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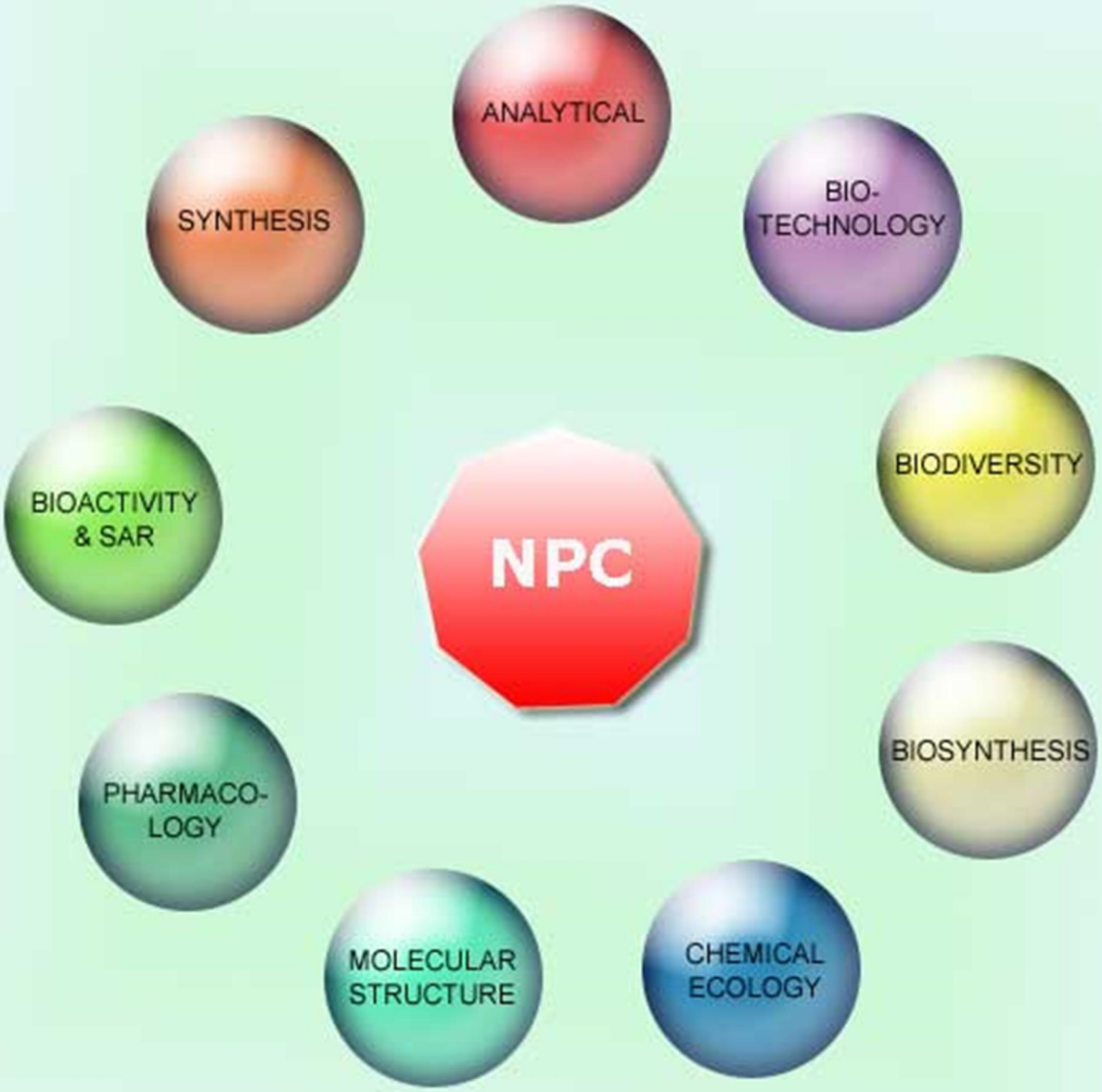

This Issue is Dedicated to
Professor Dr DHC Yoshinori Asakawa
On the Occasion of his 75th Birthday

