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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]