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Analysis of phenolic compounds and antifungal potential of arabica coffee pulp extract using acidified ethanol extraction

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Abstract Extraction of *Coffea arabica* pulp with 70% ethanol acidified with 1% HCl yielded the highest recovery of bioactive compounds, including TPC (41.82±0.99 mg GAE/g DW), TFC (424.69±7.19 mg QE/g DW), and TTC (40.87±2.10 mg TAE/g DW), alongside superior antioxidant activity (The DPPH and ABTS radical scavenging assays demonstrated the lowest IC_{50} values, whereas the FRAP assay revealed the highest antioxidant capacity). The highest chlorogenic acid content (13.17±0.04 mg/g DW) occurred in the 50% ethanol, whereas the 70% ethanol yielded the greatest caffeine concentration (39.45±0.09 mg/g DW). Antifungal assays demonstrated that immersion of *Penicillium digitatum* mycelial plugs in coffee pulp extract (0.1 g/mL) for 5–10 min completely inhibited growth (100%), whereas shorter immersion times (1–3 min) produced only partial inhibition (9.18–23.47%). These findings confirmed that acidified ethanol extraction efficiently recovered phytochemicals and caffeine from coffee pulp, and that the resulting extract exhibited strong antifungal activity against green mold in tangerine fruits, highlighting its potential as a sustainable postharvest disease management strategy.

Keywords: Tangerine, *Penicillium digitatum*, Chlorogenic acid, Caffeine, Ultrasound-assisted extraction

Introduction

Generated in large volumes during wet coffee processing, coffee pulp accounts for nearly half of the berry's mass and represents a significant agro-industrial by-product (Duangjai *et al.,* 2016; Marín-Tello *et al*., 2020). However, by-products from green bean production are often discarded, contributing to environmental pollution and inefficient resource. Although often discarded as waste, coffee pulp contains a rich composition of phytochemicals, notably

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chlorogenic acid, caffeic acid, ferulic acid, protocatechuic acid, catechin, epicatechin, and caffeine (Ramirez–Martinez, 1988; Sangta *et al*., 2021). Recent studies have explored the valorization of coffee pulp across multiple fields, e.g., agriculture, biotechnology, pharmaceuticals, and food processing (Geremu *et al.,* 2016; Loukri *et al*., 2020; Marín-Tello *et al*., 2020). In agriculture, coffee pulp can be used in biocontrol strategies against plant disease, such as the treatment of fungal infections like leaf spot in lettuce (*Alternaria brassicicola*), leaf spot in oil palm (*Pestalotiopsis* sp.), and leaf spot in coffee (*Paramyrothecium breviseta*), highlights its potential for disease control in agricultural practices (Sangta *et al*., 2021). Published studies have reported that phenolic constituents like chlorogenic acid, caffeic acid, and tannins, along with caffeine, exhibit inhibitory effects on several fungal pathogens, notably *Aspergillus*, *Penicillium*, *Colletotrichum*, *Fusarium*, *Rhizopus*, and *Rhizoctonia* species (Ansari *et al*., 2013; El–Khateeb *et al*., 2013; Konuk and Ergüden, 2020).

Multiple extraction strategies have been utilized to isolate phenolic compounds from coffee pulp, with outcomes varying according to methodological design. Traditional techniques like distillation and pressing, though widely adopted, often suffer from limited efficiency and extended processing times (Marín–Tello *et al*., 2020; Pyrzynska, 2024), which may compromise phenolic integrity through oxidative or thermal degradation (Stoica *et al*., 2013). Crucial factors such as solvent polarity, thermal input, and extraction duration lead to notable changes in the yield and stability of extracted constituents (Le *et al*., 2024). Among all techniques, solvent extraction stands out as the most advanced and widely adopted method. It delivers high yields of phenolic compounds and is considered reliable for large–scale recovery. However, modern non-conventional methods are gaining attention for their improved performance and sustainability (Marín–Tello *et al*., 2020; Pyrzynska, 2024). The application of ultrasound in extraction processes has shown considerable promise, particularly in improving mass transfer and reducing solvent usage. Ultrasound-assisted extraction (UAE) utilizes the cavitation effect to generate localized high temperatures and pressures, which facilitate the breakdown of cellular structures and promote solvent penetration. Studies have shown that UAE can achieve up to 85% yield while consuming significantly less energy – about 3.4 times lower than traditional methods (Bondam *et al*., 2022; Jabbari *et al*., 2024). This makes it an attractive option for extracting bioactive compounds from coffee pulp (Sangta *et al*., 2021). Another effective technique involves using acidified ethanol. A 70% ethanol solution, especially when acidified, has proven to extract higher total phenolic content (TPC) compared to pure ethanol or lower concentrations. The acidic environment helps break down cell walls, facilitating the release of polyphenols (Duangjai *et al*., 2016).