Volume 11. Issue 8. Pages 1057-1206. 2016
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

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NPC Natural Product Communications

2016
Vol. 11
No. 8
1061 - 1064

A Puna Collection of *Senecio punae*, Main Source of a Versatile Eremophilane-type Ketone

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Received: March 22nd, 2016; Accepted: June 27th, 2016

Senecio punae Cabrera (Asteraceae–Senecioneae) is an endemic species from Puna semi-desert region of Argentina.The major compound, 4β,5β-eremophil-7(11)9-dien-8-one, also known as dehydrofukinone (D), was isolated from the diethyl ether extract (E) of the plant. The phytochemical constituents from *S. punae* are presented here for the first time, as well as bioassays employing D, a molecule with versatility to carry out many different biological activities. E and D showed acute molluscicidal activity against the bilharzia vector snail *Biomphalaria peregrina* with LD_{50} values of 68.6 and 16.7 µg/mL, respectively. D exerted moderate and strong effects against promastigotes of *Leishmania amazonensis* and *L. braziliensis*, with IC_{50} values of 34.3±1.2 and 9.93±0.17 µg/mL, respectively. Antibacterial effects were also found. Diethyl ether extract (E) and dehydrofukinone (D) were slightly active against *Pseudomonas aeruginosa* and *Staphylococcus aureus* at 50 and 25 µg/mL, respectively. However, D and E improved the effectiveness of oxacillin at 6 µg/mL against *S. aureus* growth and its biofilm, but did not affect beneficial lactobacilli strains. Synergistic effects were also measured between D (23 µg/mL) and ciprofloxacin (0.25 µg/mL) against two *P. aeruginosa* strains (FIC index = 0.38), as well as against two *S. aureus* strains (FIC indexes = 0.38 and 0.25). Finally, when 250 µg of either dehydrofukinone (D) or diethyl ether extract (E) were incorporated into the diet of the pest *Oryzaephillus surinamensis*, an alteration in the feeding behavior of the insect (Repellency indexes = +60 and +10, respectively) was observed.

Keywords: *Senecio punae*, Dehydrofukinone, Molluscicides, Leshmanicides, Bio-repellents, Antibacterial activity, Selective synergistic interactions.

Senecio is one of the largest and most complex Asteraceae genera. More than 1500 species have been reported and they are spread all over the world. The genus is characterized by the occurrence of toxic pyrrolizidine alkaloids, eremophilane and furanoeremophilane-type sesquiterpenes [1a]. *S. punae* Cabrera is an endemic high mountain plant that grows in the north of Argentina at 3500-4600 m above sea level. Since this flora is particularly adapted to extreme climatic conditions, the study of their defensive secondary metabolites is very attractive. The present investigation was performed to identify the volatile metabolites from *S. punae* diethyl ether extract. The molluscicidal, leishmanicidal, feeding deterrent, antibacterial and biofilm-reducing activities against different biological models were also evaluated.

Molluscicides have a history of success and failure in the control of schistosomiasis, reported in as many as 78 countries The high cost of imported synthetic compounds, along with increasing concern over their toxicity to non-target organisms, have given a new force to the study of plants with molluscicidal properties. Furthermore, investigation of plants used in traditional medicine provides a ready means of increasing the diversity of available molluscicides and simplifying the choice of selective, ecologically safe snail-controlling compounds [1b,2a]. The fresh water mollusk *Biomphalaria peregrina* is considered a potential vector of schistosomiasis in Argentina [2b].

Leishmaniasis, considered one of the most important neglected diseases, is caused by protozoa of the genus *Leishmania*. Current strategies to control this disease are mainly based on chemotherapy; treatment not well tolerated because of the persistence of side effects. Natural products, primarily plant-derived compounds of diverse structural classes, have been described in the literature showing anti-leishmanial properties *in vitro* and may, thus, be of potential utility in drug discovery and for producing new antileishmanial medicines [3].

