v), 45% (v/v) and 60% (v/v); ethanol 18.75% (v/v), 22.5% (v/v), 30% (v/v) and 36 % (v/v); methanol 18.75% (v/v), 22.5% (v/v), 30% (v/v), 36% (v/v), 45% (v/v), 60% (v/v) and 80% (v/v); and methanol-water-acetic acid (30:69:1, v/v/v). To prepare each of these solutions, the corresponding volume of the pure solvent was placed in a volumetric flask and made up with sterilized water. Fresh solvent solutions were prepared daily.

Broth microdilution method

The experimental design carried out to study the organic solvent effect on the antimicrobial activity obtained by the broth microdilution method was the following. A first set of experiments were performed on four *P. larvae* strains (BAI, BAII, CI and IT.II) to obtain the solvent concentration range where the MIC and MNIC values were included. Then, a second set of experiments were performed on the eight *P. larvae* strains to determine the MIC and MNIC for each solvent, as well as the repeatability within-day and between days. The MIC value corresponds to the minimum concentration of the solvent at which *in vitro* bacterial growth inhibition is observed (De Graaf et al., 2013); and the MNIC is

Table 1
Final concentrations of the solvent (%, v/v) in contact with the bacteria in the broth microdilution method*

		Final solvent concentrations in contact with bacteria in plate (%, v/v)									
Solvents	C**	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
DMSO	18.75	6.25	3.13	1.56	0.78	0.39	0.20	nt	nt	nt	nt
	22.5	7.50	3.75	1.88	0.94	0.47	0.23	nt	nt	nt	nt
	27	9	4.5	2.25	1.13	0.56	0.28	nt	nt	nt	nt
	30	10	5	2.5	1.25	0.63	0.31	nt	nt	nt	nt
Acetonitrile	18.75	6.25	3.13	1.56	0.78	nt	nt	nt	nt	nt	nt
	22.5	7.5	3.75	1.88	0.94	nt	nt	nt	nt	nt	nt
	30	10	5	2.5	1.25	nt	nt	nt	nt	nt	nt
	36	12	6	3	1.5	nt	nt	nt	nt	nt	nt
	45	15	7.5	3.75	1.88	nt	nt	nt	nt	nt	nt
	60	20	10	5	2.5	nt	nt	nt	nt	nt	nt
Ethanol	18.75	6.25	3.13	1.56	0.78	nt	nt	nt	nt	nt	nt
	22.5	7.5	3.75	1.88	0.94	nt	nt	nt	nt	nt	nt
	30	10	5	2.5	1.25	nt	nt	nt	nt	nt	nt
	36	12	6	3	1.5	nt	nt	nt	nt	nt	nt
Methanol	18.75	6.25	3.13	1.56	0.78	nt	nt	nt	nt	nt	nt
	22.5	7.5	3.75	1.88	0.94	nt	nt	nt	nt	nt	nt
	30	10	5	2.5	1.25	nt	nt	nt	nt	nt	nt
	36	12	6	3	1.5	nt	nt	nt	nt	nt	nt
	45	15	7.5	3.75	1.88	nt	nt	nt	nt	nt	nt
	60	20	10	5	2.5	nt	nt	nt	nt	nt	nt
	80	26.67	13.33	6.67	3.33	1.67	0.83	nt	nt	nt	nt
Methanol-water-acetic acid***	30:69:1	10:89.67:0.33	5:94.84:0.16	2.5:97.42:0.08	1.25:98.71:0.04	nt	nt	nt	nt	nt	nt
n-Butanol	100	10	9	8	7	6	5	4	3	2	1

* Abbreviations: C, concentration (%, v/v); nt: not tested.
** Initial concentration of aqueous solvent solution tested (%, v/v).
*** Concentration expressed as % (v/v/v).

the maximum concentration of the solvent that can be used without observing *in vitro* bacterial growth inhibition. All assays were performed in triplicate.

Miscible solvents with the culture media

a) Slightly volatile solvent: DMSO

Using a polystyrene 96-well culture plate vertically-oriented as shown in Fig. 1, 100 µL of brain-heart infusion (3.7 %, w/v) was placed in each well of the microtitre plate, and 100 µL of the DMSO solution only in the wells corresponding to column A. Two-fold serial dilutions were then performed starting from column A to column F. Afterwards, 50 µL of the bacterial suspension were added to all wells. Columns G and H, which only contained culture media (100 µL) and bacterial inoculum (50 µL), were used as strain viability control (SVC). The plate was incubated at 37°C for 48 h under microaerophilic conditions (O_2 10% (v/v)). Bacterial growth was detected after the plates were incubated for 1 h with resazurin sodium salt at 0.01% (w/v), which is a blue redox indicator that turns pink in the presence of aerobic growing bacteria. A different plate was used to test each DMSO solution.

	A	B	C	D	E	F	G	H	Strain
1	10	5	2.5	1.25	0.63	0.31			
2	10	5	2.5	1.25	0.63	0.31			BAI
3	10	5	2.5	1.25	0.63	0.31			
4	10	5	2.5	1.25	0.63	0.31			
5	10	5	2.5	1.25	0.63	0.31			BAII
6	10	5	2.5	1.25	0.63	0.31			
7	10	5	2.5	1.25	0.63	0.31			
8	10	5	2.5	1.25	0.63	0.31			CI
9	10	5	2.5	1.25	0.63	0.31			
10	10	5	2.5	1.25	0.63	0.31			
11	10	5	2.5	1.25	0.63	0.31			IT.II
12	10	5	2.5	1.25	0.63	0.31			
	SSD						SVC		

Fig.1. Example of the final concentration tested in a serial microdilution plate (vertically-oriented) for slightly volatile solvents: DMSO 30% (v/v) in water. Abbreviations: SVC, strain viability control; SSD, concentration of the solvent serial dilution (%, v/v).

	A	B	C	D	E	F	G	H	Strain
1	10			5	2.5	1.25			
2	10			5	2.5	1.25			BAI
3	10			5	2.5	1.25			
4	10			5	2.5	1.25			
5	10			5	2.5	1.25			BAII
6	10			5	2.5	1.25			
7	10			5	2.5	1.25			
8	10			5	2.5	1.25			CI
9	10			5	2.5	1.25			
10	10			5	2.5	1.25			
11	10			5	2.5	1.25			IT.II
12	10			5	2.5	1.25			
	SSD	SEC		SSD			SVC		

Fig. 2. Example of the final concentration tested in a serial microdilution plate (vertically-oriented) for volatile solvents at 30% (v/v) in water. Abbreviations: SVC, strain viability control; SSD, concentration of the solvent serial dilution (%, v/v); SEC, solvent evaporation control.

b) Volatile solvents: acetonitrile, ethanol, methanol and acidified hydroalcoholic solutions

Using a polystyrene 96-well culture plate vertically-oriented as shown in Fig. 2, 100 µL of brain-heart infusion (3.7 %, w/v) was placed in each well of the microtitre plate, and 100 µL of the volatile solvent solution only in the wells corresponding to column A. Then two-fold serial dilutions were performed starting from column A to column F, but skipping columns B and C. Afterwards, 50 µL of the bacterial suspension were added to each well. Columns B and C were used as solvent evaporation control (SEC), and columns G and H as SVC. Columns B, C, G and H only contained culture media (100 µL) and bacterial inoculum (50 µL). The plate was incubated and the bacterial growth detected as described for slightly volatile solvents, except for the methanol-water-acetic acid solution. In this case, bacterial growth was detected visually through the observation of turbidity in the wells, because resazurin reacted immediately with acetic acid, turning the culture media pink and giving a false positive result. A different plate was used to test each solvent solution.

