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Soybean hulls, an alternative source of bioactive compounds: Combining pyrolysis with bioguided fractionation

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ABSTRACT

The trend for material and energy recovery from residues along with the need to reduce greenhouse gases has led to an increased interest in the thermal exploitation of biomass and/or their wastes. Due to the enormous quantity generated every year, agro-industrial byproducts have an attractive potential to be recycled. One way to do this is by means of pyrolysis, a thermal decomposition of high molecular weight polymers into simpler compounds. In this study, we applied an autographic assay to analyze the biological effect of bio-oils produced by pyrolysis of soybean hulls. The discovery of a compound with antimicrobial activity validated this novel approach as a tool for the generation of bioactive compounds.

1. Introduction

Sustainable use of renewable natural resources through value addition using chemical, biological or thermal processes is an ideal transition from a petroleum based economy to a bioresource economy, and in addressing climate change.

Widely available, renewable, and virtually free, biomass wastes or residues are an important resource that could cause disposal problems when not used. The challenge, therefore, is to convert biomass as a resource for energy, chemicals and other productive uses. As the debate on food versus fuel intensifies, the use of biomass residues can provide added income to farmers without interfering with food production and biodiversity (Trautmann et al., 2014). Furthermore, from a chemical point of view, its oxygenated nature, chemical diversity, and chirality render biomass a highly suitable raw material to manufacture a wide array of high added-value compounds (Corne et al., 2013).

Soybean [*Glycine max* (L.) Merr.] is a species of legume and one of major source of energy and nourishment for the worlds population. World production of soybean for the campaign 2015/2016 is estimated to be 315.8 million tons (USDA, 2016). Soy hulls, accounting for 5% of the soybean seed, are a residue of postharvest soy processing. This soybean component consists mainly of cellulose (38%), hemicelluloses (10%), lignin (2.8%), protein (10%) and ash (1–4%) (Mielenz et al., 2009). The hulls constitute an important source of low-value products in soy producing countries. One way to add value to these biomass wastes is through a pyrolytic process.

Several research groups have investigated the pyrolysis of different types of biomass (Yanik et al., 2007; Qu et al., 2011). Pyrolysis can transform low-grade biomass into high-quality liquid fuel or high added-value chemicals. On the other hand, bioguided fractionation of plant extracts has been applied in the area of natural products to identify bioactive compounds (Borras-Linares et al., 2015; Sbai et al., 2016; Malmir et al., 2015; Ansante et al., 2015), however there are only a few literature precedents regarding the biological applications of bio-oils derived from biomass pyrolysis, such as: the wood preservation effect of bio-oils obtained from pine wood and oak wood pyrolysis (Mohan et al., 2008) and the antifungal activity of tobacco bio-oil (Booker et al., 2010). Another example was the work reported by Friedmańs group concerning biological activities of bio-oils obtained from pyrolysis of rice hulls (Friedman, 2013; Kim et al., 2012). Among the effects observed by this group are antioxidant properties and the inhibition of *Salmonella enterica* serovar *typhimurium* (*S. typhimurium*)

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infection *in vivo* when mice were fed with a diet containing a small proportion of rice hull bio-oil.

All these studies reported interesting biological activities; however, the identity of the compound/s responsible/s for the observed effects remained a mystery. Nevertheless, should be desirable to identify the compound responsible for a given biological activity in a mixture, not only for elucidation purposes but also in order to make more detailed biological studies and to obtain synthetic derivatives with improved activities. With respect to this point, autographic assays are of great versatility: they combine the separative ability of thin layer chromatography (TLC) along with the *in situ* determination of a certain biological activity of the compounds present in the mixture. There are several autographic assays reported in the literature that were designed to detect antimicrobial compounds or enzyme inhibitors in complex mixtures (Marston, 2011; Choma and Grzelak, 2011).

Recently, a bioautography has been developed with the aim to detect compounds that affect the activity of the PhoP/PhoQ two-component regulatory system (TCS), the main pathogenicity regulator system of *Salmonella enterica*. This method involves the use of *lacZ* gene as a reporter of the transcriptional expression of specific genes regulated by this TCS (Salazar et al., 2014; Viarengo et al., 2013). *Salmonella* is a gastrointestinal pathogen and the etiological agent of the disease called salmonellosis. The lifestyle of bacterial pathogens requires them to establish infection in the face of host immunity. To do this, *Salmonella* deploys a battery of mechanisms which are under control of the PhoP/PhoQ two component system. Among the genes activated by PhoP (the response regulator) are those involved in the survival inside macrophages, resistance to acid pH, biliary salts and microbicidal peptides (Garai et al., 2012; Fábrega and Vila, 2013). These two component systems are present in bacteria and lower eukaryotes but not in humans, so they constitute a promising target for the search of new antibiotics.

Herein we highlight the bactericidal effect observed in bio-oils produced by the pyrolysis of soy hulls over *S. typhimurium* and the importance of using the aforementioned autographic assay to isolate the compound responsible for the activity. A set of derivatives from the isolated compound were also obtained to pinpoint the chemical functional groups involved in the inhibitory action.

2. Methods

2.1. Materials

Soybean hulls and flour were provided by Bunge Argentina. All reagents and solvents were used directly as purchased or purified according to standard procedures. Pyrolysis experiments were performed in a bench scale tubular electrical furnace with a digital temperature controller and a total power of 1.5 Kw. Analytical thin layer chromatography was carried out using commercial silica gel plates (Merck, Silica Gel 60 F254) and visualization was effected with short wavelength UV light (254 nm) and a *p*-anysaldehyde solution (2.5 mL of *p*-anysaldehyde + 2.5 mL of H_2SO_4 + 0.25 mL of AcOH + 95 mL of EtOH) with subsequent heating. Once isolated or synthesized, compounds were re-purified by column chromatography or recrystallization in order to test their biological activities. Column chromatography was performed with silica gel 60 H (Merck), slurry packed, run under low pressure of nitrogen.

2.2. Physical data and structure elucidation

NMR spectra were recorded at 300 MHz for ^1H, and 75 MHz for ^{13}C on a Bruker Avance-300 DPX spectrometer with $CDCl_3$ as solvent and Si $(CH_3)_4$ (^1H) or $CDCl_3$ (^{13}C, 76.9 ppm) as internal standards. In order to make the best comparisons, ^1H NMR spectra of bio-oils were obtained dissolving 30 mg of each sample in 0.5 mL of $CDCl_3$ and, all experiments were carried out using 64 scans and 13 ppm of spectral width.

The GC/MS analyses were conducted on a Shimadzu GCMS-QP2012 Plus gas chromatograph mass spectrometer. The column was a SPB™-1 (30 m × 0.25 mm id x 025 μm film thickness) from Supelco. The oven temperature program was started at 60 °C, then ramped at 3 °C/min to 246 °C and subsequently ramped at 15 °C/min to 280 °C. The pyrolysis oil was diluted to 1 mg/mL in dichloromethane and 1 μL was injected under split conditions into the injector port. The carrier gas was helium (99.999%). The identification of chromatographic peaks was made by comparison with NIST library.

Infrared spectra were recorded on a Shimadzu IR Prestige-21 spectrometer. Solid samples were measured like dispersions on KBr tablets, obtained compressing a solid mixture finely sprayed containing approximately 1 mg sample and 100 mg KBr. Oil samples were determined by making a thin film supported in NaCl tablets. Absorbance frequencies are recorded in reciprocal centimeters (cm^{-1}). Optical rotations were determined using a JASCO DIP-1000 digital polarimeter in 100 mm cells and the sodium D line (589 nm) at room temperature in the solvent and concentration indicated. The melting points were taken on a Leitz Wetzlar Microscope Heating Stage Model 350 apparatus and are uncorrected. The structure of the products were determined by a combination of spectroscopic methods such as IR, 1D and 2D NMR (including NOE, DEPT, COSY, HSQC and HMBC experiments) and HRMS.