Four biofilm phenotypic variants of *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and three probiotic lactobacilli strains were assayed. It is evident that biofilm formation is an ancient and integral component of the prokaryotic life cycle, and it is a key factor for survival in diverse environments. Biofilm formation represents a protected mode of growth that allows cells to survive in hostile environments and also disperse to colonize new niches. The implications of these survival and propagation mechanisms in the context of both the natural environment and infectious diseases have been previously studied [4a]. Indeed, bacterial biofilm phenotype is a major virulence factor contributing to the chronicity of infections [4b]. In this work the antibacterial properties of dehydrofukinone (D) and the diethyl ether extract (E) were determined; both were active against all pathogenic strains, and their mixtures with clinical antibiotics improved the antibiotic effectiveness against all pathogenic strains, being harmless on probiotic *Lactobacillus,* in agreement with previous results [5,6].

Antifeedant and repellency indexes of dehydrofukinone (D) and diethyl ether extract (E) were also determined against the coleopteran *Oryzaephilus surinamensis.* This cosmopolitan pest mainly attacks cereals, causing important economic losses because of quality deterioration. Since coleoptera have become resistant to organophosphorus pesticides, plant metabolites would be an effective control strategy [7].

The diethyl ether extract of *S. punae* (yield of 4%) was submitted to GC-MS analysis with electron ionization/ion trap detection. The TIC showed a few volatiles present in low percentage and a main peak at t_R = 40.35 min (Table 1) with a high concentration (94.8%),

Table 1: GC-MS profiling of *S. punae* extract.

RT (min)	RI	Compounds	Formula	MW	Main fragmentation peaks (*m/z*)	Area (%)
8.54	1018	Limonene	$C_{10}H_{16}$	136	136, 121, 107, 94, 93, 91, 79 and 67	0.26
27.75	-	Unknown	-	-	-	0.21
28.08	-	Unknown	-	-	-	0.31
28.97	1440	α-Cadinene	$C_{15}H_{24}$	204	204, 189,175, 161, 147,134, 119, 115, 105, 91, 81, 69 and 55	0.19
31.47	1536	Spathulenol	$C_{15}H_{24}O$	220	220, 205, 187, 177, 159, 147, 131, 119, 105, 91, 79, 69 and 55	0.48
38.08	-	Unknown	-	-	-	0.37
40.35	1678	Dehydrofukinone	$C_{15}H_{22}O$	218	218, 203, 189, 175, 161, 147, 133, 121, 105, 91, 83, 77, 65 and 53	94.83
40.78	1888	2(1H)-Naphthalenone, 4a,5,6,7,8,8a-hexahydro-6-[1-(hydroxymethyl)ethenyl]-4,8a-dimethyl-, [4ar-(4aà,6â,8aá)]-	$C_{15}H_{22}O$	234	234, 216, 206, 201, 188, 173, 159, 147, 135, 121, 108, 95, 93, 79*, 69, 65 and 55	1.07
52.46	1916	6-(1-Hydroxymethylvinyl)-4,8a-dimethyl-3,5,6,7,8,8a-hexahydro-1H-naphthalen-2-one	$C_{15}H_{22}O$	234	234, 216, 201, 187, 173, 159, 145, 131, 119, 105, 93, 79, 67 and 55	2.04

t_R: Retention time. RI: Kovats retention indexes.

and a fragmentation pattern that correlated with dehydrofukinone (NIST library). The presence of a (M+H) peak in the spectrum obtained was attributed to self-chemical ionization (self-CI) [8a].

Chromatographic fractionation of E gave a main fraction that yielded D as pale yellow oil with a strong odor and high percentage (1.7% related to the fresh plant material). IR, UV, EI-MS, ^{1}H and ^{13}C NMR spectra were identical with previously reported data. D was first isolated from *Arcticum lappa* [8b], later from *Cacalia hastata* [9], *Senecio humillimus* [10], *S. aureus* [11], *Petasites hybridus* [12a], and recently from the aerial parts of *S. viridis* var. *viridis* [12b].