Non miscible solvents with the culture media

a) Volatile solvents: n-butanol

Polystyrene 96-well culture plates, horizontally-oriented (Fig. 3) and vertically-oriented (Fig. 4) were divided into sectors for the final tested n-butanol concentrations (FTC), and for SEC and SVC controls. The n-butanol solutions of different concentrations were prepared directly in the plate wells in the culture media; the corresponding solvent and brain-heart infusion volumes were added to the plate wells in order to obtain the desired final n-butanol concentration. Then, 50 µL of the bacterial suspension were added to each well in the plate. The final volume in each well was 150 µL: 100 µL corre-

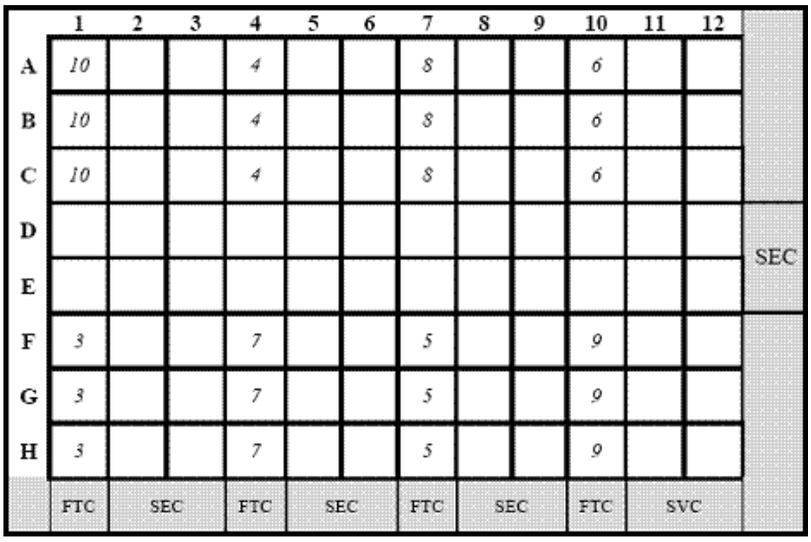

Fig. 3. Example of the final concentration tested in a microdilution plate (horizontally-oriented) for non miscible volatile solvents, e.g. n-butanol. Abbreviations: SVC, strain viability control; SSD, concentration of the solvent serial dilution (%, v/v); SEC, solvent evaporation control; FTC, final tested concentration (%, v/v).

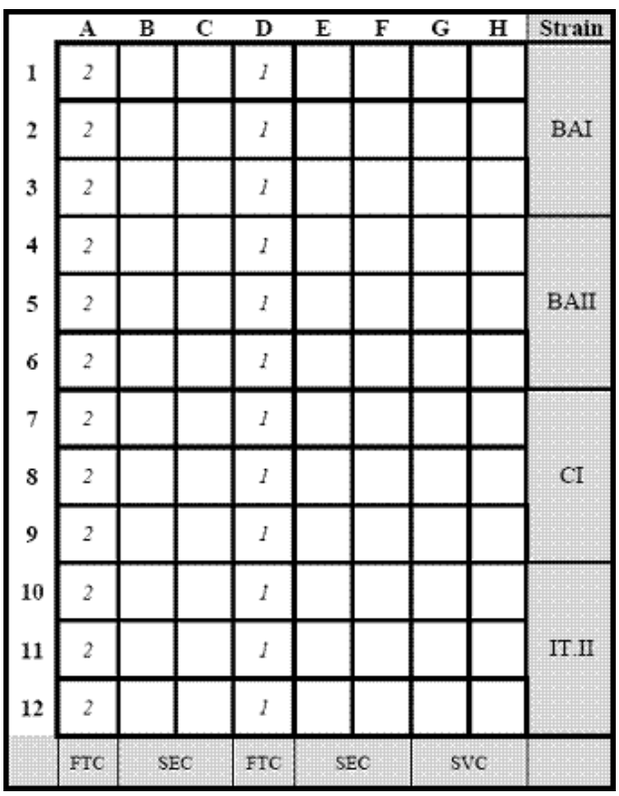

Fig. 4. Example of the final concentration tested in a microdilution plate (vertically-oriented) for non miscible volatile solvents, e.g. n-butanol. Abbreviations: SVC, strain viability control; SSD, concentration of the solvent serial dilution (%, v/v); SEC, solvent evaporation control; FTC, final tested concentration (%, v/v).

sponding to the sum of solvent and the culture media and 50 µL to the bacterial inoculum. For instance, in order to obtain a final n-butanol concentration of 10 % (v/v) in the plate well, 15 µL of pure n-butanol, 85 µL of brain-heart infusion (3.7 %, w/v) and 50 µL of the bacterial inoculum were placed in the well. The SEC and SVC wells contained 100 µL of brain-heart infusion (3.7 %, m/v) and 50 µL of the bacterial inoculum. Ten different n-butanol concentrations were tested. Final concentrations of n-butanol ranging between 3 and 10 % (v/v) were tested in horizontally-oriented plates (Fig. 3), and n-butanol 1 and 2% (v/v) in vertically-oriented plates. In the former, only one strain per plate was tested, while in the latter four strains were studied at a time. The plate was incubated and the bacterial growth detected as described for slightly volatile solvents.

Repeatability assays

Repeatability studies were performed using those aqueous solvent solutions with concentrations close to the MIC and MNIC values obtained in the broth microdilution assays described above. Repeatability assays within-day (n = 3) and between days (n = 4) were performed following the plate schemes presented in Figs. 1 and 2 for miscible solvents with the culture media, and in Fig. 4 for non miscible volatile solvents with the culture media.

Statistical analysis

Statistical analyses were performed using the statistical package Statistica 6.1 (StatSoft Inc., Tulsa, OK, USA, 1984–2004). Repeatabilities of the assays were evaluated in terms of percentage of relative standard deviation.

RESULTS

This paper presents a systematic study to

determine the MIC and MNIC of organic solvents and their aqueous mixtures, in order to establish the maximum contents of the organic solvent in the medium once the bacteria is put in contact with the extract or the pure compound.

