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ORIGINAL ARTICLE

Antifungal Activity in Ethanolic Extracts of *Carica papaya* L. cv. Maradol Leaves and Seeds

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Abstract Bioactive compounds from vegetal sources are a potential source of natural antifungic. An ethanol extraction was used to obtain bioactive compounds from *Carica papaya* L. cv. Maradol leaves and seeds of discarded ripe and unripe fruit. Both, extraction time and the papaya tissue flour:organic solvent ratio significantly affected yield, with the longest time and highest flour:solvent ratio producing the highest yield. The effect of time on extraction efficiency was confirmed by qualitative identification of the compounds present in the lowest and highest yield extracts. Analysis of the leaf extract with phytochemical tests showed the presence of alkaloids, flavonoids and terpenes. Antifungal effectiveness was determined by challenging the extracts (LE, SRE, SUE) from the best extraction treatment against three phytopathogenic fungi: *Rhizopus stolonifer*, *Fusarium* spp. and *Colletotrichum gloeosporioides*. The leaf extract exhibited the broadest action spectrum. The MIC_{50} for the leaf extract was 0.625 mg ml^{-1} for *Fusarium* spp. and >10 mg ml^{-1} for *C. gloeosporioides*, both equal to approximately 20% mycelial growth inhibition. Ethanolic extracts from *Carica papaya* L. cv. Maradol leaves are a potential source of secondary metabolites with antifungal properties.

Keywords Papaya harvest by-products · Antifungal compounds · Phytopathogen inhibition · Ethanolic extraction

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Introduction

Papaya (*Carica papaya* L.) is prized worldwide for its flavor and nutritional properties. As a crop, papaya is characterized by high yield and precociousness since it begins scaled production before the first year [1]. Fruit harvest produces various by-products as plants are removed after finishing the production cycle (approximately 2 years of continuous production), and immature or sub-quality fruit are discarded (2–5% of total production) [2].

Papaya production and quality in Mexico are limited by post-harvest diseases, which are directly responsible for losses of up to 40% during transport and storage [3]. These diseases include anthracnose, white rot and fruit dry rot, all largely caused by different fungi, mainly *Colletotrichum gloeosporioides*, *Rhizopus stolonifer* and *Fusarium* spp. Synthetic fungicides are essential to effectively controlling fungus attacks on fruit. However, some of these fungicides are toxic in the environment and to humans or animals that come into contact with them, and their efficiency can be reduced as fungi develop resistance due to improper application [4].

Papaya is not the only crop affected by fungal diseases, and there is currently a huge demand for natural and biodegradable fungicides. This demand has generated increased interest in the potential of biological control of pathogens using vegetal extracts containing secondary metabolites. Use of vegetal extracts with antifungal properties has been a common practice for thousands of years, for example, powders or extracts of acacia, garlic, eucalyptus and mint all function as fungicides capable of controlling different diseases. This recent focus on natural management of phytopathogenic fungi has been reflected in extensive research on biological fungicides. Many authors (Many authors [5, 6]), have highlighted the importance of identifying the chemical profiles of secondary metabolites in vegetal extracts with

pesticide or fungicide effects. Aqueous extracts from the leaves of *Pithecellobium dulce*, *Achras sapota*, *Annona cherimola*, *Casimiroa edulis*, *Citrus limon*, *Crataegus mexicana*, *Carica papaya*, *Psidium guajava*, *Persea americana* and *Spondias purpurea* effectively control in vitro development of the post-harvest fungi *Alternaria* spp., *Fusarium* spp., *Pestalotiopsis* spp. and *Rhizopus* spp. [7]. Powders and aqueous and ethanolic extracts of *Pithecellobium dulce* seeds and leaves have been shown to reduce infection of the phytopathogenic fungi *Botrytris cinerea*, *Penicillium digitatum* and *Rhizopus stolonifer* in strawberries during storage [6].