Mortality rates of *B. peregrina* were registered after 24 h of exposure. E affected the snail population with a LC_{50} value of 68.6 µg/mL when added into an experimental aquarium. In comparison, D (LC_{50} = 16.7 µg/mL) showed a more acute toxicity than E. According to the WHO (1983), plant extracts showing LC_{50} values lower than100 µg/mL have potential for the control of vector snails currently controlled by the highly toxic Baylucide®, a synthetic molluscicide (LC_{50} = 0.1 µg/mL on *B. glabrata*) that is an environmental xenobiotic [12c].

The antileishmanicidal capacity of dehydrofukinone was measured. The activity results for *Leishmania amazonensis* and *L. brasiliensis* were expressed as 50% inhibitory concentration, the values being 34.3±1.2 µg/mL and 9.93±0.17 µg/mL, respectively. The present study was made using the promastigote form of the parasite because it is easier to maintain under *in vitro* conditions. However, since this is not the infective form of the parasite in vertebrate hosts, evaluations made with promastigotes have an indicative value of the possible leishmanicidal activity of the ketone tested.

Biofilm biomass and growth quantification of *P. aeruginosa* after 24 h incubation are shown in Figure 2. D produced a strong biofilm decrease of 66% at 25 µg/mL. No correlation was detected between this effect and growth inhibition. In contrast, E at 50 µg/mL only inhibited 22% of *P. aeruginosa* biofilm, and this behavior was correlated with the same growth inhibition (Figure 1). The MIC determinations of D against two strains of *P. aeruginosa* (27853 ATCC, and a clinical isolate) were high (6 mg/mL). Nevertheless, their associations with ciprofloxacin (C, MIC = 1 µg/mL) showed the same synergistic interaction against both strains (MIC of D = 23 µg/mL and MIC of C = 0.25 µg/mL in the mixture, FIC index = 0.38). The MIC values were the same as the MBC values determined by plating onto agar.

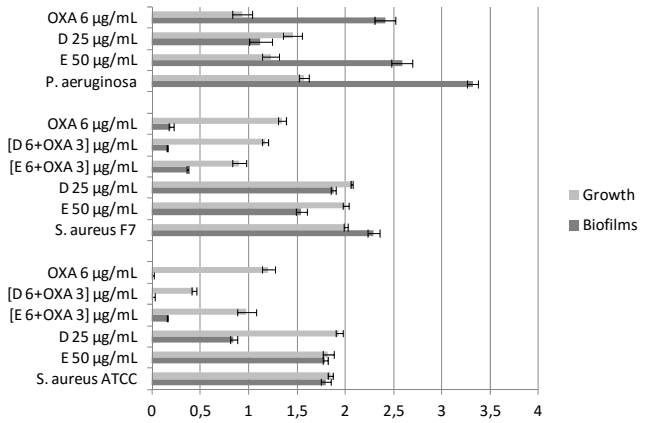

Figure 1: Effects of E and D on growth and biofilms of *S. aureus* and *P. aeruginosa*. E50: Diethyl ether extract of *S. punae* at 50 µg/mL. D25: dehydrofukinone at 25 µg/mL. E6+OXA3: Diethyl ether extract of *S. punae* at 6 µg/mL plus oxacillin at 3 µg/mL. D6+OXA3: dehydrofukinone at 6 µg/mL plus oxacillin at 3 µg/mL. All experiments showed significant differences with respect to controls (n = 8, P< 0.05), except for E against *S. aureus* ATCC 6538P.