During preliminary studies, bacterial growth inhibition was observed in the SVC columns as well as in wells containing the organic solvent in concentrations that had been previously tested and found to be innocuous to *P. larvae*; hence the antimicrobial activity results were not being repeatable. This phenomenon only occurred when relatively high concentrations of the volatile organic solvents were tested. It was explained by the evaporation of the volatile organic solvent during incubation from wells with higher concentrations and its partial dissolution in contiguous SVC wells or wells containing solutions with lower concentrations of organic solvent. This way the initial solvent concentration in these wells increased, and hence the antimicrobial activity results were distorted. Therefore, different experimental designs on the plates (Figs. 1 - 4) were carried out to study the different solvents, depending on their miscibility with aqueous culture media and volatility (Tab. S1, in the supplementary information). For volatile solvents, solvent evaporation controls (SEC) in adjacent columns to the wells containing the highest concentration of the solvent were included (Fig. 2, 3 and 4).

Minimum inhibitory concentration and maximum non inhibitory concentration of organic solvents

DMSO

Aqueous DMSO 18.75 % (v/v) (i.e. DMSO 6.25 % (v/v) in column A of the plate in Fig. 1) or lower concentrations allowed the growth of *P. larvae* in the entire plate (Tab. 2). Aq. DMSO 22.5 % (v/v) inhibited the bacterial growth in column A (DMSO 7.5 % (v/v) in plate) of strains BAI, BAII and CI, while strain IT.II grew normally in the whole plate. Aq. DMSO solutions of 27 % (v/v) and 30 % (v/v) inhibited bacterial growth in column A (DMSO 9 % (v/v) and 10 % (v/v) in plate, respectively) and allowed bacterial growth of the four strains tested from column B (DMSO 4.5 % (v/v) and 5 % (v/v) in the plate (Fig. 1), respectively) to F. Aq. DMSO 18.75 % (v/v), 22.5 % (v/v) and 30 % (v/v) were chosen to continue with the repeatability assays on the eight *P. larvae* strains to determine the MIC and MNIC values (Tab. 3). Aq. DMSO 18.75 % (v/v) allowed the growth of strains BAI, BAII and CI from column A (DMSO 6.25 % (v/v) in plate (Fig. 1)) in 50% of the assays; the remaining 50 % of the assays yielded bacterial growth from column B (DMSO 3.13 % (v/v) in plate) and next ones. Strains BAIII, ERI and IT.I grew from column A (DMSO 6.25 % (v/v) in plate) in 100 % of the repeatability assays. Strain IT.II grew from column A (DMSO 6.25 % (v/v) in plate) in 75 % of the assays, while strain BAIV grew from column B (DMSO 3.13 % (v/v) in plate) in 100 % of the assays. Aq. DMSO 22.5 % (v/v) and 30 % (v/v) inhibited the growth of strains BAI, BAII, CI, BAIII, ERI, IT.II and BAIV in column A (DMSO 7.5 % (v/v) and 10 % (v/v) in plate, respectively) in 100 % of the repeatability assays, and allowed the bacterial growth from column B (3.75 % (v/v) and 5 % (v/v) DMSO in plate, respectively). Strain IT.I behaved in the same manner as the rest of the strains tested when incubated with aq. DMSO 30 % (v/v), but aq. DMSO 22.5 % (v/v) solution inhibited strain IT.I growth in column A (7.5 % (v/v) DMSO in plate) in 25 % of the repeatability assays. According to these results, the MNIC of DMSO against *P. larvae* was 5 % (v/v) in plate, while the MIC of DMSO ranged between 6.25 % (v/v) (inhibition in 34.37 % of the assays) and 10 % (v/v) (inhibition in 100% of the assays) in plate (Tab. 4).

Acetonitrile

Aqueous acetonitrile solutions with concentrations of 18.75 % (v/v), 22.5 % (v/v), 30 % (v/v), 36 % (v/v) and 45% (v/v) allowed the growth of all *P. larvae* strains from column A (i.e. acetonitrile 6.25 % (v/v), 7.5 % (v/v), 10 % (v/v), 12 % (v/v) and 15 % (v/v) in plate (Fig. 2), respectively) (Tab. 2). Aq. acetonitrile 60 % (v/v) inhibited growth in column A (acetonitrile 20 % (v/v) in plate) and allowed bacterial growth from column D (acetonitrile 10 % (v/v) in plate). Aq. acetonitrile 36 % (v/v), 45 % (v/v) and 60 % (v/v) were chosen to perform the repeatability assays with eight *P. larvae* strains (Tab. 3). Aq. acetonitrile 36 % (v/v) allowed the bacterial growth of all strains from

Table 2

MIC and MNIC (%, v/v) for each solvent against *Paenibacillus larvae* obtained from the set of experiments performed using different initial solvent concentrations*

			MIC				MNIC			
Solvent	C**	Strain	BAI	BAII	CI	IT.II	BAI	BAII	CI	IT.II
DMSO	18.75		ni	ni	ni	ni	6.25	6.25	6.25	6.25
	22.5		7.5	7.5	7.5	ni	3.75	3.75	3.75	7.5
	27		9	9	9	9	4.5	4.5	4.5	4.5
	30		10	10	10	10	5	5	5	5
Acetonitrile	18.75		ni	ni	ni	ni	6.25	6.25	6.25	6.25
	22.5		ni	ni	ni	ni	7.5	7.5	7.5	7.5
	30		ni	ni	ni	ni	10	10	10	10
	36		ni	ni	ni	ni	12	12	12	12
	45		ni	ni	ni	ni	15	15	15	15
	60		20	20	20	20	10	10	10	10
Ethanol	18.75		ni	ni	ni	ni	6.25	6.25	6.25	6.25
	22.5		7.5	ni	7.5	ni	3.75	7.5	3.75	7.5
	30		5	10	5	ni	2.5	5	2.5	10
	36		12	12	12	12	6	6	6	6
Methanol	18.75		ni	ni	ni	ni	6.25	6.25	6.25	6.25
	22.5		ni	ni	ni	ni	7.5	7.5	7.5	7.5
	30		ni	ni	ni	ni	10	10	10	10
	36		ni	ni	ni	ni	12	12	12	12
	45		15	ni	15	15	7.5	15	7.5	7.5
n-Butanol	100		2	2	2	3	1	1	1	2

* Abbreviations: MIC, minimum inhibitory concentration (%, v/v); MNIC, maximum non inhibitory concentration (%, v/v); ni, non inhibitory.