Carica papaya L. leaves and seeds are known to contain proteolytic enzymes (papain, chymopapain), alkaloids (carpain, carpasemine), sulfurous compounds (benzyl isothiocyanate), flavonoids, triterpenes, organic acids and oils [8, 9]. Extracts from different papaya tissues have been shown to be bioactive. Aqueous extracts of leaves and seeds are known to have antifungal activity against *Colletotrichum gloeosporioides* [10], and aqueous and organic extracts of seeds have antihelminthic activity against *Caenorhabditis elegans* [11, 12]. Alcoholic extracts of the epicarp, endocarp, roots and seeds from ripe and unripe papaya fruit have antidiarrheic, antidysenteric and antibacterial properties [9, 13, 14], and aqueous extracts of seeds have contraceptive effects in male albino rats [15].

In Yucatan, Mexico, papaya by-products are generally disposed of in open areas, although the fruit is occasionally used as animal feed, particularly in the dry season when forage is scarce. High transport costs seriously limit any secondary uses and in most cases this waste is left to rot, allowing phytopathogens growth that cause ecological problems and pose a risk to human health [2]. One potential alternative use for these wastes is extraction of biologically-active metabolites. Likewise very little research has been done to determine extraction yields using commonly available solvents in an effort to develop simple, cost-effective methods for extracting biologically-active metabolites from non-conventional sources like papaya harvest by-products.

The present study objective was to describe the in vitro antifungal activity of ethanolic extracts of papaya harvest by-products (leaves, seeds from ripe and unripe fruit), determine the minimum inhibitory concentration of these extracts and qualitatively identify some of the bioactive groups of compounds present in them.

Materials and Methods

Vegetal Material

Plant material was collected at Rancho San Pedro (Km 139.5, Carretera Mérida-Tizimín), Yucatan state, Mexico. Collected vegetal material consisted of harvest by-products such as plants removed after completing the production cycle, immature fruit and fruit that did not meet quality specifications (e.g. size, shape). By-products were classified by tissue type: leaves; seeds from ripe fruit; and seeds from unripe fruit. Samples were dried at 55°C in a convection oven, ground in a mill to reduce particle size and the flour screened through # 20 mesh.

By-product Proximate Analysis

By-product proximate composition was determined following standard procedures (AOAC): nitrogen (954.01); crude fat (920.39); ash (923.03); crude fiber (962.09); and moisture content (925.09). Protein percentage was calculated as nitrogen × 6.25 and carbohydrate content estimated as nitrogen free extract (NFE).

Ethanolic Extracts

A 2^2 factorial design was used with four replicates of the central trial to determine best extraction conditions. The evaluated factors were extraction time (4, 14 and 24 h) and the flour:solvent ratio (1:8, 1:10 and 1:12), with yield being the response variable. Bioactive compound extraction was done using ethanol under constant agitation [14]. Extracts were recovered by vacuum filtration, centrifugation at $5{,}000 \times g$ for 30 min and elimination of remnant alcohol in a rotary evaporator. Ethanolic extraction yield was calculated using the formula:

$$\% \text{ Yield} = (\text{OW} - \text{MW}/\text{SW})100$$

where OW is the overall weight of matrass with dry sample; MW the constant weight of ball matrass; and SW is the sample weight.

Phytochemical Tests to Identify Active Principles

Extracts with the highest and lowest extraction yields were analyzed with phytochemical tests. Alkaloids were tested for by dissolving a portion of extract in diluted hydrochloric acid, mixing and filtering it. The filtrate was then assayed with Dragendorff and Wagner reagents. Flavonoids were tested for with the Shinoda test [16] and saponins were determined with the foam test [13]. Presence of triterpenes was determined by dissolving a portion of extract in 1 ml chloroform in a test tube, adding 1 ml acetic anhydride along the tube walls and leaving it to rest under refrigeration. Appearance of red, rose, green, purple or blue colors in the interphase after adding 1–2 drops sulfuric acid is considered a positive reading [16].