Figure 1 shows that D (25 µg/mL) also inhibits 53% of *S. aureus* ATCC 6538P biofilm. High reductions of 94% and 82% were observed when either oxacillin or OXA (3 µg/mL) was mixed with D (6 µg/mL) or E (6 µg/mL). All biofilm inhibitions were growth-dependent. D MIC determinations against both strains of *S. aureus* were, as expected, lower than those against *P. aeruginosa*, 3.00 mg/mL (for *S. aureus* ATCC 6538 P) and 1.50 mg/mL (for the methicillin-resistant *S. aureus* F7 isolated from a hospital sample). Nevertheless, their associations with ciprofloxacin (C, MIC = 1 µg/mL) showed synergistic interactions. In both mixtures, the MIC of D was 23 µg/mL and of C 0.25 µg/mL for the ATCC strain (FIC index = 0.38*)*, as well as for a hospital isolate (FIC index = 0.25) at non-inhibitory concentrations for beneficial lactobacilli strains (data not shown). It is important to note that E and D improved the effectiveness of both antibiotics C and OXA with different mechanisms of action against the pathogenic bacteria tested and their main virulence factor, the biofilm. The lipophilic properties of these natural products, E, and D with a theoretical partition coefficient o/w value: 4.814, and log P o/w = 3.3135 (both descriptors were calculated using Chem3D Ultra after minimizing D energy by MM2 program) suggest that their principal targets are cell membranes and their toxicity would be caused by loss of chemiosmotic control. In fact, terpenes might also synergize antibiotic effects by acting as solvents to facilitate their passage through membranes. Similar results were observed in our previous reports [5,6].

On the other hand, E administration of 250 µg per g of diet produced an alteration of the feeding behavior of *O. surinamensis* adult population (Repellency index = +60); so did D (Repellency index = +10). Food preference indexes (PI) were also measured. They indicated that these natural products were repellents, as shown in Table 2 (E: PI = -0.6, and D: PI = -0.1). Neither E nor D were able to produce mortality after a week of contact. Therefore, they could be considered less toxic natural strategies for coleopteran control, and less likely to generate resistance than synthetic fumigants. The gaseous fumigant methyl bromine was extensively used as a tool to control post-harvest stored grain pests [12d], but it was phased out in developed countries by 2005 because of its contribution to stratospheric ozone depletion.

The moderate to potent *in vitro* activities studied herein should encourage more extensive investigations of dehydrofukinone and

Table 2: Preference and repellency indexes.

Mean number of insects			Indexes		
Control diet	Treated diet		Statistic *P* values	PI[1]	RI[2]
32.0 ± 0.0	E	8.0 ± 0.0	0.0000	-0.6	+60
22.0 ± 1.0	D	18.0 ± 1.0	0.0080	-0.10	+10

[1] PI values between -1.00 to -0.10 indicate that the extract is repellent; between -0.10 to +0.10 the extract is neutral. If the PI is between +0.10 to +1.00 the extract is attractive. [2] Positive values indicate repellency. E: diethyl ether extract. D: dehydrofukinone. Significant differences ($P< 0.05$) in the mean number of adults of *O. surinamensis* in the treated diet compared with the control diet were measurements in all cases.

related compounds present in *S. punae* extract that would provide an understanding of the structural requirements for their biological activities.

Experimental

General: MS, Thermo Electron Trace^{TM}GC Ultra coupled to a Thermo Electron Polaris Q ion trap mass spectrometer with a 30 m x 0.25 mm id DB-5 MS column.The identification of volatile components was based on computer matching with the NIST08 GC/MS library (USA) and data reported in the literature [13a].

Plant material: *S. punae* was collected in Mina Pirquitas, Jujuy province, Argentina, at 3600 m.a.s.l. during the flowering stage in February 2010. A voucher specimen (LIL 609967) is deposited at the Herbarium of Fundación Miguel Lillo, Tucumán, Argentina.

Preparation of diethyl ether extract and chromatographic fractionation: Fresh aerial parts (163 g) were extracted at room temperature for 3 days with diethyl ether (3 x 1300 mL). The extract was dried in a rotary evaporator under vacuum at 30ºC to give 6.56 g of residue. The extract (6 g) was subjected to column chromatography on silica gel (180 g) employing *n*-hexane and increasing amounts of EtOAc (0−100%), and finally MeOH as a mobile phase. Fractions eluted with *n*-hexane-EtOAc 85:15 were combined (3.72 g) and further separated by silica gel CC (186 g) employing *n*-hexane and increasing amounts of EtOAc (0−100%), furnishing 2.80 g of compound D.