2.3. Bacterial strains, cell culture, and growth conditions

Bacterial strains used in this work are listed in Table 1. Bacteria were grown at 37 °C in Luria-Bertani (LB) broth with shaking, with or without the addition of levoglucosenone or its derivatives, at the concentrations indicated in each assay. Ampicillin and kanamycin were used at a final concentration of 100 and 50 μg mL^{-1}, respectively.

2.4. Samples pyrolysis

Soybean hulls and flour were treated with different concentrations of H_3PO_4 before pyrolysis. Treatment was made by suspending 100 g of material in 1 L of an aqueous solution of H_3PO_4 1% or H_3PO_4 10% overnight at room temperature. The samples were then filtered and dried at room temperature.

A tubular furnace (40 cm length) was used with an inclination angle of 30 °. A 60 cm length glass tube with an outer diameter of 35 mm and inner diameter of 30 mm was placed inside the furnace and used as pyrolysis chamber. The samples were introduced through the upper part of the glass chamber which was fitted with a removable release valve. The lower part of the glass tube was connected to a collecting flask cooled in an ice bath. Once the sample was loaded, the system was connected through the collecting flask to a trap in line with a water aspirator pump that provided a 50 mm vacuum.

Six batches of 2 g of each sample were placed in an aluminum container inside a glass tube under vacuum. Pyrolysis experiments were

Table 1
Bacterial strains used in the present work.

Strain	Genotype
MS14028s	*Salmonella enterica* serovar *typhimurium*
PB2069 (Aguirre et al., 2006)	14028s Δ*phoPQ*:Sp^R
PB2790 (García Véscovi et al., 1996)	14028s *virK*:MudJ
PB3062 (Gibson et al., 1987)	14028s *tppB*:MudJ
E. coli BL21(DE3)	F- ompTgaldcmlonhsdSB(rB- mB-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])
Serratia marcescens RM66262 (Castelli et al., 2008; Bruna et al., 2015)	Non-pigmented clinical strain
Bacillus subtilis (Aguilar et al., 2001)	trpC2 pheA1 amyE:[pdes(-269 to +31)-lacZ]

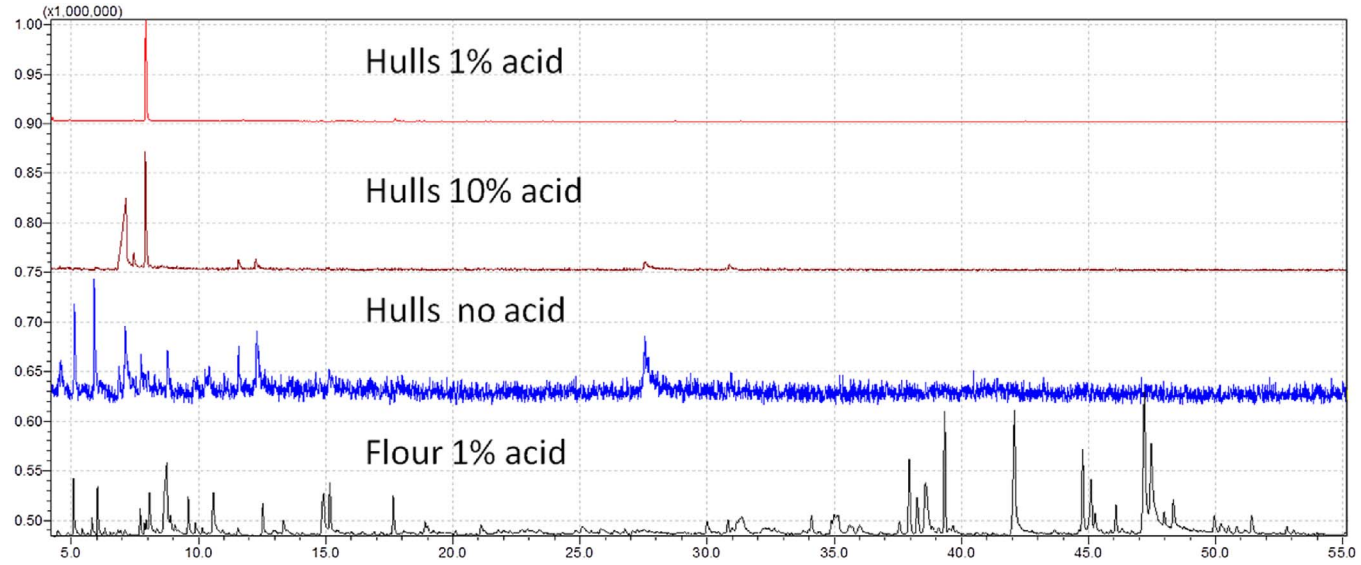


Fig. 1. GC–MS of bio oils.

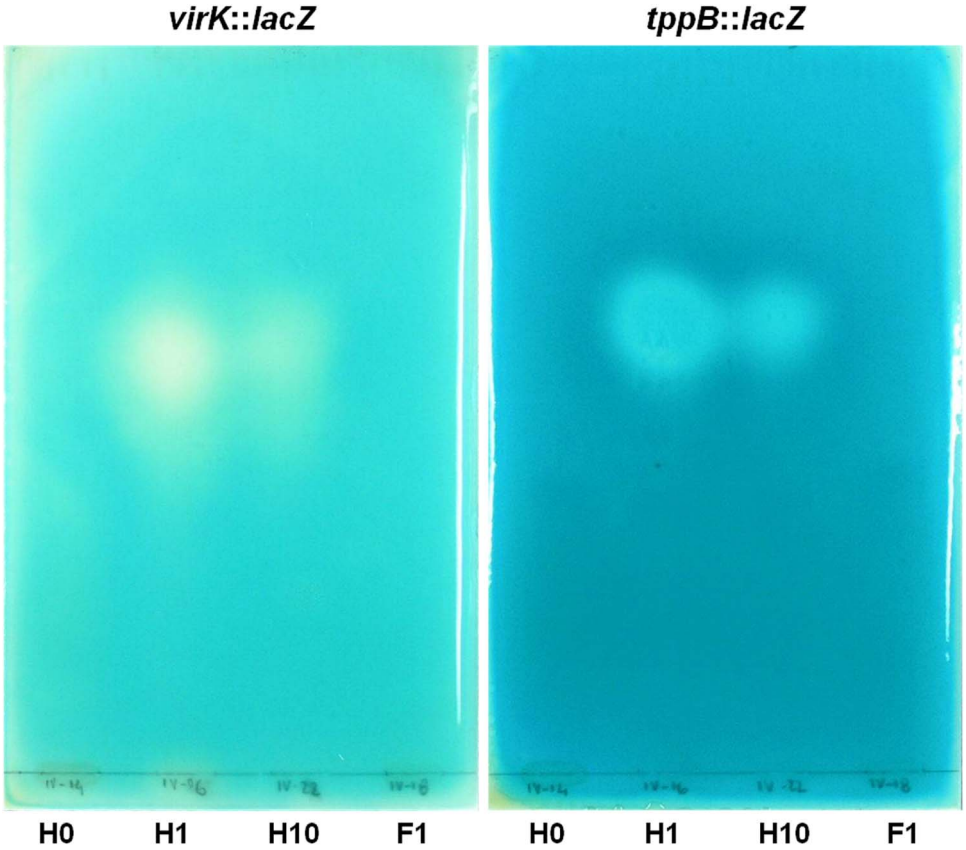


Fig. 2. Autographic assays with genes *virK* and *tppB*. *Samples preparation:* **H0:** soy hulls without acid pre-treatment. **H1:** soy hulls pre-treated with H_3PO_4 1%. **H10:** soy hulls pre-treated with H_3PO_4 10%. **F1:** soy flour pre-treated with H_3PO_4 1%. **Pyrolysis T:** 300 °C. **Sample load:** 100 μg each.

performed in an isothermal manner at 300 °C during 20 min each run. The condensable volatile compounds were collected as two phases (organic and aqueous) which were separated. In the present work, the incondensable gas products and the aqueous phase were not considered for this study.