Additionally, lower pH conditions stabilize compounds like catechins, reducing their risk of oxidation during extraction (Nonthakaew *et al*., 2015; Duangjai *et al*., 2016).

This investigation examined the phenolic compound content in coffee pulp and its effectiveness in suppressing postharvest fungal growth on tangerines caused by *Penicillium digitatum*.

Materials and methods

Plant material

Arabica coffee pulps harvested during 2022–2023 from Chiang Mai, Thailand, were preserved in sealed polyethylene bag at 28±2 °C until processing. Prior to extraction, samples were re-exposed to drying conditions at 45 °C for 5 h using a hot air oven and subsequently milled into fine powder with a mechanical mill.

Effect of acidified ethanol extraction on phenolic compounds and antioxidant activity in coffee pulp

Extraction was determined using a modified method based on Chen *et al*. (2021). Coffee pulp powder (5 g) was weighed and transferred to beaker and mixed with 50 mL of ethanol at concentrations of 0%, 50%, 70%, and 99.7%, each containing 1% hydrochloric acid (HCl). The mixtures were subjected to UAE using an ultrasonic bath (Power Sonic 410, Hwashin Technology, Korea) at 45 °C for 60 min. After sonication, the extracts were centrifuged at 5,000 rpm for 10 min, and the resulting supernatants were filtered through 0.45 µm syringe filters. Solvent removal was performed at 40 °C using a rotary evaporator (Büchi Rotavapor R–205, Germany), yielding concentrated coffee pulp extracts. These were stored in dark brown glass bottles at –20 °C until analysis. Extraction yield was determined, and the samples were subsequently analyzed for TPC, total flavonoid content (TFC), total tannin content (TTC), and antioxidant activity.

Total phenolic content

The Folin–Ciocalteu colorimetric assay, with slight modification from the method of Shao *et al*. (2014), was employed to determine TPC in the extracts. The reaction mixture consisted of 0.1 mL of diluted sample extract and 1.5 mL of Folin–Ciocalteu reagent (10-fold dilution) and incubated at 28±2 °C for 15 min in darkness. After incubation, 1.5 mL of 7.5% sodium carbonate (Na_2CO_3) solution was added. The reaction mixture was incubated at 28±2 °C in

the dark for 60 min to allow color development, and absorbance was measured at 725 nm using a spectrophotometer (Genesys 30, Thermo Scientific, USA).

Total flavonoid content

The aluminum chloride assay, with slight modification from the procedure of Haile and Kang (2019), was employed to quantify TFC. In brief, 0.1 mL of the appropriately diluted extract was mixed with 0.2 mL of 5% sodium nitrate ($NaNO_2$) solution and incubated for 5 min. Afterward, 0.3 mL of 10% aluminum chloride ($AlCl_3$) solution was added, and the mixture was kept at 28±2 °C for 6 min. Then, 2.5 mL of 1 M sodium hydroxide (NaOH) solution was introduced, followed by incubation in the dark for 10 min to allow color development. Absorbance was subsequently recorded at 510 nm using a spectrophotometer.

