# Supercritical Fluid Extraction of *Persea indica* leaves

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*Persea indica* is a relict tree from the Macaronesian laurel forest with reported insecticidal effects due to its content in ryanodane diterpenes. *P. indica* leaf extracts are obtained by operating a pilot plant which uses supercritical $CO_2$ as solvent, with and without ethanol as modifier. This work is focused on the supercritical extraction of the leaves, the chromatographic analysis of these extracts, and their bioactivity. The biological assays were carried out with larvae of the insect *Spodoptera littoralis* and juveniles of the nematode *Meloidogyne javanica*.

## INTRODUCTION

"Green Chemistry" is the future of current chemistry. Through clean and economical processes and safe products, a sustainable development is tried to be achieved (1). The potential advantages of supercritical fluid extraction for food, pharmaceutical and related substances are well known (2). On the other hand, the need of developing ecological pesticides is urgent, due to the increasing pesticide safety requirements (3).

Biopesticides based on natural products are traditionally extracted by organic solvents, while plant essential oils, which are also used as biopesticides or for human consumption, are traditionally obtained by hydrodistillation. These extraction techniques have several disadvantages, such as the heat instability of essential oils and the difficulty to remove the solvent from the solute, in the extraction with organic solvents. Furthermore, organic solvents are a source of VOCs that contribute to elevated $CO_2$ levels that could enhance climate change. These disadvantages can be overcome by the use of supercritical fluid extraction with $CO_2$ as solvent. $CO_2$ is stable, inert, non toxic, cheap and can be reused. The main limitation of this supercritical solvent is its total apolarity, which becomes a disadvantage when polar compounds have to be extracted. In this case, the use of an entrainer or modifier is desirable, being ethanol the most widely used. In our work, ethanol as modifier is used and both supercritical extractions, with and without entrainer, are compared. (1quit)

*Persea indica* leaves are chosen as working material because of the composition of their extracts. The major components of this plant are ryanodane diterpenes such as ryanodol/isoryanodol with interesting insecticidal properties. Beyond their toxic nature, their interest lies in the higher selectivity towards insects than towards mammals (4).

The chemical characterisation of the extracts was done by means of HPLC-MS chromatography, and we then tried to correlate their bioactivity with their composition.

*S. littoralis* larvae, a polyphagous crop pest, are the target insects. *M. javanica* is a plant pathogenic nematode. Nowadays, half of the world production of nematicides is used to fight against it, with its associated environmental risk.

Therefore, the aim of this work is to characterise chemically the supercritical extracts of *Persea indica* leaves, and to evaluate their possible application as biopesticides.

## MATERIALS AND METHODS

*Plant material:*
*Persea indica* branches were collected in Monte de las Mercedes, Tenerife, and its aerial part air dried. The dried material was supplied to the University of Zaragoza, where it was pre-treated by milling and sieving. Milling was accomplished in a domestic food processor with addition of liquid nitrogen to avoid loss of volatiles and sieving was carried out in a vibratory shaker. A particle size of 0.555mm was selected to carry out the extraction experiments.

*Target pests:*
*S. littoralis* larvae were reared on artificial diet and maintained at 24±1 ºC, >70% relative humidity with a photoperiod of 16:8 h (light:dark) in a growth chamber.
*M. javanica*: From infected marmande tomato, *Lycopersicon esculentum*, egg masses of *Meloidogyne javanica* were manually isolated. From their emerging, infective juveniles (J2) of 24 to 48 hours life time, were selected.

*Supercritical extraction:*
Two consecutive supercritical $CO_2$ extractions were done to the same plant material, one without entrainer and the other with ethanol as entrainer. The extraction vessel conditions of the run without entrainer were 135 bar and 40 ºC. For the exhaustion of the matrix, a second extraction with ethanol as entrainer and conditions of 180 bar and 40 ºC, was completed. $CO_2$ was 99.995% (mass) in purity, supplied by Abelló Linde. The experiments were carried in a pilot plant built from scratch by GATHERS. The plant is described fully elsewhere (5), being its main components the following: a compression pump, a cylindrical extraction vessel of 1 L, and two separators of 180 mL. The pilot plant is completed with a 0.5 L cylindrical refrigerating jacket, a heat exchanger, a back pressure and a flowmeter-totalimeter.
The experimental procedure is similar to the one described by Langa et al.(6). 80 g of triturated *P. indica* leaves were introduced in the extraction vessel and embedded symmetrically by inert porous materials, allowing a homogenous $CO_2$ flow to be achieved. Once the temperature in the vessel and separators was reached, the pump compressed the $CO_2$ up to the desired pressure, and by the action of the heat exchanger, temperatures above critical temperature were obtained. When this supercritical fluid in the selected *pT* working conditions (135 bar and 40 ºC for the first extraction) filled the reaction vessel, the extraction could be started. The pressure in these separators was controlled by manipulating different valves and needle valves, and the low temperatures in the first separator were reached due to the action of the refrigerating jacket. The extract in the second separator was collected, weighed and stored at different time intervals in order to obtain an extraction curve. Once this curve reached its asymptote, after 500 minutes of effective extraction, the vessel was decompressed and the extraction finished. Once this first extraction experiment was completed, over the same matrix, a new experiment was fulfilled, differing in the pressure conditions (reaching 180 bar) and in the addition of 50 ml of ethanol by soaking the material prior to the extraction. The process for the second extraction was the same as the one described above. The asymptote appeared before in this extraction, with 400 minutes of effective extraction.

