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Antifungal Activity of Seed Powders, Extracts, and Secondary Metabolites of *Pachyrhizus erosus* (L.) Urban (Fabaceae) Against Three Postharvest Fungi

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Failure to control postharvest pathogenic fungi can result in...
serious economic losses to worldwide horticultural production. Fungi such as Colletotrichum gloeosporioides (Penz.) Penz. y Sacc., Fusarium oxysporum Schlechtend.:Fr., and Rhizopus stolonifer (Ehrenb.:Fr.) Vuill. cause diseases on different fruits and vegetables, and all are considered major plant pathogens (Farr et al., 1989). There is a world need to develop new and acceptable postharvest disinfection methods. To minimize the adverse effects of synthetic products on agro-ecosystems and because of the emergence of plant pathogens resistant to the currently used fungicides, it is necessary to evaluate other alternatives such as natural products. Plants synthesize a vast array of organic compounds, commonly called secondary metabolites, that play an important role in the complex interactions between plants and other organisms. One of their many functions is a chemical defense against pathogens and herbivores (McLaren, 1986). Important roles of secondary metabolites are to maintain pathogenicity.

In vitro bioassay. Seed powders were prepared at four concentrations (0.5, 2.0, 5.0, and 10.0 mg/ml), added to 24 ml of potato-dextrose agar (PDA), and autoclaved (15 lb/cm², 15 min). After sterilization media were poured into Petri plates (60 x 15 mm). A five mm agar disc containing the respective pathogen was placed at the center of each plate which was then incubated at 25°C as follows: One day for R. stolonifer, four days for F. oxysporum, and C. gloeosporioides. Mycelial growth (colony diameter) was measured at the end of the incubation time. Six replications were run simultaneously for each concentration of seed powder. Control Petri plates contained only PDA. Tests were finished when mycelium of the control plates reached the edge of the dishes. The experiment was repeated twice. Growth inhibitory effects were calculated as follows: % I = (MGC - MGT)/MGC x 100 where: % I = % Inhibition; MGC = Mycelial growth in control, and MGT = Mycelial growth in treatment.

Extraction. Seed powders (1.5 kg) were extracted with hexane, dichloromethane, and acetone for 48 h in each solvent system at room temperature. After each extraction step, the seed extracts were concentrated in a rotary evaporator (Büchi R-114). The hexane extract was rich in yellow oil (14.3% average yield of dry weight). From the dichloromethane extract, a dark yellow precipitate was obtained with 4.88% yield, and acetone extract had 1.21% yield. Three concentrations (2.0, 5.0, and 10.0 mg/ml) of each extract were used to amend 24 ml of PDA, which was then autoclaved and poured into Petri plates. Growth of each test fungus was recorded at the end of each incubation time. Each treatment was replicated six times.

Isolation, separation and identification of secondary metabolites. Organic extracts were subjected to column chromatography (CC) on silica gel 60 (Merck 0.2-0.5), at the ratio (1:20). Elution was carried out with a mixture of hexane-dichloromethane in order to increase polarity in a linear gradient. The identity of components was determined by infrared, ultraviolet, nuclear proton magnetic resonance, and carbon¹³ spectroscopic analysis. The melting point was determined in a Fisher-Johns apparatus Mod. 12-144 (Fisher, USA). The yellow dark precipitate and supernatant obtained with dichloromethane extract, and the acetone extract, were subjected to column chromatography to isolate different isoflavonoids. The isolated compounds and certificate rotenone (1) were used as reference to determine their presence in dicyclomethane and acetone extract by thin layer chromatography (TLC) using silica gel plates (Merck, 0.25 mm), and eluted with dichloromethane. Developed TLC plates sprayed afterwards with a reagent (Cerium IV dehydrate sulphate-Baker 2% in 2N H₂SO₄) and warmed up on a hot plate (150°C, 1 min) were evaluated in UV light. The RF-value = Solute front/ solvent front of six compounds was determined in these conditions. The secondary metabolites amounts were then dissolved in 1 ml of acetone to obtain a final concentration of 250 µg/ml, added to the PDA media, and tested for fungicidal activity as described previously. Two different controls were used: the first containing only PDA, and the second containing PDA amended with 1.0 ml of acetone. Petri dishes were incubated in darkness at 25 ± 1°C, and the antifungal properties of the six compounds eluted from CC were tested against C. gloeosporioides, F. oxysporum, and R. stolonifer measuring mycelial growth as previously described. Three plates were run per treatment.