2.5. TLC bioautography

The inhibitory activity of the assayed compounds on PhoP/PhoQ-dependent reporter gene expression was studied by TLC bioautography (Salazar et al., 2014; Viarengo et al., 2013).

The amount of sample loaded on the TLC plate was 50 or 100 μg/spot. After elution with a mixture of chloroform/methanol (95:5), the solvents were removed under an air current, and the plate was subjected to autographic analysis. Chromatograms were placed in sterile Petri dishes with covers. TLC plates were overlaid with soft agar medium (LB with 0.6% agar) containing an inoculum of the reporter bacterial cell suspension from an overnight culture at a final concentration of $50 \times 10^5 CFUmL^{-1}$, 0.33 $mgmL^{-1}$ X-gal, and the addition of the adequate antibiotic. After solidification of the medium, the TLC

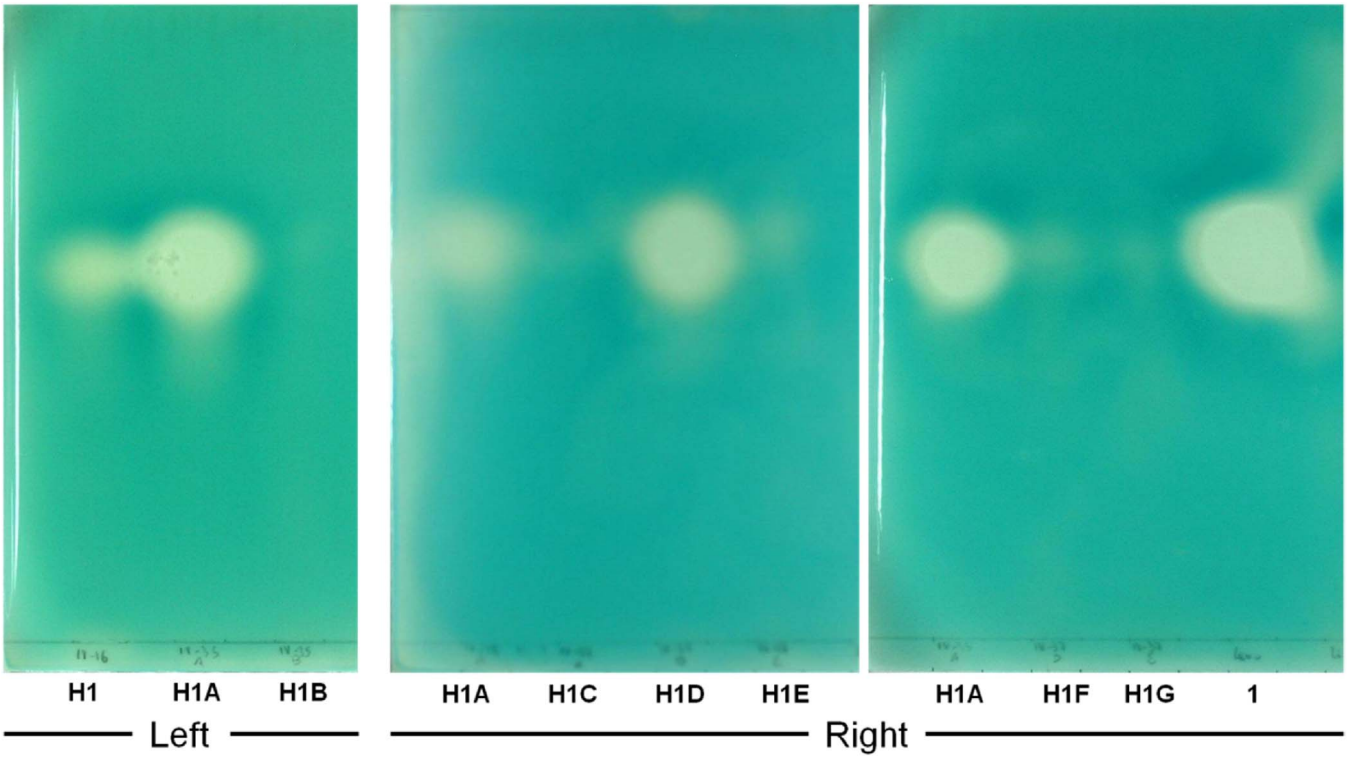


Fig. 3. *Bioguided fractionation*. Left: Fractionation of sample **H1**. Right: Fractionation of sample **H1A**. In all cases sample load was 50 μg.

plates were incubated overnight at 37 °C for 18 h. Inhibition of the reporter gene expression was detected as light blue or white areas on a blue background given by the β-galactosidase activity-mediated cleavage of the X-gal substrate. Concomitantly, growth inhibition could also be detected by the presence of a transparent halo in contrast of the turbid appearance of the LB-agar layer that denoted bacterial growth.

2.6. Bacterial growth curves

Overnight cultures of *S. typhimurium* wild-type, *S. typhimurium phoPQ*, *Escherichia coli*, *Serratia marcescens* or *Bacillus subtilis* strains were diluted 1:100 in a sterile 96-well microplate (Greiner Bio-one) with fresh LB medium with or without the addition of levoglucosenone or its derivatives at a final concentration of 5, 10, 20, 40, 80, 160, 320 or 640 μg mL^{-1}, and the optical density at 600 nm was measured every 60 min with BioTek Synergy 2 multimode microplate reader. Samples were processed by duplicates, and proper blank controls were used in each microplate.

2.7. Bacterial viability assay

Overnight culture of *S. typhimurium* wild-type strain was diluted 1:100 in 2 mL of fresh LB medium with or without the addition of levoglucosenone or 3-bromolevoglucosenone. At the indicated time points, the optical density at 600 nm was measured and aliquots were taken to determine the colony-forming units (CFU) by performing serial dilutions on LB-agar plates. Samples were processed by duplicates in each assay, and mean and standard deviation from 2 independent assays were calculated.

Statistical analysis was performed using one-way ANOVA and Holm-Sidak test with an overall significance level = 0.05.

2.8. Synthetic procedures

2.8.1. Levoglucosenone (1) preparation

Levoglucosenone was obtained by cellulose pyrolysis according to the procedure described in literature (Sarotti et al., 2007; Morin, 1994). The product was purified by vacuum distillation and column chromatography. 1 is a yellow oil, $[\alpha]_D^{25} = -562.3$ (*c* 1.04; $CHCl_3$); physical properties and spectroscopic characterization are in agreement with the data reported in the literature (Shafizadeh and Chin, 1977; Halpern et al., 1973).

2.8.2. Synthesis of 3-bromolevoglucosenone (2)

Levoglucosenone (0.86 g, 6.86 mmol) was dissolved in dichloromethane (17 mL) and cooled at 0 °C in an ice bath. Bromine was added dropwise while stirring until the solution turned into red colour indicating excess of bromine. Subsequently, Et_3N (1.4 mL) was added and the reaction mixture was stirred during 30 min at room temperature. The reaction was diluted with distilled water (20 mL) and extracted with dichloromethane (3 × 15 mL). The combined organic extracts were dried with Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography in order to obtain 2 (1.30 g, 1.91 mmol, 92%). (Nishikawa et al., 2001). 2 is a colorless solid. mp = 45–46 °C, $[\alpha]_D^{25}$ −435.3 (*c* 0.89, $CHCl_3$); physical properties and spectroscopic characterization are in agreement with the data reported in the literature (Ward and Shafizadeh, 1981).