In Vitro Antifungal Activity

Micelial Inhibition Assay

Three phytopathogenic fungus strains obtained from Colección Nacional de Cepas Microbianas y Cultivos Celulares (CINVESTAV-IPN) were used: *Rhizopus stolonifer* (NRRL-2710), *Fusarium* spp. (CDBBH-1172) and *Colletotrichum gloeosporioides* (CDBBH-1340). Strains were kept in potato dextrose agar (PDA) or broth (PDB), and the cultures stored at room temperature. Strains were allowed to grow for 4–7 days before use in the antifungal activity tests. Inhibition of mycelial growth was determined by cutting approximately 5 mm diameter discs from the edge of a young fungus culture colony, and placing the disc in the center of a Petri dish on PDA containing 20 mg ml^{-1} of previously sterilized extracts from papaya leaves and seeds [17]. The dishes were left to incubate at room temperature and the experiment terminated when the control culture (PDA without extract) completely colonized the agar surface. Radial growth was measured with a digital vernier and based on three replicates per experiment. Results were expressed as the percentage of radial growth inhibition in the extract-containing media versus the control medium according to the formula of Bautista-Baños et al. [10]. Additionally, the efficiency of the commercial fungicide Ridomil Bravo (Chorothalonil 72%–Metalaxyl 9%) was evaluated employing 1 g l^{-1} in PDA medium.

Minimum Inhibitory Concentration 50 (MIC_{50})

Leaf extract (LE) was selected for determination of MIC_{50} because it exhibited the broadest inhibitory activity against the tested fungi strains. The MIC_{50} was not determined for *R. stolonifer* because it had no sensitivity to any of the tested extracts. Growth curves were generated for *Fusarium* spp. and *C. gloeosporioides* by comparing biomass content and time, and these used to standardize the amount of inoculum to be used in later tests. Raw leaf extract was dissolved in 0.5% Tween 80 solution to create a 20 mg ml^{-1} mother solution. This solution (5 ml) was mixed into the same volume of PDB to produce a 10 mg ml^{-1} concentration and then diluted in series to produce concentrations of 5, 2.5, 1.25 and 0.625 mg ml^{-1} [18]. Tubes containing the different concentrations were inoculated with a suspension of spores from the fungi to be tested, and the tubes incubated at 180 rpm and 30 ± 3°C for 5–10 days. Dry weight was determined by centrifuging the culture medium at 10,000 rpm and 5°C for 5 min and then drying at 80°C for 12 h to obtain the cells. Total biomass was evaluated using a cell count in a Petit Salumbeni chamber under a microscope. Total cell population (X) was calculated with the formula:

$$X = \frac{\text{cells counted}}{\text{quadrants counted}} \times \text{Dilution} \times \left(\frac{1}{1.6 \times 10^{-6}}\right) = \text{Total population}\left(\frac{\text{cel}}{\text{ml}}\right)$$

The dry weight/cell population correlation was determined by taking samples at different times during growth to calculate the percentage of inhibition.

Statistical Analysis

Data were analyzed with an analysis of variance and means compared with a Duncan test. All analyses were done with the Statgraphics Plus v. Centurion XV for Windows system.

Results and Discussion

Proximate Analysis

The seeds from ripe fruit had proximate composition different from those reported for other papaya varieties (Table 1). For seeds from ripe fruit in *Carica papaya* L. var. Batek Batu, Puangsri et al. [19] reported 28.3% protein, 30.7% fat, 19.1% fiber, 25.6% carbohydrates (by difference) and 8.2% ash. Likewise, Singh [20] reported a proximate composition of 27.8% protein, 28.2% fat, 22.6% fiber, 11.7% carbohydrates and 3.5% ash. These differences are probably due to use of a different variety in these studies. The seeds from unripe fruit analyzed here had higher ash and carbohydrate contents, but lower fiber and fat contents than the seeds from ripe fruit. Protein content in the leaves was high (29.5%), which coincides with reports of low molecular weight proteins in this tissue as part of the plant defense mechanism [21]. It is worth noting, however, that this content is considerably higher than reported by Galindo-Estrella et al. [2] for the same tissue (5.6%). The discrepancies between the present results and

