RESEARCH NOTE

Postharvest Biology and Technology 55 (2010) 129–132

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Research Note

Fungicidal activity of compounds extracted from the pericarp of *Areca catechu* against *Colletotrichum gloeosporioides in vitro* and in mango fruit

Punnawich Yenjit[a,*], Montree Issarakraisila[a], Warin Intana[a], Kan Chantrapromma[b]

[a] *Walailak University, School of Agricultural Technology, Nakhon Si Thammarat 80160, Thailand*
[b] *Walailak University, School of Science, Nakhon Si Thammarat 80160, Thailand*

ARTICLE INFO

Article history:
Received 7 January 2009
Received in revised form
10 September 2009
Accepted 10 September 2009

Keywords:
Anthracnose
Antifungal
Areca
Colletotrichum
Mango

ABSTRACT

Areca catechu L., a member of the Palmaceae family, is one of the most commonly used drugs in the world. Compounds obtained from the hexane, ethyl acetate and methanol extracts of the pericarp of *A. catechu* L. were assessed *in vitro* and in mango fruit for antifungal activity against *Colletotrichum gloeosporioides* Penz. *In vitro* studies also indicated that three triterpenes, namely fernenol (fern-9(11)-en-3α-ol), arundoin (fern-9(11)-en-3α-ol ME), and a mixture of stigmasterol and β-sitosterol, and one fatty acid, lauric acid, could inhibit the mycelial growth of *C. gloeosporioides* with EC_{50} values of 36.7, 47.5, 56.7 and 111.5 mg L^{-1}, respectively. Furthermore, fernenol, arundoin, and the mixture of stigmasterol and β-sitosterol highly inhibited spore germination and germtube elongation. Mango fruit studies suggested that fernenol, arundoin and the mixture of stigmasterol and β-sitosterol were significantly more effective than benomyl for controlling postharvest anthracnose disease when used at 100 and 200 mg L^{-1}.

1. Introduction

Mango (*Mangifera indica* Linn.) is an important tropical fruit crop because of the popularity of its fresh and processed products among consumers (Maneepun and Yunchakad, 2004). The most serious problem for mango growers is anthracnose disease caused by *Colletotrichum gloeosporioides* (Penz.) because it can attack various parts of trees and can grow as latent infections on fruit. Synthetic fungicides are commonly used to control pre- and postharvest anthracnose diseases. Hot benomyl (methyl [1-[(butylamino) carbonyl]-1H-benzimidazol-2-yl]carbamate) dips effectively control postharvest anthracnose diseases, but a buildup of pathogen resistance may occur (Pitkethley and Conde, 2007). Recently, synthetic fungicides have been found unacceptable for consumers due to the presence of fungicide residues (Sanders et al., 2000). As a result, the use of natural antifungal compounds from plants to control mango anthracnose disease has been investigated (Kumpoun et al., 2005).

Areca catechu L., belonging to the Palmaceae family, grows in much of the tropical Pacific, Asia, and parts of east Africa. It is one of the most commonly used drugs in the world, containing alkaloids, tannins, polyphenols, sugars, and lipids that have anthelmintic, antifungal, antibacterial, anti-inflammatory, and antioxidant activities (Staples and Bevacqua, 2006). Particularly, the pericarp of *A. catechu* L. is known to also contain β-sitosterol, leukocyanidins, tannins and lipids that exhibit antibacterial and antifungal properties (Kapoor, 2001); however, little is known about the antifungal property of *A. catechu* L. on plant diseases. Therefore, the aim of this work is the development of new methods to control postharvest mango anthracnose disease using compounds extracted from the pericarp of *A. catechu* L.

2. Materials and methods

2.1. Plant material and fungal pathogen

Mature fruit of *A. catechu* L. were collected from the southern part of Thailand. The pericarp was separated from the fruit, cut into small pieces and dried in a hot air oven at 40 °C for 48 h. The phytopathogenic fungus *C. gloeosporioides* was isolated from diseased tissues of symptomatic mango fruit. The virulence and pathogenicity of the isolates were verified by inoculating mango fruit with 5 μL of a spore pathogen suspension. The pathogen was purified via single spore isolation on potato dextrose agar (PDA) and maintained in PDA slants at 10 °C (Cloh, 1999).

2.2. Isolation of compounds

Dried pericarp of *A. catechu* was soaked for 5 d in three organic solvents: hexane, ethyl acetate, and methanol. The crude extracts were separated by flash column chromatography over silica gel 60 (0.040–0.063 mm) and elution with 10% dichloromethane in

* Corresponding author. Tel.: +66 084 0542017; fax: +66 075 672302.
E-mail address: pyenjit@yahoo.com (P. Yenjit).