** Initial concentration of aqueous solvent solution tested (%, v/v).

column A (acetonitrile 12 % (v/v) in plate (Fig. 2)) in 100% of the assays. With aq. acetonitrile 45 % (v/v), strains BAI, BAII, CI, ERI and IT.II grew from column A (acetonitrile 15 % (v/v) in plate) in 75 % of the repeatability assays; and in the remaining 25% of the assays, bacterial growth was observed from column D (7.5 % (v/v) acetonitrile in plate). Strains BAIII and BAIV grew from column A as well (acetonitrile 15 % (v/v) in plate) but only in 50 % of the assays, while strain IT.I grew in 25 % of the assays when that same concentration was present. Aq. acetonitrile 60 % (v/v) inhibited the growth of strains BAI, BAII, IT.I, IT.II and BAIV in column A (acetonitrile 20 % (v/v) in plate) in 100% of the repeatability assays and allowed their growth from column D (acetonitrile 10 % (v/v) in plate). Strain BAIII growth was inhibited in column A (acetonitrile 20 % (v/v) in plate) and in column D (acetonitrile 10 % (v/v) in plate) in 50 % of the assays. Strains CI and ERI showed inhibition in column A (acetonitrile 20 % (v/v) in plate) in 100 % of the assays, while in column D (acetonitrile 10 % (v/v)) inhibition occurred in 25 % of the assays. When the aq. acetonitrile 36 % (v/v) was used, no bacterial growth inhibition was observed in column A (acetonitrile 12 % (v/v) in plate) in 100% of the assays for all strains tested. However, when the aq. acetonitrile 60 % (v/v) was tested, inhibition at 10 % (v/v) acetonitrile in plate was observed in 12.5 % of the assays. Thus, differences in the inhibiting effective concentration were observed depending on the concentration of the initial aq. acetonitrile solution tested, indicating that these acetonitrile concentrations represent the limit between the growth and

Table 3

MIC and MNIC (%, v/v) for each solvent against *Paenibacillus larvae* obtained from the set of experiments performed to study the repeatability of the broth microdilution method*

			MIC								MNIC							
Solvent	C**	Day Strain	BAI	BAII	CI	BAIII	ERI	IT.I	IT.II	BAIV	BAI	BAII	CI	BAIII	ERI	IT.I	IT.II	BAIV
DMSO	18.75	1	ni	6.25	ni	ni	ni	ni	ni	6.25	6.25	3.13	6.25	6.25	6.25	6.25	6.25	3.13
		2	6.25	ni	6.25	ni	ni	ni	ni	6.25	3.13	6.25	3.13	6.25	6.25	6.25	6.25	3.13
		3	ni	ni	ni	ni	ni	ni	ni	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	3.13
		4	6.25	6.25	6.25	ni	ni	ni	6.25	6.25	3.13	3.13	3.13	6.25	6.25	6.25	3.13	3.13
	22.5	1	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
		2	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
		3	7.5	7.5	7.5	7.5	7.5	ni	7.5	7.5	3.75	3.75	3.75	3.75	3.75	7.5	3.75	3.75
		4	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
	30	1	10	10	10	10	10	10	10	10	5	5	5	5	5	5	5	5
		2	10	10	10	10	10	10	10	10	5	5	5	5	5	5	5	5
		3	10	10	10	10	10	10	10	10	5	5	5	5	5	5	5	5
		4	10	10	10	10	10	10	10	10	5	5	5	5	5	5	5	5
Acetonitrile	36	1	ni	ni	ni	ni	ni	ni	ni	ni	12	12	12	12	12	12	12	12
		2	ni	ni	ni	ni	ni	ni	ni	ni	12	12	12	12	12	12	12	12
		3	ni	ni	ni	ni	ni	ni	ni	ni	12	12	12	12	12	12	12	12
		4	ni	ni	ni	ni	ni	ni	ni	ni	12	12	12	12	12	12	12	12
	45	1	ni	ni	ni	ni	ni	15	ni	15	15	15	15	15	15	7.5	15	7.5
		2	ni	ni	ni	15	ni	ni	ni	ni	15	15	15	7.5	15	15	15	15
		3	ni	ni	ni	15	15	15	ni	15	15	15	15	7.5	7.5	7.5	15	7.5
		4	15	15	15	ni	ni	15	15	ni	7.5	7.5	7.5	15	15	7.5	7.5	15
	60	1	20	20	20	10	20	20	20	20	10	10	10	5	10	10	10	10
		2	20	20	20	10	10	20	20	20	10	10	10	5	5	10	10	10
		3	20	20	10	20	20	20	20	20	10	10	5	10	10	10	10	10
		4	20	20	20	20	20	20	20	20	10	10	10	10	10	10	10	10
Ethanol	22.5	1	ni	ni	ni	ni	ni	ni	ni	ni	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
		2	ni	ni	ni	ni	ni	ni	ni	ni	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
		3	ni	ni	ni	ni	ni	ni	ni	ni	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
		4	ni	ni	ni	ni	ni	ni	ni	ni	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
	30	1	ni	10	10	10	ni	10	ni	ni	10	5	5	5	10	5	10	10
		2	10	ni	10	ni	ni	ni	ni	10	5	10	5	10	10	10	10	5
		3	10	10	10	ni	10	10	ni	10	5	5	5	10	5	5	10	5
		4	10	10	10	ni	ni	ni	10	10	5	5	5	10	10	10	5	5
	36	1	12	12	12	12	12	12	12	12	6	6	6	6	6	6	6	6
		2	12	12	12	12	12	12	12	12	6	6	6	6	6	6	6	6
		3	12	12	12	12	12	12	12	12	6	6	6	6	6	6	6	6
		4	12	12	12	12	12	12	12	12	6	6	6	6	6	6	6	6

			MIC								MNIC							
Solvent	C**	Day Strain	BAI	BAII	CI	BAIII	ERI	IT.I	IT.II	BAIV	BAI	BAII	CI	BAIII	ERI	IT.I	IT.II	BAIV
Methanol	36	1	ni	ni	ni	ni	ni	ni	ni	ni	12	12	12	12	12	12	12	12
		2	ni	ni	ni	ni	ni	ni	ni	ni	12	12	12	12	12	12	12	12
		3	ni	ni	ni	ni	ni	ni	ni	ni	12	12	12	12	12	12	12	12
		4	ni	ni	ni	ni	ni	ni	ni	ni	12	12	12	12	12	12	12	12
	45	1	ni	ni	ni	ni	ni	ni	ni	ni	15	15	15	15	15	15	15	15
		2	ni	ni	15	ni	ni	ni	ni	15	15	15	7.5	15	15	15	15	7.5
		3	15	15	15	ni	ni	ni	15	15	7.5	7.5	7.5	15	15	15	7.5	7.5
		4	ni	15	ni	ni	ni	ni	ni	ni	15	7.5	15	15	15	15	15	15
Methanol-water-acetic acid***	30: 69: 1	1	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04
		2	2.5: 97.42: 0.08	2.5: 97.42: 0.08	5: 94.84: 0.16	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	1.25: 98.71: 0.04	1.25: 98.71: 0.04	2.5: 97.42: 0.08	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04
		3	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04
		4	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	2.5: 97.42: 0.08	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04	1.25: 98.71: 0.04
n-Butanol	100	1	2	2	2	3	2	2	2	2	1	1	1	2	1	1	1	1
		2	2	3	3	3	3	3	3	3	1	2	2	2	2	2	2	2
		3	2	2	2	2	3	2	2	3	1	1	1	1	2	1	1	2
		4	2	2	2	3	2	3	3	2	1	1	1	2	1	2	2	1

* Abbreviations: See Table 2.
** Initial concentration of aqueous solvent solution tested (%, v/v).
*** Concentration expressed as % (v/v/v).

inhibition of the bacteria. The MNIC of acetonitrile against *P. larvae* is 7.5 % (v/v) in plate, while the MIC ranges between acetonitrile 10 % (v/v) and 15 % (v/v) in plate (inhibition in 16.66 % of the assays). At acetonitrile at 20 % (v/v) in plate, inhibition occurs in 100 % of the assays (Tab. 4).