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MATERIALS AND METHODS

Plant material. Seeds of P. erosus variety Agua Dulce were donated by farmers to the Centro de Desarrollo de Productos Bióticos in Yautepec, State of Morelos, Mexico. They were dipped in 1% sodium hypochlorite solution, rinsed with distilled water, and air-dried. To obtain a better extraction of the active compound, seeds were finely ground and then stored at room temperature (26°C) in amber bottles until further use.

Microorganisms. The following postharvest pathogens were isolated from infected papaya (Carica papaya L.): C. gloeosporioides, F. oxysporum, and R. stolonifer. Each fungus was frequently inoculated and reisolated from its host in order to maintain pathogenicity.
Statistical analysis. Data were analyzed through ANOVA. Treatments were arranged in a completely randomized design, and the mean separation was carried out by Turkey's multiple range test (p < 0.05).

RESULTS AND DISCUSSION

Powders fungistatic effect. In general, a dose-effect response for the four concentrations was observed for the fungi evaluated (Fig. 1). Seed powder of P. erosus had a significant inhibitory effect of -2 to -15% (F = 12.01, **p < 0.001) on C. gloeosporioides. A stimulation effect was observed on F. oxysporum and R. stolonifer by the four concentrations tested. For these two fungi growth occurred from the lowest (0.5 mg/ml) concentration. In this study, it was observed that seed powders caused inhibition of mycelial growth of one fungus and stimulation on the other two, probably owing different susceptibility of each fungus as previously reported by Barrera-Necha et al. (2002), Bautista-Baños et al. (2000), Bravo-Luna et al. (1998), and Montes et al. (1997). It was also observed that seed powder showed specific fungicidal activity against C. gloeosporioides.

Seed extract fungistatic effect. A dose-effect response for the three concentrations was also observed on the fungus evaluated (Fig. 2). All extracts significantly inhibited from -5.4 to -64.9% (F = 88.93, **p < 0.001) the three fungi at 2.0 5.0, and 10 mg/ml, with the exception of R. stolonifer.---

![Fig 1. In vitro effect of Pachyrhizus erosus seed powder on percent growth inhibition and stimulation of three fungi.](image1)

![Fig 2. In vitro effect of Pachyrhizus erosus hexane, dichloromethane, and acetone extracts on percent growth inhibition and stimulation of three fungi.](image2)
which was significantly stimulated by the hexanic extract at 5 and 10 mg/ml. The best fungistatic effect was achieved on R. stolonifer (-64.97%), F. oxysporum (-37.8%), and C. gloeosporioides (-36.4%) when were treated with diconoromethane precipitate extract. With acetone extract, the percentage inhibition was -60.08 on R. stolonifer, -27.4 on F. oxysporum, and -21.4 on C. gloeosporioides. These results suggest that fungistatic compounds might be extracted in these solvent systems and the best fungicidal activity was attained with that solvent of medium polarity as previously demonstrated by Barrera-Necha et al. (2002). These crude extracts are a complex mixture of chemicals and chief among them is rotenone as well as other isoflavonoids (Table 1) (Alavez-Solano et al., 1998).