0925-5214/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.postharvbio.2009.09.003

hexane with the polarity increased in 10% increments using dichloromethane, ethyl acetate, and methanol. The compounds obtained from *A. catechu* pericarp were repeatedly purified by eluting with 5% dichloromethane in hexane with the polarity increased in 5% increments using dichloromethane, ethyl acetate, and methanol. The purification of compounds was assessed on TLC plates (Silica gel 60 F_{254} pre-coated TLC aluminum sheets, Merck) using UV λ_{max} 254 and 316 detector, vanilline-H_2SO_4, and anisaldehyde standard spray reagents. The chemical structures of compounds were determined by spectroscopic methods, ^1H and ^{13}C-nuclear magnetic resonance spectroscopy (NMR) and gas chromatography–mass spectrometry (GC–MS). ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX-500 MHz spectrometer, with deuterochloroform ($CDCl_3$) and tetramethylsilane (TMS) as internal standards for the chemical shift parameter (δ) (^{13}C 77.0 mg kg^{-1}, residual ^1H 7.24 mg kg^{-1}). All 2D NMR data were recorded using standard pulse programs. GC–MS analyses in the scan mode were performed on a HP-5890 series II GC, equipped with EPC coupled to a HP-5972 MS. Samples were injected using a splitless injector at 250 °C on an Rtx-5MS column (30 m × 0.25 μm × 0.25 mm). The carrier gas was helium. The temperature program was as follows: initial 170 °C with a 1-min hold; ramp 0.083 °C s^{-1} to 185 °C, 1-min hold; ramp 0.25 °C s^{-1} to 330 °C; 10-min hold; and scanning from 29 to 500 u. The compounds were characterised by comparisons of their spectral data with known samples (Lall et al., 2006).

2.3. In vitro experiments

To assess mycelial growth inhibition, the purified compounds (5 mg) were dissolved in 5 mL of 10% dimethyl sulfoxide (DMSO) and diluted serially with sterile distilled water. The diluted compounds were added to PDA at 48 °C and the final concentrations were adjusted to 10.25, 25, 50, 100 and 200 mg L^{-1}. The media were poured into 5-cm-diameter Petri plates. Then, a 5-mm-diameter plug of the fungi, removed from the margin of a 4-d-old colony on PDA, was placed in the centre of the agar plates. The compound treatments were compared with the treatment of a known fungicidal standard: benomyl (benlate 50%), and 0.5% DMSO served as control. Each treatment was tested in three replicates. The diameter of colonies was measured after incubation at 28 °C for 5 d. Data were calculated as percentage inhibition of mycelial growth using the following equation: Inhibition (%) = [(colony diameter in untreated control (0.5% DMSO) – colony diameter in treatment) × 100]/colony diameter in untreated control. The EC_{50} values (effective concentration resulting in 50% reduction in fungal growth) were determined by probit-log analysis using the statistical package for social science software (SPSS) (Hadacek and Greger, 2000). The experiment was repeated twice.

To study effects on spore germination and germtube elongation, the purified compounds (5 mg) were dissolved with 5 mL of 10% DMSO and diluted with sterile distilled water. The diluted compounds (0.1 mL) and 0.05 mL potato dextrose broth (PDB) were dropped in the holes of multi well plates. At the same time, 0.05 mL spore suspension (10^5 spores mL^{-1}) was added to final concentrations 10.25, 25, 50, 100 and 200 mg L^{-1}. The compound treatments were compared with benomyl, and 0.5% DMSO served as control. Each treatment consisted of three replicates. After incubation at 28 °C for 12 h, the treated fungi were strained with lactophenol cotton blue. The number of germinated spores and the length of germtubes were examined by counting and measuring a total of 100 spores in each field of three randomly selected fields under a dissecting microscope at 400× magnification. The results were calculated as the percentage inhibition of spore germination and germtube elongation using the following equation: Inhibition (%) = [(the number of germinated spores or the length of germtubes in untreated control (0.5% DMSO) – the number of germinated spores or the length of germtubes in treatment) × 100]/the number of germinated spores or the length of germtubes in untreated control. EC_{50} values were determined by using a previously described method (Hadacek and Greger, 2000). The experiment was repeated twice.