Table 1 Proximate composition of *Carica papaya* L. cv. Maradol by-product flours

Composition (%)	By-product		
	Leaf	Seed unripe fruit	Seed ripe fruit
Ash	10.3 ± 0.0^{a}	9.9 ± 0.0^{a}	8.5 ± 0.0^{b}
Crude protein	29.5 ± 0.1^{a}	21.1 ± 0.3^{b}	21.2 ± 0.0^{b}
Crude fiber	22.9 ± 0.3^{a}	36.6 ± 0.2^{b}	45.2 ± 0.0^{c}
Crude fat	2.8 ± 0.1^{a}	8.5 ± 0.1^{b}	18.1 ± 0.1^{c}
NFE	35.9 ± 1.1^{a}	22.8 ± 1.5^{b}	6.9 ± 0.4^{c}

Different letter superscripts in the same row indicate statistical difference ($P < 0.05$)

previously reported values are probably due to differences in soil type, climate, variety, fruit type and seasonality, among other factors [22].

Extraction Yields

The highest yields were produced with the longest extraction time (12 h) and the highest flour:solvent ratio (1:12), and both these factors had a significant ($P < 0.05$) effect on yield (Table 2). Seeds from both ripe and unripe fruit produced higher yields than leaf tissue. This agrees with the results of Carmona et al. [23], who reported that in aqueous extracts of *Calendula officinalis* L. factors such as agitation time and the flour:solvent ratio had a significant influence on response variables. They also reported a positive effect of temperature on bioactive compound extraction yield. Higher temperatures were not used in the present work because they influence in degradation of secondary metabolites such as alkaloids and flavonoids [24, 25].

Detection of Secondary Metabolites

The phytochemical tests showed the highest presence of compounds with antibacterial and antifungal activity (e.g. alkaloids, triterpenes, flavonoids, and saponins) to be in the leaf extract from the best treatment (Table 3). The extract from seeds from unripe fruit in the best treatment had the highest triterpene and saponine contents, but no alkaloids or flavonoid contents, probably due to the unripe condition of the fruit. These results are similar to those of Oloyede [26], who reported saponins and cardenolides in pulp from unripe *C. papaya*. Also, Marfo et al. [27] reported presence of phytates, glucosinolates and tannins in papaya seeds from Nigeria, but these compounds were not evaluated in the present study. Phytochemical analyses have also shown the presence of compounds such as alkaloids, tannins, saponins, steroids and flavonoids, among others, in methanolic extracts from plants such as *C. sendtnerianum*, *P. barbatun*, *B. latifolia*, *B obtusifolia*, *S. muricata*, *G. verrucosa* and *C. repens*, and other native species from southern Ecuador [28].

Using qualitative tests of *Carica papaya* leaves, Hadi and Bremner [24] identified the presence of alkaloids, which are quite probably an important element in defense against plant pathogens [29]. For example, several alkaloids isolated from *Ruta graveolens* L. leaves have fungicidal activity against *Colletotrichum* and *Fusarium* species [29]. In addition, two or more phytoconstituents (e.g. alkaloids, tannins, phenols, glycosides and flavonoids) were detected in alcoholic extracts of *Mangifera indica* L., *Murraya koenigii* leaves and *Caesalpinia bonducella* F seeds [30].

Latex is another constituent in papaya leaves [21] and contains chitinases, some of which have been shown to have strong antifungal activity in biochemical analyses [31]. No analyses were done in the present study to identify chitinase in the extracts, but these enzymes may play an important role in the antifungal activity identified in the LE.

