Antimicrobial activity of *Melia azedarach* fruit extracts for control of bacteria development in inoculated *in-vitro* shoots of ’MRS 2/5’ plum hybrid and calla lily and extract influence on the shoot cultures

By Grazia Marino¹*, Francesca Gaggia¹, Loredana Baffoni¹, Chiara Toniolo², Marcello Nicoletti²

¹Department of Agricultural Sciences, Bologna University, viale Fanin, 44 - 40127 Bologna, Italy.
²Department of Environmental Biology, Sapienza University of Rome, Piazzale A. Moro 5 - 00185 Rome, Italy

*e-mail address of corresponding author: grazia.marino@unibo.it

HPTLC ANALYSIS

The HPTLC system (CAMAG, Muttenz, Switzerland) and materials have been described in the ‘Material and Methods’ section of the paper.

Some of the plates obtained from the fruit extracts, and showed in the Figures of the paper, were further processed for densitometric analysis, as reported below. Additional HPTLC analysis was performed to compare the couple of filter-sterilized, H₂O/EtOH preparations PEE2 and PEE3 with the crude methanol extract, PM1 MeOH, and the autoclaved PM4, and also with the more widely studied neem cake (NC). A methanol extract (Nicoletti *et al.*, 2012) of a commercial de-oiled NC was used in present trials in order to evidence its different composition with *M. azedarach*, as the two species are often confused in raw material of derived products.
Standards

Standards used in the HPTLC analysis were isolated from neem cake (i.e., salannin) in previous research (Nicoletti et al., 2011 and 2012) or were bought in the market, like catechin (Aldrich) and chlorogenic acid (Extrasynthese). Purity (> 98.5%) was detected by NMR (Bruker 400 MHz),

Sample Application

Filtered solutions were applied with nitrogen flow. The operating conditions were: syringe delivery speed, 100 nl s⁻¹; injection volume, 2 μl; band width, 6 mm; distance from bottom, 15 mm.

Development

The HPTLC plates were developed in the automatic and reproducibly developing chamber ADC 2, saturated with the same mobile phase for 20 min at room temperature. The length of the chromatogram run was 80 mm from the point of application. The developed layers were allowed to dry in air for 5 min, derivatized with a selected solution, including anhysaldehyde (1.5 ml p-anisaldehyde, 2.5 ml H₂SO₄, 1 ml AcOH in 37 ml EtOH) and/or Natural Product Reagent (NPR) (1 g diphenylborinic acid aminoethylester in 200 ml of ethyl acetate), dried in the open air and then dipped into Macrogol reagent (1 g polyethylene glycol 400 in 20 ml of dichloromethane). Finally, the plates were warmed for 5 min at 120 °C before inspection. All treated plates were inspected by a CAMAG TLC visualizer under a UV light at 254 or 366 nm or under reflectance and transmission white light (WRT), respectively before and after derivatization. Phenolic nature of compounds at the starting line was confirmed by positive reaction at Ferric chloride and Ferricyanide tests (Marini-Bettolo et al. 1981; Graham, 1992). For the densitometric analysis, the scanner was set at 366 nm, after a multi-wavelength scanning between 190 and 800 nm in the absorption mode had been preliminarily tried. Minimum background compensation was performed on the x-axis during the scanning. The sources of radiation were deuterium and tungsten lamps. The slit dimension was kept at 6.00 x 0.45 mm and the scanning speed used was 100 mm s⁻¹.
CAMAG DigiStore2 digital system with win CATS software 1.4.3 was used for the documentation of derivatized plates.

**Stability and repeatability**

Sample solutions of the extracts were found to be stable at 4 °C for at least 1 month and for at least 3 days on the HPTLC plates. They were prepared and stored at room temperature for 3 days and then applied on the same HPTLC plate, and the chromatogram was evaluated for additional bands. Similarly, band stability was checked by keeping the resolved peaks and inspecting at intervals of 12, 24 and 49 h.

Overlapping of bands is a typical analytical challenge for complex mixtures like multi-ingredient products. HPTLC allowed a good separation and visualization of the constituents. Repeatability was determined by running a minimum of three analyses. RF values for main selected compounds varied ± 0.02 %. The effects of small changes in the mobile phase composition, mobile phase volume, duration of saturation were minute and reduced by the direct comparison. On the contrary, the results were critically dependent on prewashing of HPTLC plates with methanol.

**RESULTS**

The HPTLC fingerprint analysis of total fruit extracts and derived fractions was reported in Fig. 5 of the paper, and widely described. The densitometric conversion of the plate (Fig. SM1) more clearly shows that the differences between PEE2 and the H2O/EtOH extract (PE) are mainly quantitative, in particular for constituents at the starting line.
Fig. SM1. Fig. 6. Densitometric analysis of PEE2 (November 2011), PM1 MeOH (crude methanol extract) and PE (H₂O/EtOH extract), respectively tracks 1, 3 and 5 of plate in Fig. 5 of the paper.

The HPTLC analysis reported in Fig SM2 evidences several differences in composition between PEE2, PEE3 and the MeOH extracts, including NC; this testifies the importance of the solvent used in the extraction. The differences are very strong in the spots at the starting line, that can be assigned to polar polyphenols. On the contrary, track 3 (autoclaved PEE) evidences a strong similarity with tracks 1 and 2, but the presence of some additional spots; while the spots in the middle of the plate are concentrated in PM1. Therefore, the effect of preparation of PEE2 and PEE3 accounts for a prevalence of polar polyphenols in comparison with the crude methanolic PM1 extract. The comparison with catechin standard allows to determine the absence of this compound in the fruit extracts, that suggests the consequent possible absence of condensed tannins. The presence of chlorogenic acid in PEE extracts was assumed by the comparison with the spots in the tracks, as well as by separation from PE obtained by Column Chromatography in the silica gel using the same solvent of the plate and identifying the isolated product by analysis and comparison of Nuclear Magnetic Resonance (NMR) data. Finally, salannin was used as a standard of Meliaceae limonoids: in PM1 it is possible to hypothesize the presence (in any case in very low quantity) of
this type of compounds, although further analyses are necessary to ascertain the complete composition of PEE, besides the main constituents.

Fig. SM2. HPTLC fingerprint analysis of *Melia azedarach* fruit extracts. Mobile phase: Toluene: AcOEt 6:4 (v/v). Visualization: 366 nm. Derivatization: Natural Product Reagent (NPR) and Anysaldeide. Tracks 1 to 5 are respectively: PEE2, PEE3, PEE4 (autoclaved), NC (MeOH extract) and PM1 MeOH; 6 to 8 are selected standards as reported in the figure.

**Literature cited**