2.4. Mango fruit experiments

Freshly almost ripe mango fruit (*Mangifera indica* L.), cv. 'Nam-Dork Mai' were harvested from fields in the southern part of Thailand. The surface of the mango fruit was sterilised by immersion in 70% ethanol for 1 min and prepared for inoculation by inflicting one 1-mm-deep wound in the middle of each fruit with a sterile needle. Each wound was then inoculated with the pathogen *C. gloeosporioides* by placing 5 μL of spore pathogen suspension (10^4 spores mL^{-1}). The inoculated fruit were incubated in a sterile box overnight at 28 °C before dipping in the diluted compounds, which were prepared by dissolution in 5 mL of 10% DMSO and dilution with sterile distilled water to final concentrations 50, 100, and 200 mg L^{-1}. The compound treatments were compared with benomyl treatment and 0.5% DMSO served as a negative control. Each treatment consisted of five replicates. Then, the treated fruits were incubated in a moist plastic box at 28 °C for 5 d and disease developments were assessed by measuring the diameter of the anthracnose lesion on mango fruits. Statistical differences were determined by the least significance difference (LSD) test at $P \leq 0.05$ using SPSS software (Regnier et al., 2008). The experiment was repeated twice.

3. Results

Three triterpenes and five fatty acids were obtained from the crude extracts of *A. catechu* pericarp using flash column chromatography. Structural analysis by ^1H, ^{13}C NMR and GC–MS resulted in identification of the three triterpenes as fernenol (fern-9(11)-en-3α-ol), arundoin (fern-9(11)-en-3α-ol ME) and a mixture of stigmasterol and β-sitosterol. The five fatty acids were identified as lauric, myristic, pentadecanoic, palmitic and stearic acids. The potential antifungal activity of the compounds against *C. gloeosporioides* was demonstrated in an *in vitro* study. The results showed that the compound fernenol had the highest activity against the mycelial growth of *C. gloeosporioides*, whereas the compounds, arundoin, the mixture of stigmasterol and β-sitosterol, and lauric acid were less active. When the compounds were tested for their effects on spore germination and germtube elongation of *C. gloeosporioides*, the results indicated that fernenol, arundoin and the mixture of stigmasterol and β-sitosterol substantially inhibited both spore germination and germtubes elongation. In particular,

Table 1
EC_{50} values (mg L^{-1}) of triterpenes and fatty acids from *Areca catechu* pericarp against *Colletotrichum gloeosporioides*.

Treatments	EC_{50} (95% FL)		
	Mycelial growth	Spore germination	Germ tube elongation
Fernenol	36.7	45.8	26.7
Arundoin	47.5	62.3	40.0
Mixture of stigmasterol and β-sitosterol	56.7	86.9	50.0
Lauric acid	111.5	>200.0	108.3
Myristic acid	>200.0	>200.0	>200.0
Pentadecanoic acid	>200.0	>200.0	>200.0
Palmitic acid	>200.0	>200.0	>200.0
Stearic acid	>200.0	>200.0	>200.0
Benomyl	110.2	153.3	75.0

EC_{50} was determined by probit-log analysis.
FL, fiducial limits.

Table 2
Efficacy of triterpenes and fatty acids from *Areca catechu* pericarp in controlling anthracnose disease.

Dip treatments	Diameter of lesion (mm)		
	50 mg L^{-1}	100 mg L^{-1}	200 mg L^{-1}
Fernenol	14.2 Ac	10.0 Ab	0.5 Aa
Arundoin	16.0 Bc	12.2 Bb	3.2 Ba
Mixture of stigmasterol and β-sitosterol	16.4 Bc	12.8 Bb	4.7 Ba
Lauric acid	17.2 Bc	15.0 Cb	9.2 Da
Benomyl	17.2 Bc	14.7 Cb	6.2 Ca
Control (0.5% DMSO)	19.6 Ca	19.7 Da	19.5 Ea

Means within the same column or row followed by the same uppercase or lowercase letter are not significantly different by the LSD test ($P \leq 0.05$).

fernenol, arundoin, and the mixture of stigmasterol and β-sitosterol had a greater effect on germtube elongation than benomyl (Table 1). Then, the efficacies of the compounds on control of anthracnose developments were demonstrated by comparison with a commercial fungicide, benomyl. The results, shown in Table 2, revealed that fernenol, arundoin and the mixture of stigmasterol and β-sitosterol were more effective than benomyl. The effects on reduction of disease developments were dose-dependent for all the compounds, with maximum effects being observed at the highest concentration of 200 mg L^{-1}.