**Characterization of isoflavonoids.** Diconoromethane extract was eluted with a mixture of hexane-diconoromethane (95:5). Fractions 28-29 from this mixture yielded needles with a melting point (mp) of 235°C that corresponded to the diconorone compound (6). Fractions 49-57 eluted with the same mixture yielded a crystalline compound with mp 195°C identified as erosone (3). Fractions 68-72 eluted with a 90:10 mixture of hexane-diconoromethane afforded two compounds identified as pachyrrizone (4), and pachyrrizone (5) with mp 200-202°C. The acetone extract was also subjected to CC and eluted with the hexane-diconoromethane. Fractions 16-17 yielded a crystalline solid with a mp of 240-242°C that corresponded to the dehydroncotonone compound (2). The acetone extract and the yellow dark precipitate and floated of diconoromethane subjected to column chromatography yielded the following isoflavonoids; erosone, pachyrrizone, and dolineone; also one arilcoumarine, pachyrrizone was isolated. The isoflavone dehydroncotonone was obtained from the supernatant of diconoromethane and acetone extracts. The characteristics of these compounds in TLC plates and the rotenone (1) isolated from all extracts are shown in Table 1, while their chemical structure are shown in Fig.3. The acetone extract presented the isoflavonoids 1 to 6. By TLC, the supernatant of diconoromethane was different in their composition with respect to the yellow dark precipitate. The supernatant had the isoflavonoids with more polarity; rotenone, dehydroncotonone, erosone, and paquirrizone. The precipitate of diconoromethane had the isoflavonoids with less polarity, paquirrizone, and dalineone together with compounds (1) and (4). Preliminarily, the diconoromethane and acetone extract were similar in their chemical composition (Alavez-Solano et al., 1998).

**Fungistatic effect of isoflavonoids.** Secondary metabolites isolated from seed of yam bean significantly inhibited -2.0 to -56.2% (F = 61.73, ***p < 0.001) the three fungi at 250 μg/ml (Fig. 4). However, C. gloeosporioides was significantly stimulated by erosone (7.9%), pachyrrizone, and dolineone (2.81%). The best fungistatic effects were achieved on R. stolonifer (-56.2%) treated with rotenone, on F. oxysporum (-48.0%) treated with pachyrrizone, and on C. gloeosporioides (-21.6%) treated with dehydroncotonone. These results suggest that the highest fungistatic activity was in those compounds of medium polarity. Hansberry et al. (1947) found similar effects when testing the toxicity of seed extract and

<table>
<thead>
<tr>
<th>Compound</th>
<th>RF</th>
<th>DMTf</th>
<th>DMTp</th>
<th>Acetone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotenone (1)</td>
<td>0.17</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dehydroncotonone (2)</td>
<td>0.15</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Erosone (3)</td>
<td>0.26</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pachyrrizone (4)</td>
<td>0.35</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Pachyrrizone (5)</td>
<td>0.45</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dolineone (6)</td>
<td>0.65</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Solvent systems: Diconoromethane. RF values = Solute front/solvent front. DMTf = floated of diconoromethane; DMTp = precipitate diconoromethane.

**Fig. 3.** Chemical structures of constituents isolated from *Pachyrhizus erosus*.
secondary metabolites against different insects. Rotenone had the highest activity compared with pachyrhizine. It has been mentioned that rotenone is poison to insects and fish, but it is not toxic to mammals (Fukami and Nakajima, 1971; Haley, 1978). Its action is to inhibit the mitochondrial respiration by blocking the reduced nicotinamide adenine dinucleotide (NADH)-dehydrogenase segment of the respiratory chain (Fukami, 1956), and to stop cellular division (Brinkley et al., 1974). This flavonoid is biodegraded by different life organisms. In mammals, the hepatic tissue can metabolize rotenone, but in insects the intestinal tissue cannot biodegrade this compound (Fukami et al., 1969). Rotenone was proposed for insect control on fruits and vegetables during storage considering its low toxicity to mammals (Dewick, 1986). Rotenone has two major advantages: humans can digest it relatively safely, and it is unstable in light and heat, losing almost all it is toxicity after 2-3 days (Leslie, A.R., 1994; Matsumura, 1985).

CONCLUSIONS
This is the first study on antifungal activity of seed powders and extracts of Pachyrhizus erosus. Rotenone, pachyrhizine, and dehydrocotonone had effective fungistatic properties. Further investigations are needed to determine the effects of these secondary metabolites in situ studies on fruit.

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LITERATURE CITED