Ethanol

Aqueous ethanol 18.75 % (v/v) allowed the growth of all strains from column A (ethanol 6.25 % (v/v) in plate (Fig. 2)) (Tab. 2). Aq ethanol 22.5 % (v/v) inhibited the growth of strains BAI and CI in column A (ethanol 7.5 % (v/v) in plate), while strains BAII and IT.II grew in the presence of this ethanol concentration. Strains BAI and CI were inhibited by aq. ethanol 30 % (v/v) in columns A and D (ethanol 10 % (v/v) and 5 % (v/v) in plate, respectively), while strain BAII was only inhibited in column A (ethanol 10 % (v/v) in plate), and strain IT.II grew even in the presence of the highest concentration (ethanol 10 % (v/v) in plate). Aq. ethanol 36 % (v/v) inhibited the growth of all strains tested in column A (ethanol 12 % (v/v) in plate). Aq. ethanol 22.5 % (v/v), 30 % (v/v) and 36 % (v/v) were chosen to perform the repeatability assays with eight *P. larvae* strains

Table 4
MIC and MNIC (%, v/v) for each solvent against *Paenibacillus larvae* established for the broth microdilution method*

Solvent	MIC			MNIC
DMSO	6.25	–	10	5
Acetonitrile	10	–	20	7.5
Ethanol	10	–	12	7.5
Methanol	15			12
Methanol-water-acetic acid**	2.5: 97.42: 0.08	–	5: 94.84: 0.16	1.25: 98.71: 0.04
n-Butanol	2	–	3	1

* Abbreviations: See Table 2.
** Concentration expressed as % (v/v/v).

(Tab. 3). Aq. ethanol at 22.5 % (v/v) allowed the growth of all strains from column A (ethanol 7.5 % (v/v) in plate (Fig. 2)) in 100 % of the assays. With aq. ethanol 30 % (v/v), strain CI growth was inhibited in column A (ethanol 10 % (v/v) in plate) in 100 % of the assays, for strains BAI, BAII and BAIV in 75 % of the assays, for strain IT.I in 50 % of the assays, and strains BAIII, ERI and IT.II in 25 % of the assays. Aq. ethanol at 36 % (v/v) inhibited the growth of all eight *P. larvae* strains in column A (ethanol 12 % (v/v) in plate) in 100% of the assays. These results indicate that the MNIC of ethanol against *P. larvae* is 7.5 % (v/v), and the MIC ranges between 10 % (v/v) (inhibition in 56.25 % of the assays) and 12 % (v/v) (inhibition in 100% of the assays) (Tab. 4).

Methanol

Aqueous methanol at 18.75 % (v/v), 22.5 % (v/v), 30 % (v/v) and 36% (v/v) allowed the growth of all strains from column A (methanol 6.25 % (v/v), 7.5 % (v/v), 10 % (v/v) and 12 % (v/v) in plate (Fig. 2), respectively) (Tab. 2). Aq. methanol 45 % (v/v) inhibited the growth of strains BAI, CI and IT.II in column A (methanol 15 % (v/v) in plate), while strain BAII grew in the presence of such methanol concentration. When aq. methanol 60 % (v/v) and 80 % (v/v) were tested (i.e. methanol 20 % (v/v) and 27 % (v/v) in column A of the plate, respectively), results were inconclusive, probably due to partial evaporation of the solvent during incubation. Indeed, complete inhibition of all strains in the SEC columns was observed, as it happened in the preliminary assays described above. When using aq. methanol 60 % (v/v) and 80 % (v/v), partial evaporation of methanol occured during incubation, and part of this methanol vapour redissolved in the solutions of the adjacent wells, resulting in the inhibition of bacterial growth also in these wells. Aq. methanol 36 % (v/v) and 45 % (v/v) were chosen to perform the repeatability assays with eight *P. larvae* strains (Tab. 3). Aq. methanol 36 % (v/v) allowed the growth of all the strains from column A (methanol 12 % (v/v) in plate (Fig. 2)). With aq. methanol 45 % (v/v), strains BAI and IT.II were inhibited in column A (methanol 15 % (v/v) in plate) in 25 % of the assays, while strains BAII, CI and BAIV in 50 % of the assays. Strains BAIII, ERI and IT.I grew normally when incubated at such concentration (methanol 15 % (v/v) in plate). Therefore, MNIC and MIC of methanol against *P. larvae* are 12% (v/v) and 15 % (v/v) (inhibition in 25% of the assays) in plate respectively (Tab. 4).

Acidified hydroalcoholic solution

The growth of strains BAI, BAII, BAIII, ERI, IT.I,

IT.II and BAIV was inhibited in 100 % of the repeatability assays in columns A (methanol-water-acetic acid, 10:89.67:0.33, v/v/v in plate (Fig. 2)), D (methanol-water-acetic acid, 5:94.84:0.16, v/v/v in plate) and E (methanol-water-acetic acid, 2.5:97.42:0.08, v/v/v in plate) (Tab. 3). Bacterial growth was only observed in column F (methanol-water-acetic acid, 1.25:98.71:0.04, v/v/v in plate). Strain CI presented the same behaviour as the rest of the strains tested in 75% of the repeatability assays, while in the remaining 25% of the assays, it grew from column E (methanol-water-acetic acid, 2.5:97.42:0.08, v/v/v in plate) to F. Hence, the MNIC of the methanol-water-acetic acid mixture against *P. larvae* is 1.25:98.71:0.04 (v/v/v) in plate, and the MIC ranges between 2.5:97.42:0.08 (v/v/v) in plate (inhibition in 96.87 % of the assays) and 5:94.84:0.16 (v/v/v) in plate (inhibition in 100% of the assays) (Tab. 4).

n-Butanol

Final n-butanol concentrations of 1% (v/v) to 10% (v/v) in plate were tested (Fig. 3 and Fig. 4). Concentrations of 3% (v/v) n-butanol or higher inhibited the growth of all strains tested (Tab. 2). Strains BAI, BAII and CI were inhibited by n-butanol 2 % (v/v), while strain IT.II grew in the presence of this n-butanol concentration. The final n-butanol concentration of 1 % (v/v) in plate allowed the growth of all strains tested. Therefore n-butanol concentrations of 3 % (v/v), 2 % (v/v) and 1 % (v/v) were chosen to continue with the repeatability assays using eight P. larvae strains (Tab. 3). In the presence of n-butanol 3 % (v/v), all strains tested were inhibited in 100 % of the assays (Fig. 4). With n-butanol 2 % (v/v), strain BAI presented inhibition in 100% of the assays, strains BAII and CI in 75 % of the assays, strains ERI, IT.I, IT.II and BAIV in 50 % of the assays, and strain BAIII in 25 % of the assays. When testing n-butanol at 1 % (v/v), all strains tested grew normally in 100 % of the assays. Thus, the MNIC of n-butanol against P. larvae is 1 % (v/v), and the MIC value ranges between 2 % (v/v) (inhibition in 59.37 % of the assays) and 3 % (v/v) (inhibition in 100% of the assays) (Tab. 4).