4. Discussion

The antifungal activity of *A. catechu* extracts was discovered by Lalithakumari and Sirsi (1965). The compounds obtained from *A. catechu* pericarp were triterpenes and fatty acids, which had antifungal property (Lall et al., 2006; Agoramoorthy et al., 2007). These natural antifungal compounds had no cytotoxicity and no phytotoxicity (Glinski and Branly, 2002; Liu et al., 2008). Consequently, the extracts of *A. catechu* pericarp are not a synthetic fungicide for safety considerations. Several triterpenes and fatty acids have potential to control phytopathogenic fungi. The triterpene saponins pitheduloside A, B, E, F and I isolated from *Pithecellobium dulce* seeds inhibited the mycelial growth of *Rhizopus stolonifer* and *C. gloeosporioides* (Necha et al., 2003), and sucrose fatty acid esters, monoglycerol, and polyglycerol fatty acid esters inhibited the mycelial growth of *Botrysis cinerea* PEAS (Yasuo et al., 2002). Besides, Regnier et al. (2008) suggested in a preliminary study the employment of monoterpenes from *Lippia scaberrima* essential oil to control postharvest pathogens. However, this is the first report on the antifungal potential of the compounds isolated from *A. catechu* pericarp to inhibit the growth of *C. gloeosporioides* and to control postharvest mango anthracnose disease.

The findings of this study are relevant for the potential enhancement of mango fruit quality by natural antifungal compounds from plants. Previously, Droby et al. (1987) found antifungal resorcinols in the peel of mango fruit and their relation to latent infection by *Alternaria alternata*. Our results indicate that three triterpenes, fernenol, arundoin and the mixture of stigmasterol and β-sitosterol (Nakane et al., 1999; Jacob et al., 2005; Koysomboon, 2006) and one fatty acid, lauric acid, (Agoramoorthy et al., 2007) obtained from the *A. catechu* pericarp had good activity inhibiting the growth of *C. gloeosporioides*. The antifungal activity of triterpenes and fatty acids is related to structural characteristics and chemical properties. Compositions containing at least one pentacyclic triterpene and one to three oxygenated substituents, such as hydroxy or carboxy groups have been found to be particularly useful as antifungal agents (Glinski and Branly, 2002). In this study, fernenol, which contains one pentacyclic triterpene and one hydroxyl group, exhibited the highest activity against *C. gloeosporioides*. Fatty acids having 10–13 carbon atoms exhibit excellent antifungal activity, while increasing the number of carbons in fatty acids results in decreased fungal suppression (Yasuo et al., 2002). Thus, in this study, lauric acid with 12 carbons inhibited the growth of *C. gloeosporioides* to a higher degree than other fatty acids that had more than 12 carbons. Walters et al. (2003) reported that lauric acid was highly active in inhibiting the mycelial growth of *Rhizoctonia solani* and *Pythium ultimum* and reduced the infection of barley seedlings by *Blumeria graminis* f. sp. *hordei*.

When the activity of these triterpenes and fatty acids on spore germination and germtube elongation was tested, we found that the triterpenes highly inhibited both spore germination and germtube elongation. For the fatty acid lauric acid, in addition to germtube elongation inhibition, it caused the irreversible disruption and the disintegration of the plasma membrane of spores and germtubes (Liu et al., 2008); it is possible that the lipophilic part of fatty acids could be absorbed by the fungus due to the lipophilic nature of the fungal tissue (Inouye et al., 1999).

The use of fernenol, arundoin and the mixture of stigmasterol and β-sitosterol resulted in better postharvest mango anthracnose control than benomyl treatment. The promising results of our *in vitro* and in mango fruit studies indicate that the compounds have potential to control postharvest mango anthracnose. Additional studies in which the compounds are used in the field or in combination with wax coatings and modified atmosphere packaging would be valuable additions to our knowledge about the efficacy of these compounds.

Acknowledgments

This work was supported from Walailak University funds and the scholarship from Nakhon Sawan Rajabhat University.