Molluscicidal assay: The bioassay was assessed against *B. peregrina* adults according to the standard World Health Organization protocol and modified by the authors [2b]. The snails, uniform in size (average diameter of the shell = 7 mm), were maintained without feeding for 24 h before the experiment. E and D were dissolved in methanol and diluted with aged tap water to reach concentrations of 100, 50 and 25 µg/mL. Solutions of each sample (20 mL) were poured into 100 mL flasks and 7 snails were put in each flask. Control experiments were performed placing 7 snails in the same volume of MeOH added to water. The proportion of MeOH did not exceed 4% (v/v). Each test concentration was set in triplicate. A 10 mg/mL H_2O solution of $CuSO_4$ was used as a positive control, since it produced 100% mortality of the snail population. After 24 h of exposure, snails were removed from the flasks and were observed in a stereoscopic microscope to record mortality. To confirm mortality, the mollusks were transferred to beakers containing aged tap water and lettuce (*Lactuca sativa*) leaves (natural diet) and after 24 h their condition was rechecked. Finally, LD_{50} were calculated by analysis of the mortality data and logarithm concentration using MINITAB *Release* 14 statistical software for Windows.

Leishmanicidal assay: Promastigotes of *L. amazonensis* complex (clon 1: Lma, MHOM/BR/76/LTB-012) and *L. braziliensis* complex (strand M2904 C192 RJA) were cultivated at 26°C in Schneider's Insect Medium, pH 6.8, supplemented with 10% calf bovine serum inactivated at 56°C for 30 min. Promastigotes were fixed with glutaraldehyde (5%, 180 µL) and counted in a Neubauer chamber. Parasites in logarithmic phase of growth, at a concentration of 1 x 10^6 parasites/mL, were seeded on a 96-well flat bottom microtiter plate and different concentrations of the test substance (0.75–100 µg/mL) dissolved in DMSO were added. The micro well plates were incubated for 72 h at 26°C. After incubation, a solution of XTT (1 mg/mL) in PBS, phosphate buffer saline (pH 7.0 at 37ºC) with PMS, and phenazine methosulfate (Sigma-Aldrich, 0.06 mg/mL) were added (50 µL/well), and incubated again for 4 h at 26°C. DMSO (1%) and Amphotericin B Sigma-A2411 (0.5 µg/mL) were used as negative and positive controls during the evaluations [13b]. Optical density of each well was obtained on a Synergy HT Microplate Reader (Biotec) at λ 450 nm. The IC_{50} value (the concentration of a substance needed to reduce 50% parasites viability) was calculated using Microsoft Excel 2007. The assay was carried out in triplicate.

Antibacterial activity: *Pseudomonas aeruginosa* ATCC 27853 from the American Type Culture Collection, and a wild-type strain of *P. aeruginosa* HNA1, isolated from a hospital infectious process, were grown in Luria-Bertani media (LB, Cabeo, Rockville, MD, USA), while *Staphylococcus aureus* ATCC 6538 P and a methicillin-resistant strain of *S. aureus* F7 from a skin infection were grown in Müller Hinton Broth (MHB, Britania). Ciprofloxacin (C) was the quinolone antibiotic used as control. It was also associated with D to determine synergism. C acts on the bacterial DNA gyrase target and is a known biofilm inhibitor of *P. aeruginosa* strains [14a]. Oxacillin (OXA), and its associations with E or D were also assayed on *S. aureus* strains to improve oxacillin antibiotic activity as a wall bacterial inhibitor. Overnight cultures of each strain were diluted to reach 10^6 CFU/mL in LB or MHB media. The diluted culture (190 µL) was placed in each of the 96 wells of a microtiter polystyrene plate. Solutions containing 50 µg/mL of E and 25 µg/ml of D in DMSO-distilled water (50:50) were prepared separately and 10 µL of each was pipetted into the plastic microtiter plate wells individually (8 replicates). Control wells (8 replicates) contained the diluted culture (190 µL) and 10 µL of a solution of DMSO-water (50:50) in which the final concentration of DMSO was 2.5%. A control medium was prepared using sterile LB or MHB. Bacteria were grown in a liquid medium at 37°C, and growth was detected as turbidity (600 or 560 nm) using a microtiter plate reader (Power Wave XS2, Biotek, VT, USA). The maximum level of DMSO to which the cells were exposed was 2.5%.