Total tannin content

The determination of TTC was determined using a modified method based on Myo *et al*. (2021). A 0.1 mL aliquot of the extract was combined with 1.5 mL of Folin–Ciocalteu reagent (10-fold dilution), and the mixture was incubated in the dark at 28±2 °C for 10 min. After the initial reaction period, 1.5 mL of 35% (w/v) sodium carbonate (Na_2CO_3) solution was introduced to the mixture. The reaction mixture was incubated in the dark at 28±2 °C for 30 min to allow color development. Absorbance was measured at 700 nm using a spectrophotometer.

Antioxidant activities assay

The antioxidant activity of the sample was evaluated using three complementary assays: DPPH, ABTS, and FRAP. The DPPH radical scavenging assay was performed by mixing the sample with a 0.1 mM DPPH solution in methanol, incubating in the dark at room temperature for 30 min, and measuring the absorbance at 517 nm (Chen *et al*., 2021). The ABTS assay involved generating the $ABTS^{\bullet+}$ radical cation by reacting ABTS with potassium persulfate, incubating the mixture for 12–16 hours, diluting to an absorbance of 0.70 ± 0.02 at 734 nm, and reacting with the sample for 6 minutes before measuring absorbance at 734 nm (Re *et al*., 1999). The FRAP assay was conducted by mixing the sample with freshly prepared FRAP reagent (containing acetate buffer, TPTZ, and $FeCl_3$), incubating at 37°C for 30 minutes, and measuring absorbance at 593 nm (Santos *et al*., 2017). DPPH and ABTS radical scavenging assay were expressed as IC_{50} values and FRAP assay was expressed as Trolox equivalent antioxidant capacity (TEAC).

Determination of chlorogenic acid and caffeine contents by High Performance Liqiud Chromatoghaphy (HPLC)

The contents of chlorogenic acid and caffeine were quantified using HPLC. Separation was performed on a Zorbax XDB-C18 column (4.6 × 150 mm, 5 µm particle size). For chlorogenic acid analysis, the mobile phase comprised 0.1% formic acid (solvent A) and acetonitrile (solvent B) in an 85:15 (v/v) ratio. The flow rate was maintained at 1.0 mL/min, and detection was carried out at 330 nm. In the case of caffeine analysis, the mobile phase consisted of deionized water (solvent A) and methanol (solvent B) in a 60:40 (v/v) ratio, with the same flow rate of 1.0 mL/min and detection wavelength set at 274 nm.

Isolation and identification of pathogenic fungus in tangerine fruit

Tangerine fruits purchased from a market located in Chiang Mai were incubated in moisture chamber at room temperature until mycelial growth appeared on the fruit surface. The fungus responsible for green mold disease was subsequently isolated on potato dextrose agar (PDA) using hyphal tip technique. Identification of the fungus was based on morphological characteristics of *Penicillium digitatum.*

Effect of coffee pulp extract in inhibiting mycelial growth

Mycelial plugs of actively growing *P. digitatum* cultured on PDA were exiced using a sterile 0.5 cm diameter cork borer. The plugs were immersed in coffee pulp extract solution at a concentration of 0.1 g/mL (the minimum effective dose capable of inhibiting mycelial growth) for 1, 3, 5, or 10 min. After immersion, the plugs were air-dried under laminar airflow and subsequently transferred onto fresh PDA plates. The plate was incubated at 28±2 °C for 7 days. Growth of fungal mycelium was evaluated on day 7 after inoculation, and growth inhibition was quantified by evaluating the difference between control and treatment groups.

Statistical analysis

All experimental procedures were repeated three times, and results were reported as mean ± standard deviation (SD). Statistical evaluation was performed under a completely randomized design (CRD) using IBM SPSS Statistics® software. Duncan's New Multiple Range Test (DMRT) was applied to compare treatment means at the 5% significance level.