The pressure and temperature difference between the separators (more than 50 bars and 10 ºC) allowed a first fractionation of the different compounds.

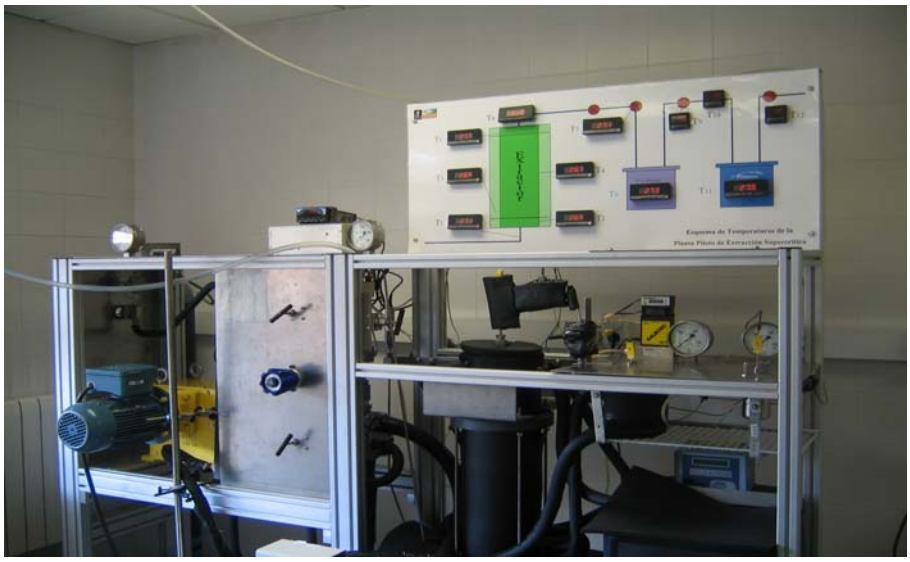


**Figure. 1.** Pilot plant of supercritical extraction GATHERS, Universidad de Zaragoza.

*Chromatography:*

Extracts pre-treatment was carried out to select the ryanodol compounds. 500 µl of each extract were solved in EtOAc (1 mg/µl), and injected in silicagel cartridges, Varian, (2cm length, 0.9 cm diameter). They were eluted at increasing polarities: EtOAc 100%; EtOAc/MeOH 50%, MeOH 100% and screened by TLC. The EtOAc 100% fraction contained the ryanodols. Standard 10mg/l solutions of ryanodol and cynnzeylanol previously isolated from *P. indica* were prepared. 10mg/ml extract solutions were also prepared.

A liquid cromatographer with quaternary gradient pump, Finnigan Surveyor Scientific, with a Hypersil HyPurity C18 column (100x2.1mm, particle size: 3µm), coupled to an ion trap mass spectrometer (Finnigan LCQ Deca ion trap), with an ESI interface, was used.

5 µl of each sample were injected. The elution method consisted in two parts: a gradient one ($H_2O$/MeOH from 10 to 60% for 30 minutes) and an isocratic one (15 minutes of $H_2O$/MeOH 90%). The used flow in both parts was of 1 ml/min.

The voltage of the source was of 4.5 kV. The capilar temperature was 325 ºC, with a Full Scan register in the negative mode (*m/z* = 50-700). The calibration was done in SIM mode over the following *m/z*, 383.3 (cinnzeylanol) and 399.3 (ryanodol).