Synergistic interactions: Synergistic effects of the mixtures of D and C were determined against *P. aeruginosa* and *S. aureus.* The combination effect can be described by means of the following equation:

FIC index = FICA + FICB = [A]/MICA + [B]/MICB

FICA, FICB: Fractional inhibitory concentration of drug A and B, respectively. MICA, MICB: Minimum inhibitory concentration of drug A and B, respectively. [A], [B]: Concentration of drug A and B, respectively. FIC index by checkerboard method is interpreted as follows: ≤ 0.5 synergy; > 0.5 and ≤ 4 additivity and > 4 antagonism [14b]. Mixtures at sub-lethal concentrations of E (6 µg/mL) or D (6 µg/mL) with OXA (3 µg/mL) were performed to measure the improvement of oxacillin activity against *S. aureus* strains.

Anti-biofilm activity against pathogenic bacteria: For biofilm quantification, a micro method based on a protocol previously

reported was employed [14c]. Biofilms formed after 24 h incubation of bacterial cultures prepared as described in the previous paragraph were stained with 20 µL of an aqueous solution of crystal violet (0.1%, w/v) for 20 min. After washing with water, the liquid was discarded from the wells and the material that remained fixed to the polystyrene (containing biofilm) was washed with PBS (thrice). Crystal violet bound to biofilm was removed from each well employing 200 µL absolute ethanol for 30 min at 37°C with shaking. Absorbance (540 nm) of crystal violet ethanol solutions was determined using a microtiter plate reader (Power Wave XS2, Biotek, VT, USA).

Impact of mixtures on probiotic lactobacilli strains: The non-pathogenic strains *Lactobacillus paracasei* ssp. *paracasei* CE 75, and *L. plantarum* CE 105 were isolated from regional cheese, while *L. plantarum* CE 358 came from high mountain soil of northwestern Argentina (Collection of CERELA, Tucumán, Argentina). These GRAS strains, generally recognized as safe, have beneficial effects on gut microbiota, and their growth under the mixtures with antibiotics were evaluated.

Antifeedant and repellent activities: E and D were subjected to insect bioassay. For the bioassay, a glass apparatus with a center cubicle symmetrically connected to another 4 cubicles was used. Two g of flour treated with 1 mL of a chloroform solution of extract was placed in 2 of them to obtain a concentration of 250 µg per g of diet (Treatment). Two g of flour impregnated with 1 mL of chloroform was placed in the 2 remaining cubicles (Control). Previously, both diets were left at room temperature for 24 h to eliminate the chloroform. In the central division, 40 adult insects of *O. surinamensis* were placed. After 24 h, food preference was assessed through the calculation of preference index (PI) with the following formula: PI = (% ITD - % ICD) / (% ITD + % ICD), where % ITD = % insect in treated diet; % ICD = % insects in the control diet. PI values between -1.00 and -0.10 indicate that the substance is repellent; between -0.10 and +0.10 the substance is neutral and is considered attractive, if the PI is between +0.10 and +1.00. Repellency was also established through the calculation of repellency index (RI), according to the following formula: RI = (C – T) / (C + T) x 100, where C = Insect in the control diet, T = Insects in the treated diet. Positive values indicate repellency [14d].

Acknowledgments - The authors would like to thank Prof. Dr Silvia N. González for providing the lactobacilli strains (CERELA, CONICET), PhD Prof. A. Gimenez and Dr E. Salamanca for the leishmanicidal bioassay (IIFB, UMSA, La Paz, Bolivia) and Dr Nora Muruaga for the identification of plant material (Fundación Miguel Lillo, Tucumán, Argentina).This research was supported with grants from CIUNT and CONICET.

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Natural Product Communications
2016
Volume 11, Number 8

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