Results

Phenolic compound and antioxidant activity of coffee pulp extract

Phenolic compounds were extracted from Arabica coffee pulp using ethanol at different concentrations (0%, 50%, 70%, and 99.7%) acidified with 1% HCl to evaluate extraction efficiency. Ethanol concentration significantly influenced all measured parameters, including extraction yield, TPC, TFC, and TTC, and antioxidant activity, as shown in Table 1. The greatest yield of extract was obtained using 70% ethanol (47.21±1.08%), which was significantly greater than other treatments, while the lowest yields were observed with 99.7% ethanol (22.40±2.47%) and distilled water (22.65±3.43%). TPC was highest in the 70% ethanol extract (41.82±0.99 mg GAE/g DW), followed by 50% ethanol (19.69±0.19 mg GAE/g DW), whereas the lowest value was found in distilled water extract (10.30±0.27 mg GAE/g DW). TFC was also highest in the 70% ethanol extract (424.69±7.19 mg QE/g DW), followed closely by 50% ethanol (411.97±5.71 mg QE/g DW), while the distilled water extract showed the lowest TFC (133.26±0.76 mg QE/g DW). TTC was highest in the 70% ethanol extract (40.87±2.10 mg TAE/g DW), which was significantly greater than other treatments, and the lowest TTC was observed in the distilled water extract (20.52±1.19 mg TAE/g DW). For the antioxidant activity assays, the extract obtained using 70% ethanol exhibited the highest radicle scavenging efficiency against both DPPH (Figure 1A) and ABTS (Figure 1B), as indicated by the lowest IC_{50} values compared to other ethanol concentrations. Additionally, the FRAP assay (Figure 2) showed that the 70% ethanol extract had the highest ferric reducing antioxidant power, followed by extracts obtained with 50%, 0% and 99.7% ethanol, respectively.

Table 1. Comparison of extraction efficiency and phytochemical profiles of coffee pulp under different acidified ethanol treatments

Ethanol conc.	Yield (%)	TPC (mg GAE/g DW)	TFC (mg QE/g DW)	TTC (mg TAE/g DW)
0%	22.65±3.43$^{c/1}$	10.03±0.27^{d}	133.26±0.76^{d}	20.52±1.19^{d}
50%	28.11±2.96^{b}	19.69±0.19^{b}	411.97±5.71^{b}	33.57±0.14^{b}
70%	47.72±1.08^{a}	41.82±0.99^{a}	424.69±7.19^{a}	40.87±2.10^{a}
99.7%	22.40±2.47^{c}	17.66±0.22^{c}	282.78±5.59^{c}	30.77±0.77^{c}

[1/] Means within the same column followed by different lowercase letters differ significantly at the 5% significance level.

Chlorogenic acid and caffeine content

The content of chlorogenic acid and caffeine in the crude extracts of coffee pulp were determined and are presented in Table 2. Extraction with 50% ethanol

yielded the highest level of chlorogenic acid (13.17±0.04 mg/g DW), followed by 0% ethanol (12.26±0.21 mg/g DW) and 70% ethanol (11.40±0.17 mg/g DW), respectively. In contrast, a negative value of chlorogenic acid was detected in the 99.7% ethanol treatment, suggesting possible degradation or analytical interference. For caffeine content, all treatments yielded comparable values ranging from 27.84±0.24 to 39.45±0.09 mg/g DW, with the highest concentration found in the 70% ethanol extract.