DISCUSSION

The organic solvents, ethanol, methanol, n-butanol and acetonitrile, which are the most commonly used to extract bioactive natural products from plants or to dissolve pure natural compounds, were studied. Hydroalcoholic solutions are usually used as well for the extraction of polyphenols, which are one of the major classes of natural bioactive products due to their antioxidant properties (Mihai et al., 2012). Acidified hydromethanolic solutions are usually used for the extraction of polyphenols from plants and agricultural food products (vegetables, fruits, beverages, beehive products) (Alonso-Salces et al., 2009; Abad-García et al., 2012; Biesaga and Pyrzyńska, 2013; Ramirez-Ambrosi et al., 2013), so the solution methanol-water-acetic acid (30:69:1, v/v/v) was particularly selected to be assayed.

Other extracting solvents such as ethyl acetate (Lokvam et al., 2000; Boligon et al., 2013), chloroform (Reyes et al., 2013), dichloromethane (Boligon et al., 2013; Hernández-López et al., 2014), and hexane (Sabaté et al., 2012) have been used to extract or dissolve bioactive compounds to be tested in antimicrobial activity assays against *P. larvae*, but they could not be assayed in the present study because the polystyrene plates commonly used for the broth microdilution method present low resistance against these solvents. The antimicrobial activity of extracts or pure natural compounds dissolved in such solvents have to be determined by using other methods such as agar diffusion (Lokvam et al., 2000; Reyes et al., 2013; Hernández-López et al., 2014) or agar dilution (Reyes et al., 2013). However, if broth microdilution is the method of choice, the solvent should be evaporated, and the extract dissolved using a polystyrene-compatible solvent prior to perform the assay (Sabaté et al., 2012). DMSO is a solvent commonly used to dissolve extracts after evaporation and so was included in this study.

Although the antimicrobial activity of natural products is widely reported in literature, controversial results have been found because of

the diversity of criteria and methods employed, as well as the omission of appropriate controls and experimental details (Allegrini et al., 1973; Pellecuer et al., 1976; Ríos and Recio, 2005). The broth microdilution method is currently one of the most widely used methods to determine the MIC value of a substance. In this regard, one of the major issues is that most natural products must be dissolved in organic solvents or their aqueous mixtures due to their lipophilic nature, in order to determine their antimicrobial activity by this method. Therefore, it is essential to know the final concentration of the organic solvent in the broth once the bacteria are put in contact with the extract or the pure compound solution, in order to assure that the antimicrobial activity observed is only due to the tested substance and not to the organic solvent. In this sense, the literature related to the antimicrobial activity against *P. larvae* usually provides little information about several important issues regarding the organic solvents present, for instance: *i*) whether the organic solvent contained in the extracts is evaporated and the extract redissolved in another suitable solvent is not reported (Gallardo et al., 2004; Boligon et al., 2013); *ii*) the nature of the solvent in which the natural pure compounds are dissolved in is not included (Feldlaufer et al., 1993; Hornitzky, 2003; Gallardo et al., 2004; Sabaté et al., 2012; Boligon et al., 2013; Hernández-López et al., 2014); *iii*) the methodology carried out to determine the antimicrobial activity is not detailed, which makes extremely difficult or even impossible to calculate the final concentration of the organic solvent in contact with the bacteria (Feldlaufer et al., 1993; Lokvam et al., 2000; Hornitzky, 2003; Gallardo et al., 2004; Flesar et al., 2010; Sabaté et al., 2012; Boligon et al., 2013; Reyes et al., 2013; Hernández-López et al., 2014); *iv)* whether control assays are performed using the organic solvent in the same concentration as it was in the extract or in the pure compound solution tested is not mentioned (Gallardo et al., 2004; Boligon et al., 2013).

All these issues have been faced and taken into account in the present work. Thus, a systematic approach was developed to establish the maximum concentration of the organic solvent in the extract or in the pure compound solution to be tested in order to determine its antimicrobial activity against *P. larvae* by the broth microdilution method. As a result, the MIC and MNIC of the studied solvents against *P. larvae* were disclosed (Tab. 4). Regarding the MNIC and MIC of methanol (12% (v/v) and 15 % (v/v) in plate respectively), the fact that methanol concentrations in plate in the assays of the acidified hydromethanolic solution were lower than the MNIC and MIC of methanol leads to the conclusion that acetic acid at 1% (v/v) in the initial acified hydromethanolic solution is responsible for the inhibition of bacterial growth, or that there is a synergic effect between acetic acid and methanol, which increases the antibacterial effectiveness of each solvent in the mixture. The antiseptic properties of acetic acid have been already reported in the bibliography (Chun et al., 2014).

From the results achieved in the present study, the maximum concentrations of organic solvents in contact with *P. larvae* are established: 5% (v/v) of DMSO, 7.5% (v/v) of acetonitrile, 7.5% (v/v) of ethanol, 12% (v/v) of methanol, 1% (v/v) of n-butanol, and methanol-water-acetic acid (1.25:98.71:0.04, v/v/v). The knowledge of the concentrations of the different organic solvents at which no inhibition of bacterial growth is observed, as well as the concentrations that produce such inhibition provides crucial and useful information for further studies on the antimicrobial activities of natural products against *P. larvae* as well as for future research on different fields related to *P. larvae*. Furthermore, the systematic study proposed and presented in this paper will contribute as a starting point to properly perform antimicrobial studies in other biological models.

ABBREVIATIONS

AFB, American Foulbrood; C, concentration; CLSI, Clinical and Laboratory Standards Institute; DMSO, dimethylsulfoxide; FTC, final tested concentration; MIC, minimum inhibitory concentration; MNIC, maximum non inhibitory concentration; n.i., non inhibitory; n.t., not tested; SEC,

solvent evaporation control; SSD, concentration of the solvent serial dilution; SVC, strain viability control; and VOC, Volatile organic compound.