2.8.3. Synthesis of ketone 3

To a solution of 1 (86.6 mg, 0.69 mmol) in ethanol (1.1 mL), a catalytic amount of Pd/C 10% was added. The system was purged three times with cycles of vacuum-hydrogen. The reaction was maintained under hydrogen atmosphere for 3 h with stirring until the total consumption of the starting material was revealed by TLC. The resulting suspension was celite-filtered and the solvent was evaporated at reduced pressure. Ketone 3 (76.5 mg, 0.60 mmol) was obtained in 87% yield. 3 is a colorless oil. $[\alpha]_D^{25} = -224.9$ (*c* 0.6, $CHCl_3$); physical properties and spectroscopic characterization are in agreement with the data reported in the literature (Flourat et al., 2015).

2.8.4. Synthesis of allylic alcohol 4

Levoglucosenone (1) (910.0 mg, 7.22 mmol) was dissolved in

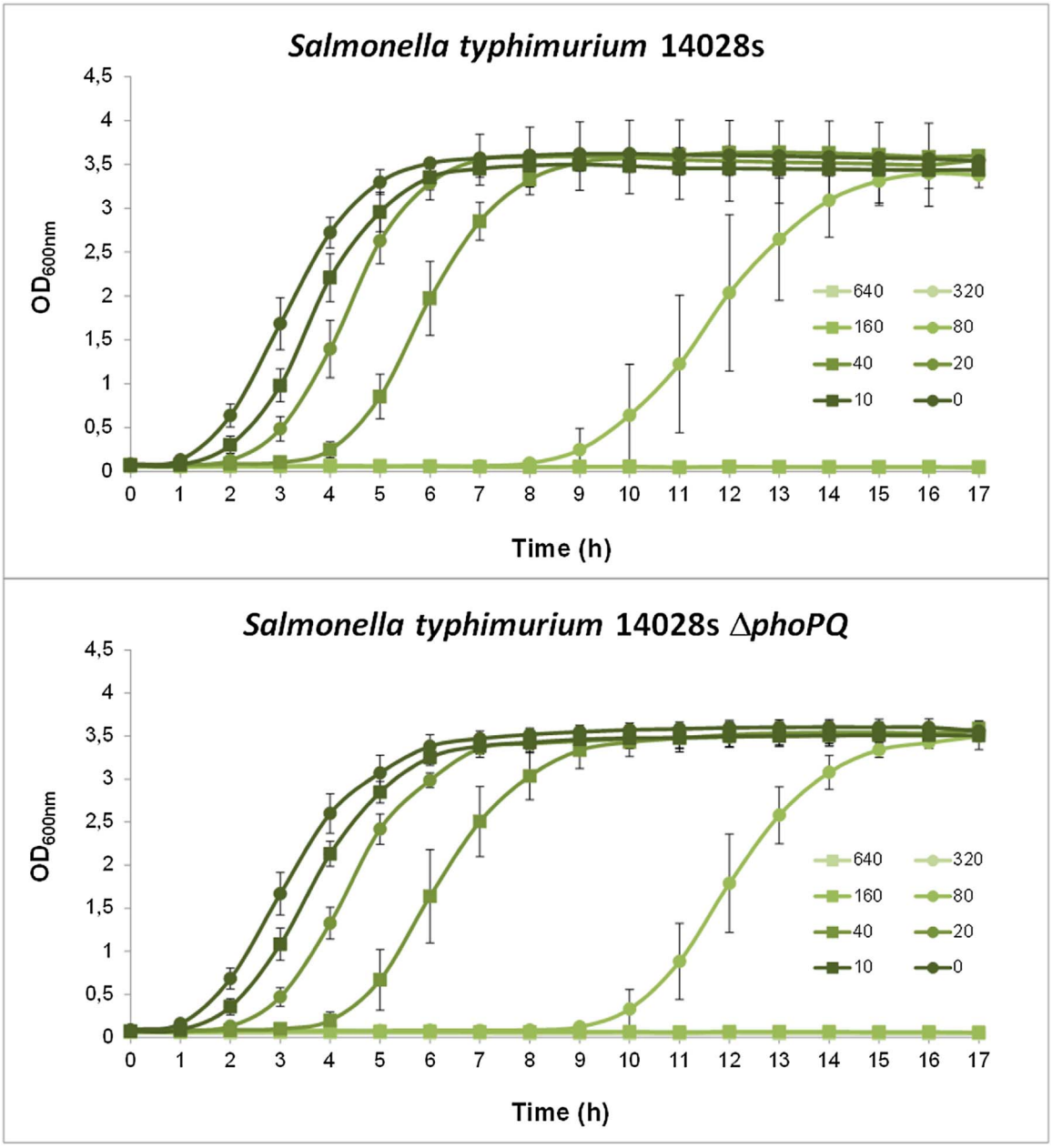


Fig. 4. Growth pattern of *Salmonella* strains in the presence of levoglucosenone. *S. typhimurium* 14028 s wild type or Δ*phoPQ* strains were grown in LB without (0) or with addition of the indicated concentrations of levoglucosenone (in μg mL^{-1}), and the OD_{600} was measured at the indicated time points. Results are average of two independent assays performed in duplicate, and error bars correspond to standard deviations.

methanol (24 mL) and cooled at 0 °C. $CeCl_3{\cdot}7H_2O$ (3.2 g, 8.59 mmol, 1.2 eq.) and $NaBH_4$ 98% (111 mg, 2.94 mmol, 0.4 eq.) were sequentially added. The mixture was stirred at room temperature for 3 h, followed by the addition of acetone (5 mL). The solvent was evaporated until almost dryness. Distilled water (10 mL) was added and the mixture was extracted with ethyl acetate (4 × 15 mL). The organic phase was dried with Na_2SO_4 and the solvent was evaporated under reduced pressure. Allylic alcohol 4 (812.5 mg, 6.43 mmol) was obtained in 88% yield (Giordano et al., 2012). 4 is a white solid. mp = 67–68 °C, $[\alpha]_D^{25} = -32.2$ (*c* 0.995, $CHCl_3$); physical properties and spectroscopic characterization are in agreement with the data reported in the literature (Brimacombe et al., 1979).

2.8.5. Synthesis of alcohol **5**

Ketone 3 (38.0 mg, 0.30 mmol) was dissolved in methanol (1 mL), cooled at 0 °C and $NaBH_4$ 98% (8 mg, 0.20 mmol) was added. The mixture was stirred at room temperature for 1 h followed by the addition of acetone (1 mL). The solvent was evaporated until almost dryness. Distilled water (5 mL) was added and the mixture was extracted with ethyl acetate (3 × 10 mL). The combined organic extracts were dried with Na_2SO_4 and solvent was evaporated under reduced pressure. Alcohol 5 (25.0 mg, 0.19 mmol) was obtained in 65% yield. (Zanardi and Suárez, 2009). 5 is a colorless oil; $[\alpha]_D^{25} = -117.3$ (*c* 2.00, $CHCl_3$); physical properties and spectroscopic characterization are in agreement with the data reported in the literature. (Trnka and Cerny, 1972).