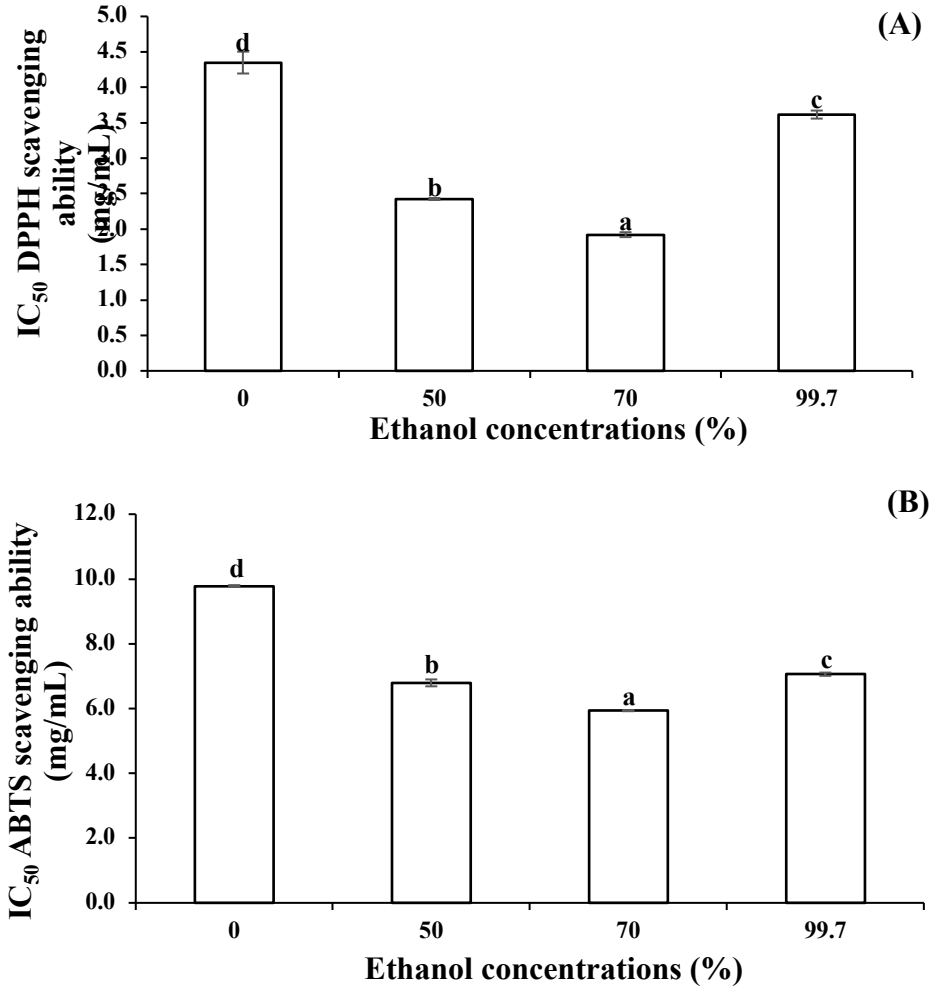

Figure 1. Antioxidant performance of crude extracts from coffee pulp under varying acidified ethanol extraction conditions; (A) IC_{50} DPPH radicle scavenging activity and (B) IC_{50} ABTS radicle scavenging activity

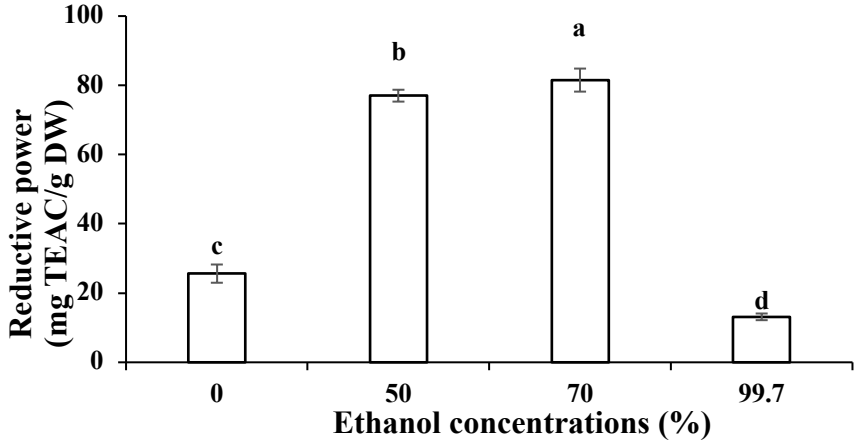

Figure 2. Effect of acidified ethanol concentration on the ferric reducing antioxidant power (FRAP) of coffee pulp crude extracts

Table 2. Variation in chlorogenic acid and caffeine content in coffee pulp extracts as influenced by acidified ethanol concentration

Ethanol concentration	Chlorogenic acid (mg/g DW)	Caffeine (mg /g DW)
0%	12.26±0.21$^{b/1}$	27.88±0.08^{c}
50%	13.17±0.04^{a}	38.37±0.10^{b}
70%	11.40±0.17^{c}	39.45±0.09^{a}
99.7%	1.75±0.47^{d}	27.84±0.24^{c}

[1/] Means within the same column followed by different lowercase letters differ significantly at the 5% significance level.