Table 2 Ethanolic extraction yields (%) from *Carica papaya* L. cv. Maradol by-product flours, obtained with the 2^2 model

Treatment	Leaf	Seed ripe	Seed unripe
1	3.1 ± 1.1^{b}	17.9 ± 1.2^{a}	16.8 ± 0.2^{a}
2	5.5 ± 0.1^{a}	19.6 ± 0.5^{a}	18.4 ± 0.9^{b}
3	4.5 ± 0.0^{b}	18.7 ± 0.4^{a}	18.0 ± 0.1^{ab}
4	7.1 ± 0.2^{a}	24.3 ± 0.1^{b}	20.2 ± 0.2^{c}
Central	5.9 ± 0.9^{a}	21.0 ± 1.0^{c}	20.0 ± 0.6^{c}

Different letter superscripts in the same column indicate statistical difference ($P < 0.05$)

In Vitro Antifungal Activity

Micelial Inhibition Assay

The LE more efficiently inhibited mycelial growth in *C. gloeosporioides* and *Fusarium* spp. compared to the extracts from seeds from ripe and unripe fruit (Table 4), although it was less effective than the commercial fungicide. The inhibition percentage of the LE versus *Fusarium* spp. was similar to the 24.2% reported for ethanolic

Table 3 Qualitative identification of phytochemical compounds in ethanolic extracts

By-product	Yield	Alkaloids	Flavonoids	Triterpenes	Saponins
Leaf	High (14 h, 1:10)	+++	++	+++	+
	Low (4 h, 1:8)	++	+	++	+
Seed unripe	High (14 h, 1:10)	−	−	+++	+++
	Low (4 h, 1:8)	−	−	+	++
Seed ripe	High (14 h, 1:10)	+	−	+	+++
	Low (4 h, 1:8)	−	−	+/−	++

Negative (−), doubtful (+/−), scarce (+), moderate (++), abundant (+++)

Table 4 Mycelial inhibition (radial diffusion) of evaluated *Carica papaya* L. by-product ethanolic extracts

Fungi strain	Mycelial inhibition (%)			
	LE	SUE	SRE	Ridomil
Fusarium spp.	18.17 ± 1.8	8.99 ± 1.5	1.60 ± 0.08	60.82 ± 0.3
Colletotrichum gloeosporioides	21.84 ± 1.3	0	0	51.20 ± 1.3
Rhizopus stolonifer	0	0	0	32.56 ± 0.9

LE Leaf extract, *SUE* seed (unripe) extract, *SRE* seed (ripe) extract

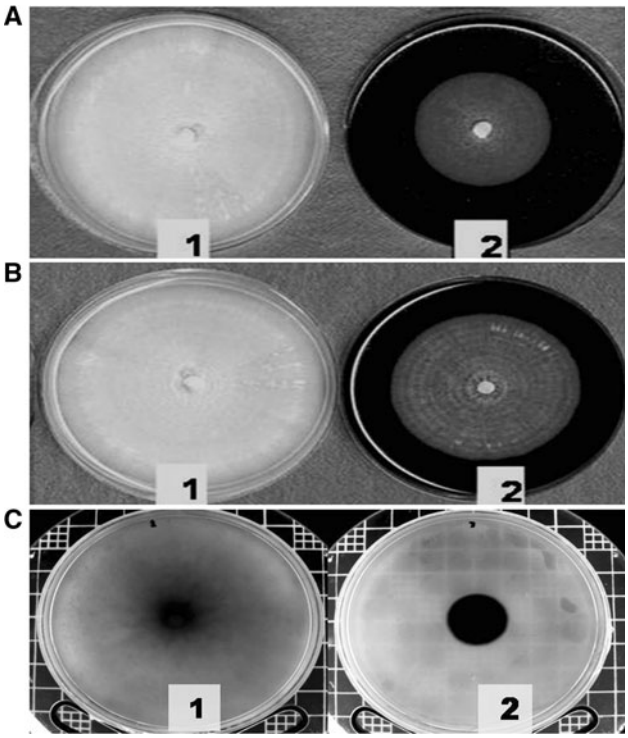

Fig. 1 Growth inhibition of phytopathogenic fungi by *Carica papaya* leaf ethanolic extract (LE) and commercial fungicide in PDA medium: A1, *C. gloeosporioides* control; A2, *C. gloeosporioides* with LE; B1, *Fusarium* spp. control; B2, *Fusarium* spp. with LE; C1, *Fusarium* spp. control; C2, *Fusarium* spp. with Ridomil

extracts of *Crupinia crupinastrum* (1,000 ppm) versus *Fusarium oxysporum* [32]. Inhibition with the ethanolic extract from seeds from unripe fruit versus *Fusarium* spp. was 9%, which is slightly higher than the 7% reported for an ethanolic extract of *Micromeria nervosa* (1,000 ppm) versus *Fusarium oxysporum* [32] (Fig. 1).