References

Agoramoorthy, G., Chandrasekaran, M., Venkatesalu, V., Hsu, M.J., 2007. Antibacterial and antifungal activities of fatty acid methyl esters of the blind-your-eye mangrove from India. Braz. J. Microb. 38, 739–742.
Cloh, T.K., 1999. Single spore isolation using a hand made glass needle. Fungal Diversity 2, 47–63.
Droby, S., Prusky, D., Jacoby, B., Goldman, A., 1987. Induction of antifungal resorcinols in flesh of unripe mango fruits and its relation to latent infection by *Alternaria alternate*. Physiol. Mol. Plant Pathol. 30, 285–292.
Glinski, J., Branly, K.L., 2002. Pentacyclic triterpenes. United States Patent 6,433,010. Available at http://www.freepatentsonline.com/6303589.html.
Hadacek, F., Greger, H., 2000. Testing of antifungal natural products: methodologies, comparability of results and assay choice. Phytochem. Anal. 11, 137–147.
Inouye, S., Uchida, K., Yamaguchi, H., 1999. *In-vitro* and *In-vivo* anti-*Trichophyton* activity of essential oils by vapour contact. Mycoses 44, 99–107.
Jacob, J., Disnar, J.R., Boussafir, M., Albuquerque, A.L.S., Sifeddine, A., Turcq, B., 2005. Pentacyclic triterpene methyl ethers in recent lacustrine sediments (Lagoa do Coço, Brazil). Org. Geochem. 36, 449–461.
Kapoor, L.D., 2001. Handbook of Ayurvedic Medicinal Plants: Herbal Reference Library. CRC Press, Inc., Lincoln, 416 pp.
Koysomboon, S., 2006. Isolation and biological activities of the chemical constituents from *Derris indica* and *Garcinia schomburgkiana* and seasonal variation in the major metabolites of the brown alga *Cystophora moniliformis*. PhD Dissertation, College, Walailak University, Nakhon Si Thammarat, Thailand, 236 pp (available at the WU Library).
Kumpoun, W., Chansri, P., Somsrivichai, J., Supyen, D., 2005. Antifungal compounds in Thai mango fruit latex. Acta Hort. 682, 2057–2061.
Lalithakumari, H., Sirsi, M., 1965. Antibacterial and antifungal activities of *Areca catechu* Linn. Indian J. Exp. Biol. 3, 66–67.
Lall, N., Weiganand, O., Hussein, A.A., Meyer, J.J.M., 2006. Antifungal activity of naphthoquinones and triterpenes isolated from the root bark of *Euclea natalensis*. J. South Afr. Bot. 72, 579–583.
Liu, S., Ruan, W., Li, J., Xu, H., Wang, J., Gao, Y., Wang, J., 2008. Biological control of phytopathogenic fungi by fatty acids. Mycopathologia 166, 93–102.
Maneepun, S., Yunchakad, M., 2004. Developing processed mango products for international markets. Acta Hort. 645, 93–105.
Nakane, T., Arai, Y., Masuda, K., Ishizaki, Y., Ageta, H., Shiojima, K., 1999. Fern constituents: six new triterpenoid alcohols from *Adiantum capillus-veneris*. Chem. Pharm. Bull. 47, 543–547.
Necha, L.B., Banos, S.B., Luna, L.B., Torres, K.B., Estrada, M.J., Chilpa, R.R., Suàrez, F.J.G., 2003. Antifungal activity against postharvest fungi by extracts and compounds of *Pithecellobium dulce* seeds (Huamuchil). Acta Hort. 628, 761–766.

Pitkethley, R., Conde, B., 2007. Mango anthracnose. Available at http://www.nt.gov.au/d/Content/File/p/Plant_Pest/604.pdf.

Regnier, T., Plooy, W.D., Combrinck, S., Botha, B., 2008. Fungitoxicity of *Lippia scaberrima* essential oil and selected terpenoid components on two mango postharvest spoilage pathogens. Posth. Biol. Tech. 48, 254–258.

Sanders, G.M., Korsten, L., Wenner, F.C., 2000. Survey of fungicide sensitivity in *Colletotrichum gloeosporioides* from different avocado and mango production areas in South Africa. Eur. J. Plant Pathol. 106, 745–752.

Staples, G.W., Bevacqua, R.F., 2006. *Areca catechu* (betel nut palm). Available at http://www.webalice.it/siamseeds/Database/Areca-catechu-betel-nut.pdf.

Walters, D.R., Walker, R.L., Walker, K.C., 2003. Lauric acid exhibits antifungal activity against plant pathogenic fungi. J. Phytopathol. 151, 228–230.

Yasuo, T., Tomomi, H., Satoko, M., Kazunori, T., 2002. Effect of sucrose fatty acid esters, monoglycerol and polyglycerol fatty acid ester on in vitro growth of *Botrytis cinerea* PEAS. and on postharvest control of rot in fruits. Food Preserve Sci. 28, 227–234.