2.8.6. Synthesis of epoxides **6** and **7**

Allylic alcohol 4 (73.8 mg, 0.58 mmol) was dissolved in dichloromethane (3 mL) at room temperature. *m*-CPBA 57% (382.3 mg 1.26 mmol, 2.2 eq.) was then added. The mixture was stirred at room temperature during 40 h until the total consumption of the starting

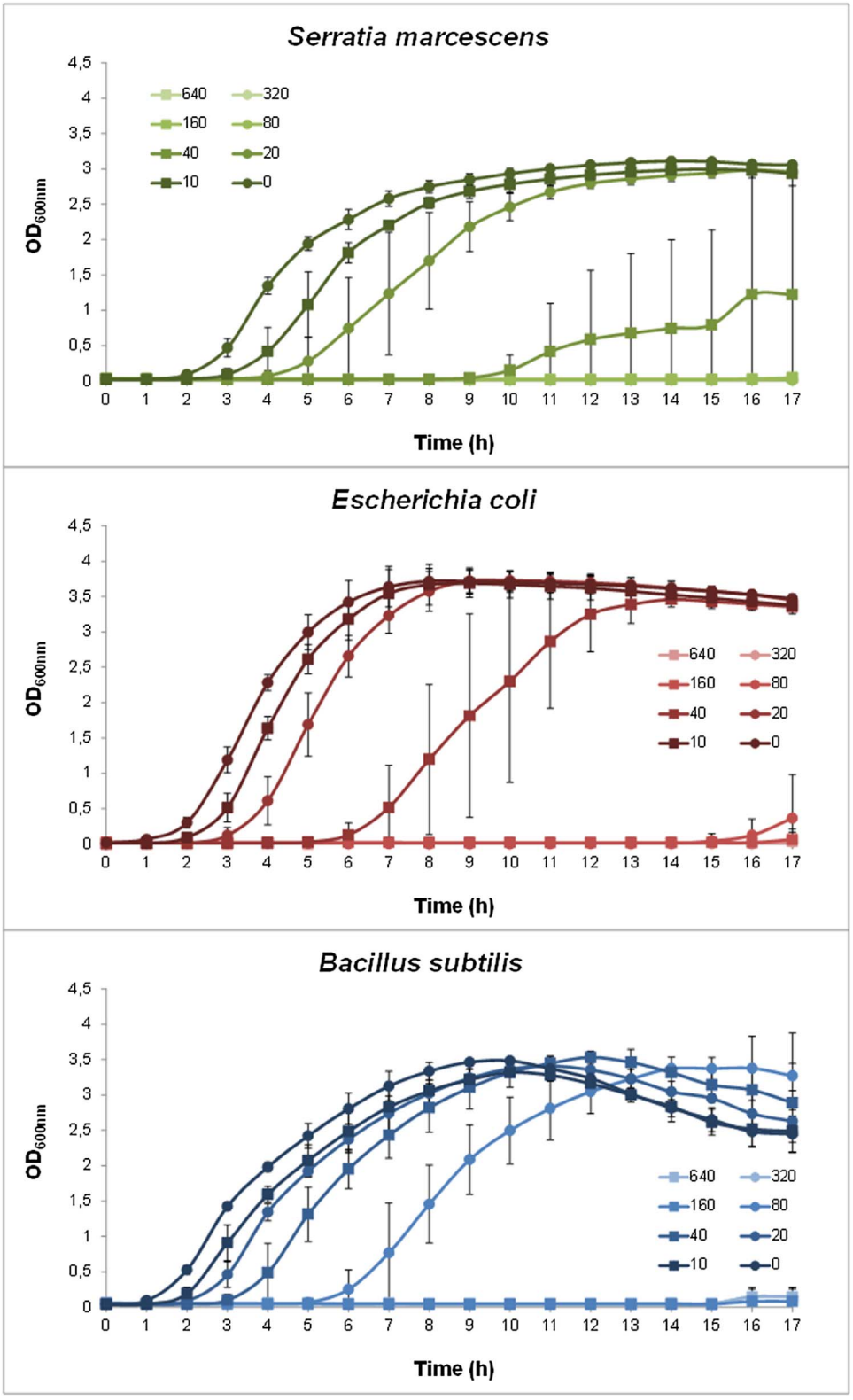
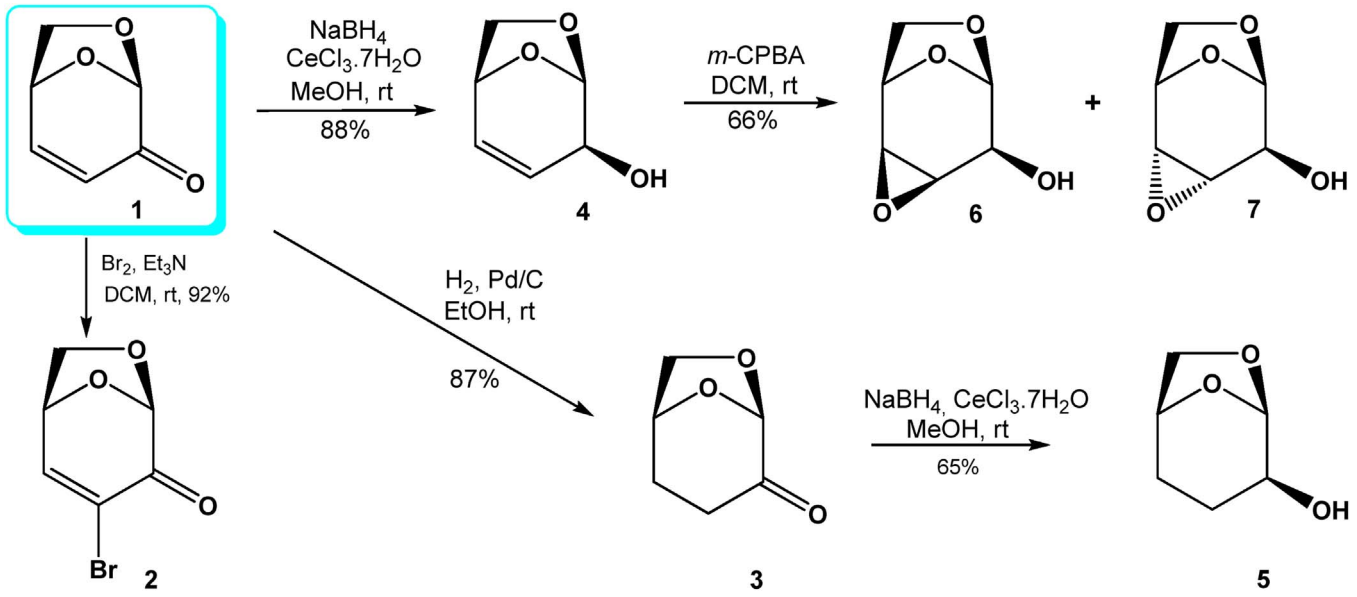


Fig. 5. Growth pattern of Gram(−) and Gram(+) bacteria in the presence of levoglucosenone. *Serratia marcescens* RM66262, *Escherichia coli* BL21(DE3) and *Bacillus subtilis* strains were grown in LB without (0) or with addition of the indicated concentrations of levoglucosenone (in μg mL^{-1}), and the OD_{600} was measured at the indicated time points. Results are average of two independent assays performed in duplicate, and error bars correspond to standard deviations.

material was revealed by TLC. Solvent was then evaporated at reduced pressure. Reaction products were separated by column chromatography to afford epoxide 6 (31.7 mg, 0.217 mmol, 37%) and 7 (24.7 mg, 0.169 mmol, 29%). Global yield: 66%, 6/7 ratio: 56/44 (Trnka et al., 1979; Ma et al., 2015). 6 is colorless solid. mp = 75–76 °C, 7 is a white solid. mp = 159–160 °C.; physical properties and spectroscopic characterization are in agreement with the data reported in the literature. (Matsumoto et al., 1993; Krohn et al., 2005)



Scheme 1. Derivatization of levoglucosenone.

3. Results and discussion

It has been demonstrated that chemical modification of mixtures (like plant extracts) can introduce changes in chemical composition that result in the generation of biological activities (Ramallo et al., 2011). However, pyrolysis has not been studied in depth with this purpose.