Using aqueous extracts of *C. papaya* leaf and seeds of mature papaya fruit (2:10 wt/v) at a 10:4 v/v proportion in PDA, Bautista-Baños et al. [33] reported no inhibition of *C. gloeosporioides* after 7 days incubation compared to a control. These results are similar to the no inhibition observed here for the SRE and SUE (Table 4), which is noteworthy because organic extracts are reported as being more effective mycelial growth inhibitors than aqueous extracts due to the greater solubility of bioactive compounds in organic solvents [13]. Inhibition of growth in *C. gloeosporioides* has also been reported with methanolic leaf extracts from *Pithecellobium albicans*, *Tribulus cistoides*, *Viguiera dentata* and *Piscidia piscipula* (all native to Yucatan) in PDA medium (2 mg ml^{-1} final concentration) (4.4, 23.1, 43.7 and 56.2% inhibition, respectively) [34]. The 21.8% observed here is similar only to the inhibition level for *T. cistoides*.

Ethanolic leaf extracts of *Silene armeria* L., a flowering plant native to Central and Northern Europe, cause growth inhibition in *Fusarium oxysporum* (49.3%), *Fusarium solani* (61.3%) and *Colletotrichum capsici* (53.0%) [35]. These are higher inhibition levels than observed in the present study for the LE against same fungi genera.

None of the extracts tested here inhibited growth in *R. stolonifer*, which coincides with the lack of inhibition observed with ethanolic extracts of *Pithecellobium dulce* leaves and seeds versus *R. stolonifer* [6].

Minimum Inhibitory Concentration 50 (MIC_{50})

The MIC_{50} of the LE was 0.625 mg extract ml^{-1} medium versus *Fusarium* spp., and >10 mg ml^{-1} versus *C. gloeosporioides*. These levels are comparable to those for a hexanic extract (0.5–25 mg ml^{-1}) and an ethanolic extract (0.25–10 mg ml^{-1}) of senescent *M. azedarach* leaves versus different fungi (*A. flavus*, *D. phaseolorum* var. meridionales, *F. oxysporum*, *F. solani*, *F. verticillioides*, and *S. sclerotiorum*) [36]. They are also similar to the 0.5 mg ml^{-1} MIC reported for a methanolic extracts of *S. armeria* L. versus *F. solani*, but different from the 1 mg ml^{-1} of the same extracts versus *C. gloeosporioides* [35]. The inhibition levels observed in the present study against *Fusarium* spp. are lower than the 1–2 mg ml^{-1} (0.1–0.2%) against *Aspergillus fumigatus* (wild type), *A. niger* (ATCC16404), *F. oxysporum* (wild type) and *Penicillium* sp. (wild type) reported by Nostro et al. [37] for diethyl ether extracts of *Nepeta cataria*. They are also lower than the 42.2% inhibition against *F. oxysporum* reported for ethanolic extracts of *Achillea santolina* (1,000 ppm) [32].

According to a MIC-based antifungal activity rating of plant extracts proposed by Maregesi et al. [38], the *C. papaya* leaf extract analyzed here would be rated a moderate inhibitor of *Fusarium* spp. and a weak inhibitor of *C. gloeosporioides*. The antifungal compounds of the LE of *C. papaya* assayed are not well known, but its flavonoid, alkaloid and terpene contents may influence its mycotoxicity by interacting with fungus membrane constituents.

Ethanolic extracts from *Carica papaya* L. cv. Maradol leaves are a potential source of secondary metabolites with antifungal properties. Developing applications for these extracts will require further research into the in vivo antifungal activity of their compounds in fruit from different plants.

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