*Bioassays:*

*Choice feeding assays*

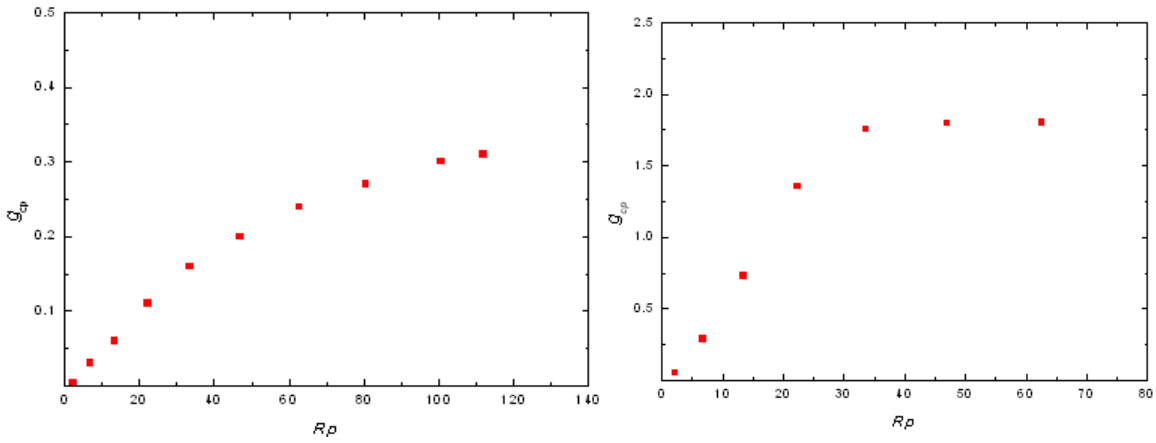
These experiments were conducted with newly emerged sixth-instar *S. littoralis* larvae. Each treatment consisted of 5-10 plates with two insects each and four leaf disks, two treated with solvent (control), and two with the extract solution (10µg/cm$^2$) as previously described (7). The uneaten leaf disk surfaces were measured according to Escoubas with a computer-interfaced scanner (8). Percent feeding reduction (% FR) was determined for each arena by the equation %FR =[1-(treatment consumption/control consumption)] x 100. (9).

*Oral Cannulation:*
This experiment was performed with preweighed newly emerged (24 h) *S. littoralis* L6-larvae. Each experiment consisted of 20 larvae orally dosed with 40 µg of the test fraction extract in 4 µL of DMSO (treatment) or solvent alone (control) as described before (10). At the end of the experiments (72 h), the weight differences between control and treatment larvae are measured.

*Nematicidal bioassay:*
*In vitro* nematicidal activity o f the *P. indica* extracts was determined. The assays were accomplished in 96 well plates, introducing about 200 *Meloidogyne javanica* J2 nematodes per well. Extract solutions in DMSO (800µg/ml) were tested four times. The nematodes mobility and vitality were analyzed 72 hours after their inoculation. Abamectin, Syngenta, was used as positive control.

**RESULTS:**

Two different extraction runs were carried over the same vegetable material, as it is described in the materials section. The supercritical extraction curves are shown in figure 2, where yield (Y axis) is depicted against used $CO_2$ (X axis). Both extraction yield (*Rp*) and used $CO_2$ ($g_{cp}$) are referred to the plant material. *Rp* is given as a percentage [100 x (g extract/g plant material)] and $g_{cp}$ is given as g $CO_2$/g plant material. Curve (a) represents the run with supercritical $CO_2$ as solvent; curve (b) represents the run with ethanol as modifier of the supercritical extraction.




(a) (b)

**Figure. 2.** Extraction curves of the supercritical runs (a) without entrainer, (b) with ethanol as entrainer. *Rp* is the extraction yield, % extract/plant material (g/g); $g_{cp}$ is the ratio used $CO_2$/plant material (g/g)

The chemical composition of different fractions collected in separator 2 during the supercritical extraction is gathered in table 1. HPLC-MS analyses were carried out as described in the material section. Separator 1 allowed a fractionation of the heavier compounds which are not the aim of this study. Some of the major compounds of the collected extracts in separator 2 are shown in figure 3.