Pyrolysis is a thermal treatment in the absence of oxygen. When this procedure is applied to polymers or large molecules, it generally produces the degradation of the molecules resulting in the generation of compounds of lower molecular weight. The influence of the pre-treatment of the starting material with acids has been studied in the past and demonstrated that it could affect the outcome of the pyrolytic process (Shafizadeh and Chin, 1977, 1976; Halpern et al., 1973). In the case of cellulose, it has been reported that an acid pre-treatment may modify the physical structure, changes its thermal stability and induces the dehydration of its pyrolysis products (Becerra et al., 2013).

With these considerations in mind, we studied the effect of the pre-treatment of soy hulls and flour with 1% or 10% aqueous solutions of phosphoric acid. The comparison between the bio-oils composition obtained from soy hulls demonstrated that the acid treatment has a pronounced influence on the organic product distribution, affecting the thermal degradation process towards the generation of a less complex mixture of low molecular weight carbohydrate derivatives. However, this behavior was not observed when the study was carried out with soy flour. The outcome became evident from the evaluation of the GC–MS of the bio oils (Fig. 1). Analysis of the ^{1}H NMR spectra (see Supplementary Data) of these bio oils were in good agreement with the GC–MS results.

3.1. Detection of bioactivity in bio-oils samples derived from soy hulls pyrolysis

With the aim to study the presence of compounds that could affect the viability of *S. typhimurium* we employed an autographic assay that has been recently developed in order to screen for substances with inhibitory action over the *Salmonella* PhoP/PhoQ signal transduction mechanism, which controls major bacterial virulence phenotypes (Salazar et al., 2014; Viarengo et al., 2013). This strategy has the advantage of allowing the simultaneous detection of bactericidal or bacteriostatic activities and of potential PhoP/PhoQ modulatory compounds. Results are shown in Fig. 2

We could detect activity against *Salmonella typhimurium* in bio-oil samples produced from the pyrolysis of soybean hulls pre-treated with 1% and 10% phosphoric acid aqueous solutions. The inhibition halos in **H1** and **H10** have identical chromatographic behavior suggesting that the same bioactive compound is present in both samples. This active compound produced growth inhibition in two bioautographic assays that measure the expression of the genes *virK* and *tppB*.[1] Concomitantly, in both assays, growth inhibition could also be qualitatively detected by the presence of a transparent halo (in contrast to the turbid appearance on the Luria-Bertani (LB)-agar layer when bacterial growth is not affected). Together, these results strongly suggested that the active compound present in the bio-oil could affect *Salmonella* survival.

Sample **H1** was subjected to bioguided fractionation. A chromatography column with hexane:ethyl acetate gradient produced one bioactive fraction, **H1A**, that contained the bioactive compound (Fig. 3 left).

Fraction **H1A** was subjected to a second column chromatography purification with hexane:ethyl acetate gradient to obtain a new fraction (**H1D**) which conserved the activity. Bioautography comparison of fraction **H1D** and pure levoglucosenone (**1**) suggested that this compound was responsible for the activity observed in the mixture (Fig. 3 right). This result was consistent with the higher activity of sample **H1** when compared to sample **H10** that contained a lower amount of **1** (Supplementary Data).

It is well known that levoglucosenone can be obtained from cellulose pyrolysis only in the presence of catalytic quantities of mild acids (Shafizadeh and Chin, 1977, 1976; Halpern et al., 1973). Therefore, we assumed that the bioactive compound obtained was derived from cellulose present in soy hulls. In agreement with this, the sample (**H0**) without acid treatment showed no activity because **1** is not present in soy hulls nor generated in the absence of acid. Additionally, the flour sample did not produced *Salmonella* growth inhibition. The fact that **1** was the compound responsible for the activity means that only the pyrolytic convertion of pre-treated material with catalytic amount of H_3PO_4 was capable to generate the bioactive compound, given that soy hulls do not contain levoglucosenone per se.

This result represents the first report of an antibacterial activity for levoglucosenone, a compound originally reported over 40 years ago and widely used among organic chemists (Halpern et al., 1973). It is

[1] *VirK* is a reporter gene that belongs to the PhoP/PhoQ-dependent regulon, while *tppB* is an unrelated reporter gene, which belongs to the OmpR/EnvZ-dependent regulon.

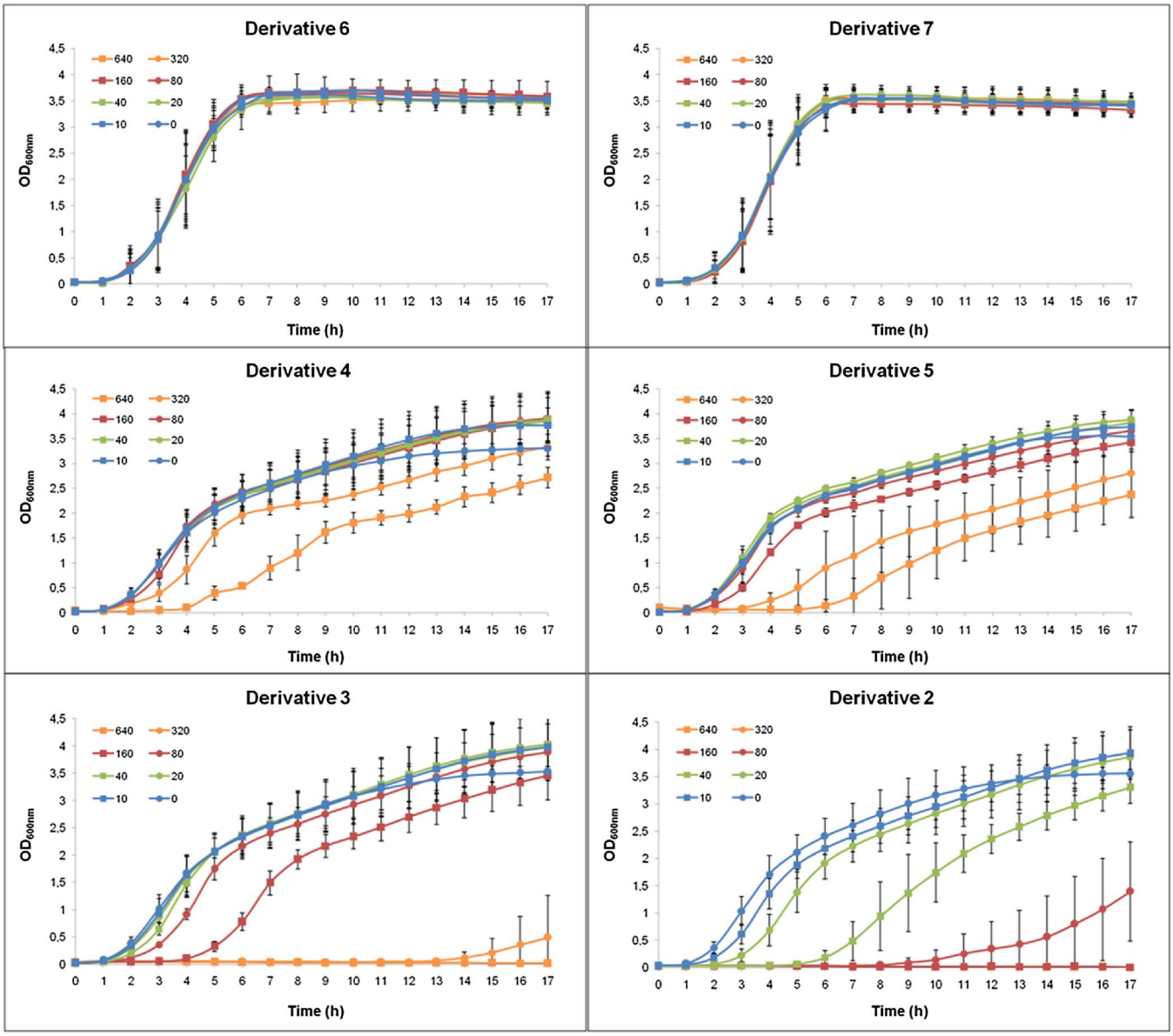


Fig. 6. Growth pattern of *Salmonella typhimurium* 14028 s in the presence of levoglucosenone derivatives. *S. typhimurium* 14028 s wild type strain was grown in LB without (0) or with addition of the indicated concentrations of levoglucosenone derivatives (in μg mL^{-1}), and the OD_{600} was measured at the indicated time points. Results are average of two independent assays performed in duplicate, and error bars correspond to standard deviations.

clear from this report that the application of the autographic assay played a key role in the easy identification of levoglucosenone as the active compound, showing its power as a bio-analytical tool and also demonstrating for the first time its use in the analysis of bio-oils.