ACKNOWLEDGEMENTS

This work was supported/funded by PICT *"Apicultura: control de loque americana mediante estrategias no contaminantes basadas en productos naturales"* project (PICT2014-3264). María Celeste Pellegrini wishes to thank the Comisión de Investigaciones Científicas (CIC) and Noelia Melina Cugnata and Elisa Guaspari to Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), for their Ph.D. Grants.

REFERENCES

Abad-García, B., Garmón-Lobato, S., Berrueta, L. A., Gallo, B., Vicente, F. (2012). On line characterization of 58 phenolic compounds in Citrus fruit juices from Spanish cultivars by high-performance liquid chromatography with photodiode-array detection coupled to electrospray ionization triple quadrupole mass spectrometry. *Talanta, 99*, 213-224. http://doi.org/10.1016/j.talanta.2012.05.042

Alippi, A. M. (1991). Evaluation of culture media for detecting the starch hydrolysis reaction in pathovars of *Xanthomonas campestris*. *Revista Argentina de Microbiologia, 23*(1), 41-47.

Alippi, A. M. (1992). Characterization of Bacillus larvae White, the causative agent of American foulbrood of honey-bees. First record of its occurrence in Argentina. *Revista Argentina de Microbiologia, 24*(2), 67-72.

Alippi, A. M. (1996). Antimicrobial activity of some essential oils against *Paenibacillus larvae*, causal agent of AFB. *Journal of Herbs, Spices and Medicinal Plants, 4*(2), 9-15.

Alonso-Salces, R. M., Guillou, C., & Berrueta, L. A. (2009). Liquid chromatography coupled with ultraviolet absorbance detection, electrospray ionization, collision-induced dissociation and tandem mass spectrometry on a triple quadrupole for the on-line characterization of polyphenols and methylxanthines in green coffee beans. *Rapid Communications in Mass Spectrometry, 23*(3), 363-383. http://doi.org/10.1002/rcm.3884

Allegrini, J., Simeon de Buochberg, M., Maillois, H., & Boillot, A. (1973). Emulsions d'huiles essentielles fabrication et applications en microbiologic. *Travaux de la Société de Pharmacie de Montpellier, 33*, 73-86.

Antúnez, K., Harriet, J., Gende, L., Maggi, M., Eguaras, M., Zunino, P. (2008). Efficacy of natural propolis extract in the control of American Foulbrood. *Veterinary Microbiology, 131*(3-4), 324-331.

Bachanová, K., Klaudiny, J., Kopernický, J., & Šimúth, J. (2002). Identification of honeybee peptide active against *Paenibacillus larvae larvae* through bacterial growth-inhibition assay on polyacrylamide gel. *Apidologie, 33*(3), 259-269.

Biesaga, M., & Pyrzyńska, K. (2013). Stability of bioactive polyphenols from honey during different extraction methods. *Food Chemistry, 136*(1), 46-54.

Bíliková, K., Mirgorodskaya, E., Bukovská, G., Gobom, J., Lehrach, H., Šimúth, J. (2009). Towards functional proteomics of minority component of honeybee royal jelly: The effect of post-translational modifications on the antimicrobial activity of apalbumin2. *Proteomics, 9*(8), 2131-2138.

Bíliková, K., Popova, M., Trusheva, B., & Bankova, V. (2013). New anti-*Paenibacillus larvae* substances purified from propolis. *Apidologie, 44*(3), 278-285.

Boligon, A. A., Brum, T. F. D., Zadra, M., Piana, M., Alves, C. F. D. S., Fausto, V. P., ... Athayde, M. L. (2013). Antimicrobial activity of *Scutia buxifolia* against the honeybee pathogen *Paenibacillus larvae*. *Journal of Invertebrate Pathology, 112*(2), 105-107.

CLSI. (2009a). Methods for dilution antimicrobial susceptibility testing for bacteria that grew aerobically. Approved Standard M7-A10. Wayne, Pennsylvania: Clinical and Laboratory Standards Institute.

CLSI. (2009b). Performance standards for antimicro-

bial disk susceptibility tests. Approved standard M2-A10. Wayne, Pennsylvania: Clinical and Laboratory Standards Institute.

Chun, A. Y., Yunxiao, L., Ashok, S., Seol, E., Park, S. (2014). Elucidation of toxicity of organic acids inhibiting growth of *Escherichia coli* W. *Biotechnology and Bioprocess Engineering, 19*(5), 858-865. http://doi.org/10.1007/s12257-014-0420-y

Damiani, N., Fernández, N., Porrini, M., Gende, L. B., Álvarez, E., Buffa, ... Eguaras, M. J. (2014). Laurel leaf extracts for honeybee pest and disease management: Antimicrobial, microsporicidal, and acaricidal activity. *Parasitology Research, 113*(2), 701-709.

De Graaf, D. C., Alippi, A. M., Antúnez, K., Aronstein, K. A., Budge, G., De Koker, D., ... Genersch, E. (2013). Standard methods for American foulbrood research. *Journal of Apicultural Research, 52*(1). http://dx.doi.org/10.3896/IBRA.1.52.1.11

Dickert, H., Machka, K., & Braveny, I. (1981). The uses and limitations of disc diffusion in the antibiotic sensitivity testing of bacteria. *Infection, 9*(1), 18-24.

Dingman, D., & Stahly, D. P. (1983). Medium promoting sporulation of *Bacillus larvae* and metabolism of medium components. *Applied and Environmental Microbiology, 46*(4), 860-869.

Eguaras, M. J., Fuselli, S. R., Gende, L. B., Fritz, R., Ruffinengo, S. R., Clemente, G., ... Ponzi, M. I. (2005). An in vitro evaluation of *Tagetes minuta* essential oil for the control of the honeybee pathogens *Paenibacillus larvae* and *Ascosphaera apis*, and the parasitic mite *Varroa destructor*. *Journal of Essential Oil Research, 17*(3), 336-340.

Feldlaufer, M. F., Knox, D. A., Lusby, W. R., & Shimanuki, H. (1993). Antimicrobial activity of fatty acids against *Bacillus larvae*, the causative agent of American foulbrood disease. *Apidologie, 24*(2), 95-99.

Flesar, J., Havlik, J., Kloucek, P., Rada, V., Titera, D., Bednar, M., Stropnicky, M., & Kokoska, L. (2010). In vitro growth-inhibitory effect of plant-derived extracts and compounds against *Paenibacillus larvae* and their acute oral toxicity to adult honey bees. *Veterinary Microbiology, 145*(1-2), 129-133.

Fuselli, S. R., García De La Rosa, S. B., Gende, L. B., Eguaras, M. J., Fritz, R. (2006a). Antimicrobial activity of some Argentinian wild plant essential oils against *Paenibacillus larvae larvae*, causal agent of American foulbrood (AFB). *Journal of Apicultural Research, 45*(1), 2-7.