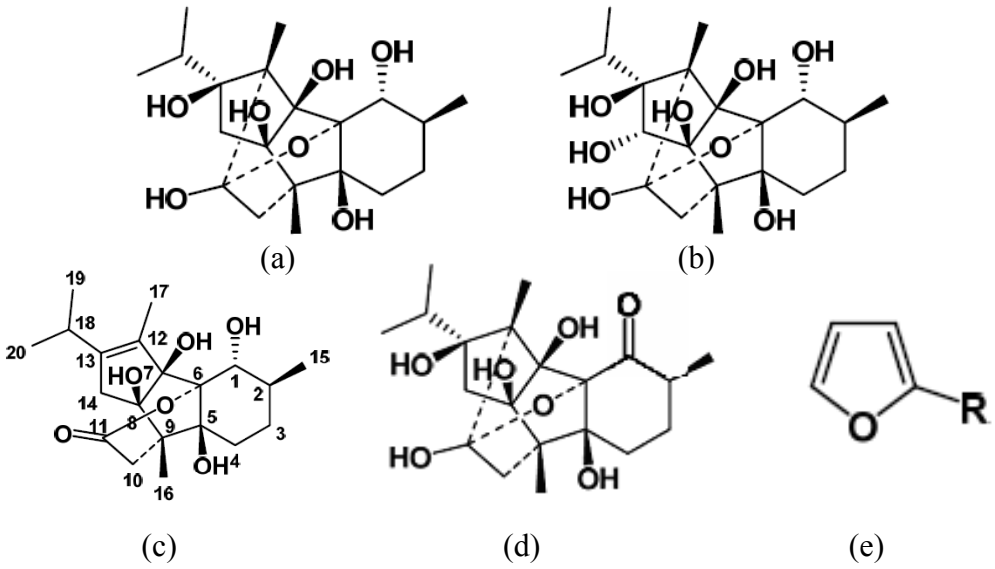



**Figure 3.** Major compounds present in the extracts. (a)Cinnzeylanol (b)Ryanodol (c) Anhidrocinnzeylanine (d) Cynnzeylanone (e) Avocadofurans

**Table 1.** Major compounds of the different fractions collected in separator 2 during the extraction.

| | | Composition (mass %) | | | | |
|---|---|---|---|---|---|---|
| | Fraction | a | b | c | d | e |
| $CO_2$ sc | 1 | - | - | - | - | 17.20 |
| | 4 | 8.68 | - | 6.97 | - | 29.57 |
| | 6 | 7.79 | - | traces | 1.44 | 31.11 |
| | 8 | 8.44 | traces | traces | 1.74 | 23.11 |
| $CO_2$+modif | 1 | 12.38 | 5.47 | traces | 1.30 | 13.50 |
| | 4 | 13.76 | 16.53 | 9.29 | 2.11 | 6.62 |
| | 6 | 18.07 | 9.04 | traces | 2.82 | 5.53 |

(a)Cinnzeylanol (b)Ryanodol (c)Anhidrocinnzeylanine (d)Cynnzeylanone (e) (f) Avocadofurans

The bioactivity of different fractions of the extracts is shown in the following table (Table 2). The antifeedant action on *S. littoralis* in choice feeding assays is expressed as %FR (percentage food consumption). The toxic action over *S. littoralis* in oral cannulation is examined by comparing weight differences. The nematicidal action over *M. javanica* juveniles is controlled by mortality percentage.

**Table 2.** Results of the different bioassays achieved with several fractions of the extraction.

| | Fraction | Choice feeding assays[a] | Oral cannulation[b] | | Nematicidal effect[c] |
|---|---|---|---|---|---|
| | | | larva | diet | |
| $CO_2$ sc | 1 | 53.04 | 65.5 | 79.1 | 19.1 |
| | 4 | 70.22 | 55.1 | 75.2 | 21.65 |
| | 6 | 59.49* | 75.9 | 80.5 | 13.58 |
| | 8 | 68.44* | 93.5 | 92.9 | 9.36 |
| $CO_2$+modif | 1 | 85.29* | 106.5 | 104.7 | 27.72 |
| | 4 | 98.18* | 104.9 | 101.6 | 23.93 |
| | 6 | 98.46* | 100.5 | 101.0 | 31.62 |

* Significantly different from the control ($p<0.05$)

a, %FR =[1-(treatment consumption/control consumption)] x 100

b, weight difference in percentage from control.

c, mortality percentage of J2.

## CONCLUSIONS:

This study focused on the extraction of leaves from the insecticidal tree *P. indica*, the chromatographic analysis of these extracts, and their bioactivity.

The supercritical extraction processes (without and with entrainer) reached the asymptotic phase of extraction. The obtained chromatographic profiles by HPLC-MS, showed as major components ryanodol, cinnzeylanol and avocadofurans, with their abundance varying with the extraction conditions. Referring to the composition, as the extraction advanced, the fractions enriched in cynnzeylanol, ryanodol, anhidrocinnzeylanine and cynnzeylanone. However, the percentage of avocadofurans in the fractions decreased as the extraction progressed. The most polar diterpene, ryanodol, is only present in the extracts when ethanol was added as entrainer of the supercritical extraction. Cynnzeylanone followed the same pattern as ryanodol, but its relative proportion in the extracts is lower.

The antifeedant effects of these fractions on *S. littoralis* can be explained by their composition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