3.2. Effect of levoglucosenone (1) on Salmonellás growth

As mentioned above, the results on the bioautography assays suggested an effect of 1 over *Salmonella* viability.

Fig. 4 shows growth curves of *S. typhimurium* wild-type and the otherwise isogenic Δ*phoPQ* mutant strain exposed to increasing concentrations of compound **1** added to LB medium from the onset of growth. In this assay, we observed an extended lag phase of bacteria grown in the presence of levoglucosenone in comparison to untreated bacteria. The extension of the lag phase delay correlated with the increase in the concentration of **1** up to 80 μg mL^{-1}. When challenged with levoglucosenone concentrations lower than 160 μg mL^{-1}, bacterial cultures resumed growth − at different time points according to the levoglucosenone concentration used- and reached a final OD_{600nm} equivalent to the one attained in stationary phase by non-treated bacteria. No growth recovery was detected when bacteria was challenged with concentrations of levoglucosenone ≥160 μg mL^{-1}. Therefore, we could estimate a minimum inhibitory concentration (MIC) of 160 μg mL^{-1} for **1**. Since the same result was obtained when the otherwise isogenic Δ*phoPQ* mutant strain was assayed, it is clear that the observed effect of levoglucosenone on the *Salmonella* growth pattern is independent of the functionality of the PhoP/PhoQ regulatory system.

3.3. Effect of levoglucosenone (1) over bacteria distinct from Salmonella

To examine whether the effect of levoglucosenone over *Salmonella* was specific for this genus, *Escherichia coli*, *Serratia marcescens* and *Bacillus subtilis* were also challenged with the compound. As shown in Fig. 5, the overall effect of levoglucosenone on growth behavior was similar for the four bacterial species tested, although susceptibility to the compound was higher for *E. coli* and *S. marcescens* followed by *S. typhimurium* and *B. subtilis*. These results suggest that levoglucosenone

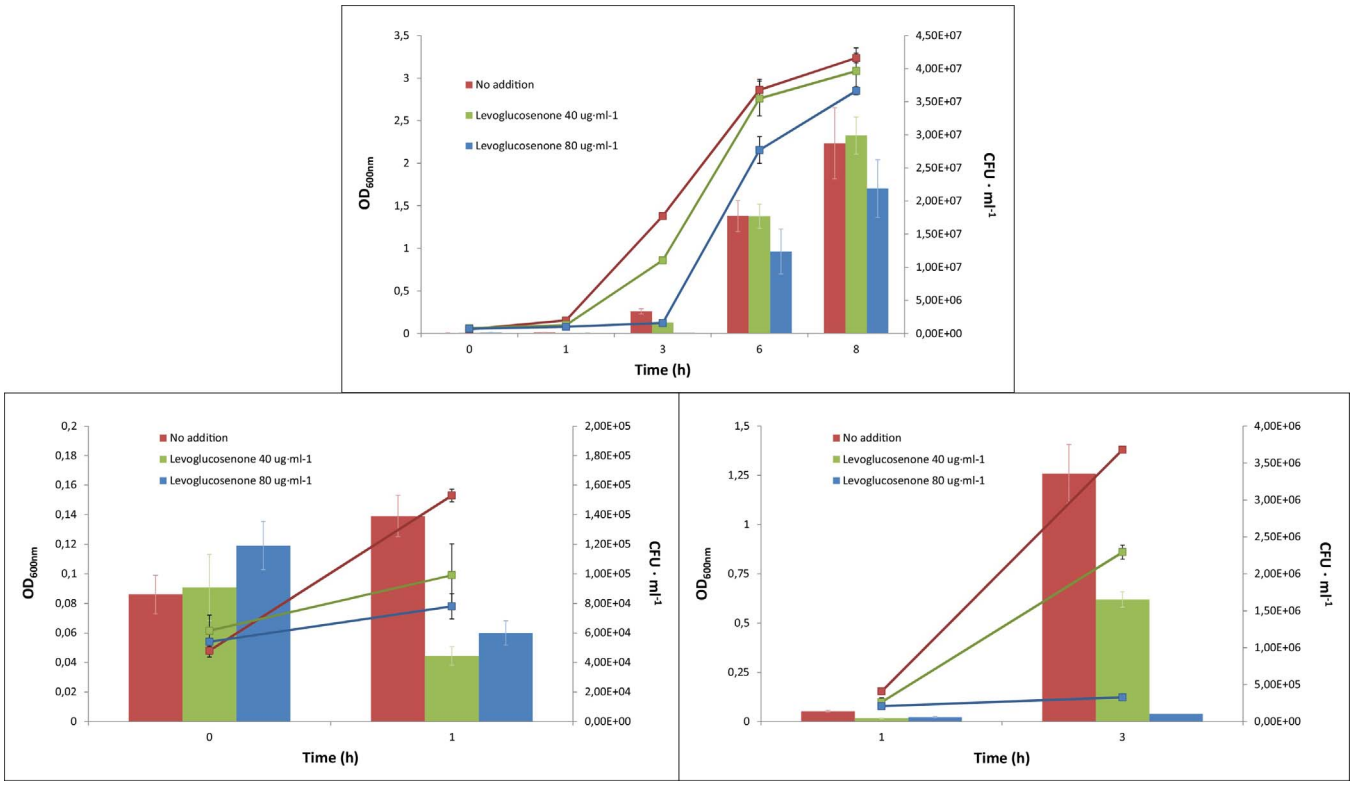


Fig. 7. Cellular viability of bacteria grown in the presence of levoglucosenone *S. typhimurium* 14028 s wild type strain was grown in LB without or with addition of the indicated concentrations of levoglucosenone (**1**). At the indicated times, the OD_{600} (lines) and the CFU/mL (bars) were determined. Results are average of two independent assays performed in duplicate, and error bars correspond to standard deviations.