Fuselli, S. R., García De La Rosa, S. B., Gende, L. B., Eguaras, M. J., Fritz, R. (2006b). Inhibition of *Paenibacillus larvae* employing a mixture of essential oils and thymol. *Revista Argentina de Microbiologia, 38*(2), 89-92.

Gallardo, G. L., Peña, N. I., Chacana, P., Terzolo, H. R., Cabrera, G. M. (2004). L-Tenuazonic acid, a new inhibitor of *Paenibacillus larvae*. *World Journal of Microbiology and Biotechnology, 20*(6), 609-612. http://doi.org/10.1023/B:WIBI.0000043175.23621.8c

Genersch, E., Forsgren, E., Pentikäinen, J., Ashiralieva, A., Rauch, S., Kilwinski, J., Fries, I. (2006). Reclassification of *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation. *International Journal of Systematic and Evolutionary Microbiology, 56*(3), 501-511. http://doi.org/10.1099/ijs.0.63928-0

Hernández-López, J., Crockett, S., Kunert, O., Hammer, E., Schuehly, W., Bauer, R., Crailsheim, K., Riessberger-Gallé, U. (2014). In vitro growth inhibition by *Hypericum* extracts and isolated pure compounds of *Paenibacillus larvae*, a lethal disease affecting honeybees worldwide. *Chemistry and Biodiversity, 11*(5), 695-708.

Hornitzky, M. A. Z. (2003). Fatty acids-an alternative control strategy for honeybee diseases: (Report 0642585962). Kingston, Australia: Rural Industries Research and Development Corporation, Autralia Government.

Lokvam, J., Braddock, J. F., Reichardt, P. B., & Clausen, T. P. (2000). Two polyisoprenylated benzophenones from the trunk latex of *Clusia grandiflora* (Clusiaceae). *Phytochemistry, 55*(1), 29-34.

Mihai, C. M., Mârghitaș, L. A., Dezmirean, D. S., Chirilâ, F., Moritz, R. F. A., Schlüns, H. (2012). Interactions among flavonoids of propolis affect antibacterial activity against the honeybee pathogen *Paenibacillus larvae*. *Journal of Invertebrate Pathology, 110*(1), 68-72.

Mutinelli, F. (2003). European legislation governing the authorization of veterinary medicinal products with particular reference to the use of drugs for the control of honey bee diseases. *Apiacta, 38*, 156-168.

Nordström, S., & Fries, I. (1995). A comparison of media and cultural conditions for identification of *Bacillus larvae* in honey. *Journal of Apicultural Research, 34*(2), 97-103.

Pellecuer, J., Allegrini, J., & Simeon De Buochberg, M. (1976). Bactericidal and fungicidal essential oils. *Revue de l'Institut Pasteur de Lyon, 9*(2), 135-159.

Ramirez-Ambrosi, M., Abad-Garcia, B., Viloria-Bernal, M., Garmon-Lobato, S., Berrueta, L. A., Gallo, B. (2013). A new ultrahigh performance liquid chromatography with diode array detection coupled to electrospray ionization and quadrupole time-of-flight mass spectrometry analytical strategy for fast analysis and improved characterization of phenolic compounds in apple products. *Journal of Chromatography A, 1316*, 78-91. http://doi.org/10.1016/j.chroma.2013.09.075

Recio, M. C., Rios, J. L., & Villar, A. (1989). A review of some antimicrobial compounds isolated from medicinal plants reported in the literature 1978-1988. *Phytotherapy Research, 3*(4), 117-125.

Reyes, M. G., Torres, M. J., Maggi, M. D., Marioli, J. M., Gil, R. R., Sosa, V. E., Uriburu, M. L., Audisio, M. C. (2013). In vitro inhibition of *Paenibacillus larvae* by different extracts and pure compounds from *Flourensia* spp. *Industrial Crops and Products, 50*, 758-763.

Ríos, J. L., & Recio, M. C. (2005). Medicinal plants and antimicrobial activity. *Journal of Ethnopharmacology, 100*(1-2), 80-84. http://doi.org/10.1016/j.jep.2005.04.025

Sabaté, D. C., Gonzaléz, M. J., Porrini, M. P., Eguaras, M. J., Audisio, M. C., Marioli, J. M. (2012). Synergistic effect of surfactin from *Bacillus subtilis* C4 and Achyrocline satureioides extracts on the viability of *Paenibacillus larvae*. *World Journal of Microbiology and Biotechnology, 28*(4), 1415-1422.

SUPPLEMENTARY INFORMATION

Table S1

Physicochemical parameters of solvents.

Solvent	Water solubility (% w/w)*	Boiling point (°C)	Vapour pressure 20 °C (kPa)**	References***
Ethyl acetate	8.7	76.5 - 77.5	9.73	Sigma-Aldrich
Acetonitrile	100 (miscible)	81 - 82	9.86	Sigma-Aldrich
Acetic acid	100 (miscible)	117 - 118	1.50	Sigma-Aldrich
Water	100 (miscible)	100	2.30	CTR scientific
Chloroform	0.81	60.5 - 61.5	21.33	Sigma-Aldrich
Dichloromethane	1.6	40	47.00	Sigma-Aldrich
Dimethylsulfoxide	100 (miscible)	189	0.05	Sigma-Aldrich
Ethanol	100 (miscible)	78	5.90	Sigma-Aldrich
Hexane	0.001	69	17.60	Sigma-Aldrich
Methanol	100 (miscible)	64.70	13.03	Sigma-Aldrich
n-Butanol	0.43	116 - 118	0.50	Sigma-Aldrich

* The data was extracted from the solvent miscibility table provided by Cienytech (Tabla de Miscibilidad de Disolventes, www.cienytech.com/ES/datos.htm, access date 16th November 2015).
** Volatile organic compound (VOC) is defined as any organic compound having at 293,15 K a vapour pressure of 0,01 kPa or more, or having a corresponding volatility under the particular conditions of use; and organic compound as any compound containing at least the element carbon and one or more of hydrogen, oxygen, sulphur, phosphorus, silicon, nitrogen, or a halogen, with the exception of carbon oxides and inorganic carbonates and bicarbonates.[1,2]
*** Solvent boiling point and vapour pressure data were extracted from the data sheet provided by these laboratories.

[1] Directive, V. S. E., Council Directive 1999/13/EC of 11 March 1999 on the limitation of emissions of volatile organic compounds due to the use of organic solvents in certain activities and installations. In Union, T. E. P. a. t. C. o. t. E., Ed. Official Journal of the European Union: 1999; Vol. 1999/13/EC, pp 0001 - 0022.
[2] Directive, V. S. E., Directive 2004/42/CE of the European Parliament and of the Council of 21 April 2004 on the limitation of emissions of volatile organic compounds due to the use of organic solvents in certain paints and varnishes and vehicle refinishing products and amending Directive 1999/13/EC. In Union, T. E. P. a. t. C. o. t. E., Ed. Official Journal of the European Union: 2004; Vol. 1999/13/EC, pp 0087 - 0096.