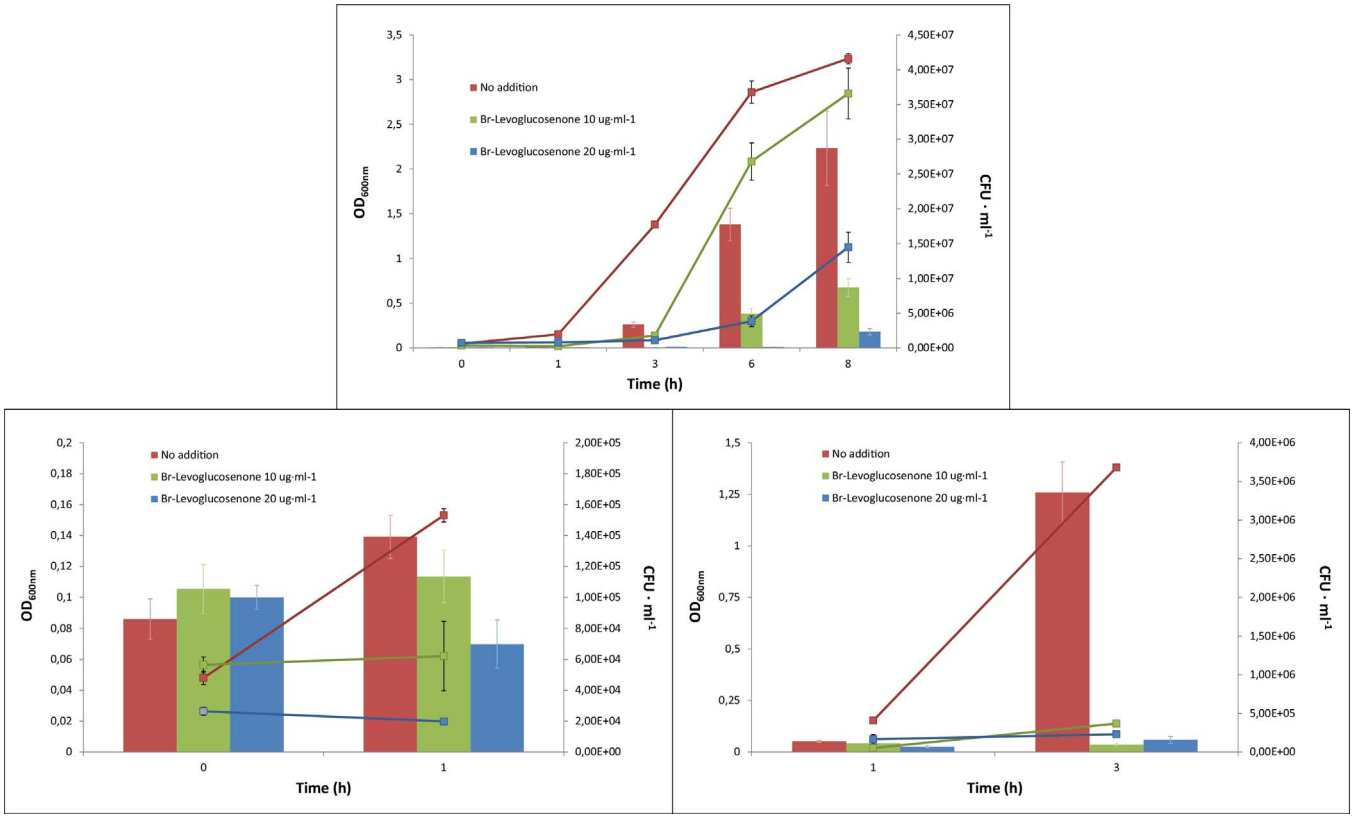


Fig. 8. Cellular viability of bacteria grown in the presence of Br-levoglucosenone. *S. typhimurium* 14028 s wild type strain was grown in LB without or with addition of the indicated concentrations of Br-levoglucosenone (**2**). At the indicated times, the OD_{600} (lines) and the CFU/mL (bars) were determined. Results are average of two independent assays performed in duplicate, and error bars correspond to standard deviations.

exerts an antibacterial effect that targets Gram-negative or Gram-positive bacteria.

3.4. Synthesis of levoglucosenone (1) derivatives and evaluation of their bioactivity

As mentioned in previous paragraphs, the results shown are of interest because they are the first evidence of an antibacterial activity for levoglucosenone. Additionally, **1** is a chiral synthon with unique structural features, which makes it a versatile compound in organic synthesis. Because of these characteristics, it is widely used as a starting material for the synthesis of a plethora of compounds with diverse structures and functions (Sarotti et al., 2012).

Taking these elements into consideration, we designed a concise set of derivatives of **1** (Scheme 1) to evaluate their effect on bacterial growth.

It has been shown that bromination of double bonds can improve certain biological activities of natural products (Méndez et al., 2011). For this reason, we performed the bromination of **1** which afforded **2** in 92% yield. Reduction of the carbonyl group or the double bond in **1** could also give us insights about the involvement of these functional groups in the bioactivity observed (compounds **3–5**). In order to generate the saturated ketone **3**, we carried out the hydrogenation of **1** using catalytic amount of Pd/C. Further reaction of **3** with $NaBH_4$ produced the saturated alcohol derivative **5**. The allylic alcohol **4** was synthesized by reducing **1** with $NaBH_4$. It was of interest to generate an epoxide, as this functional group interacts with biomolecules such as proteins and DNA (González-Pérez et al., 2012). Epoxidation of **1** with *m*-CPBA afforded the isomeric epoxides **6** and **7**. All derivatives **2–7** were obtained in straightforward manner with good to excellent yields.

Results shown in Fig. 6 demonstrate that derivative **2** has a stronger effect than **1** over *Salmonella* growth. While derivatives **3**, **4** and **5** showed a milder effect compared to levoglucosenone, compounds **6** and **7** yielded growth curves that were indistinguishable from the one obtained when untreated bacteria was assayed.

The precedent study suggests that the carbonyl group is important for the antibacterial activity. The loss of this functional group causes a significant decrease in the observed effect.

3.5. Effect of 1 and 2 on bacterial viability

To discern between a bacteriostatic versus a bactericidal effect, we explored bacterial survival after treatment with 0, 40 or 80 μg mL^{-1} of levoglucosenone. Aliquots of the growth culture were withdrawn at different time-points and colony forming units (CFU mL^{-1}) were determined. As shown in Fig. 7, at 3 h post inoculation, the increase in levoglucosenone concentration in the growth medium results in a concomitant reduction in the CFU counts. However, at 6 h, no statistical significant difference in CFU values can be detected between the samples taken from the cultures treated with 0 or 40 μg mL^{-1}. At 8 h, samples taken from the cultures treated with 0, 40 or 80 μg mL^{-1} of levoglucosenone did not show statistical significant difference in CFU values. These results suggest that upon contact with bacteria the compound exerts its action by rapid killing, and that, at concentrations below 160 μg mL^{-1} levoglucosenone in the culture medium, a viable subpopulation of bacteria is able to resume proliferation.

A similar behavior is observed when **2** is used in this experiment (Fig. 8). There is a decrease of the CFU count at an earlier time post-inoculation, and the effect extends beyond 3 h. Recovery of bacterial growth is observed at later times (6 h) in accordance with a stronger effect of this compound when compared to levoglucosenone.

The fact that the addition of **1** or **2** decreases CFU·mL^{-1}, lowering the number of viable bacteria, indicates that these compounds are able to rapidly kill a fraction of the initial bacterial population, in a dose-dependent manner. The remaining sub-population of viable bacteria would be responsible for culture growth recovery.

The results achieved in this study demonstrated the efficacy of coupling a pyrolysis procedure to a straightforward autographic assay in order to generate and identify a bioactive compound from soy hulls, a massive byproduct derived from agro-industrial activity.

4. Conclusions

Levoglucosenone was identified as the compound responsible for the bioactive effect in the bio-oil. It showed a striking biocidal action over Gram (−) and Gram (+) bacteria, being the first report of an antimicrobial activity for this molecule.

Levoglucosenone was used as molecular scaffold for the rational design of novel derivatives that allowed to assess the structure/activity relationship for the observed antibacterial effect and to find a product with enhanced bioactivity.

Finally, this work proofs the usefulness of a combined strategy that can be applied for the discovery of bioactive entities from waste materials of economic significance.

Author contributions

G. F. Giri and G. Viarengo contributed equally to the present work. E. García Véscovi directed the biological studies. The manuscript was written through contributions of all authors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2017.05.005.

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