Fungal isolation and identification

Disease symptom was observed in tangerine fruit after incubated in high humidity and temperature conditions. Initial sign included water-soaked lesion at the stem-end, which subsequently developed into fungal mycelial structure and sporulation. Green mold infection was evident by the appearence of green colonies on the fruit surface, as shown in Figure 3A. For fungal identification, a pure culture was isolated on PDA using hyphal tip technique. Morphological characteristics were identified based on conidiophore, conidia shape, and colony appearance. The green mold isolate exhibited branched conidiophores with terminal phialides, producing globose to subglobose conidia arranged in chains (Figure 3B). Colony on PDA were fast-growing, initially white, and later turned green with a powdery texture (Figure 3C), consistent with the morphology features of *Penicillium digitatum.*

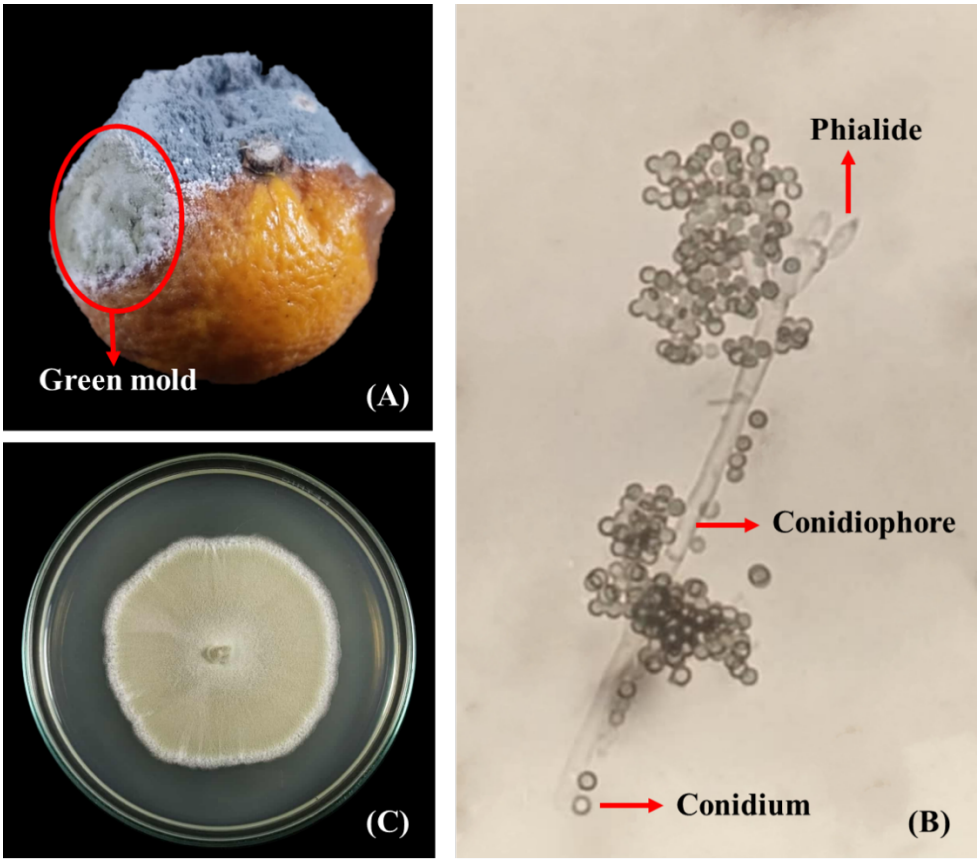

Figure 3. Morphological characteristocs of *P. digitatum*; (A) disease symptom, (B) fungal structure, and (C) colony appearance

Mycelial growth inhibition assay

The effect of immersion time in 0.1 g/mL of coffee pulp extract on mycelial growth inhibition was evaluated and is presented in Figure 4. The inhibitory effect on growth intensified with increasing immersion time. Specifically, immersion for 5 and 10 min both resulted in nearly complete inhibition (100.00%), with no significant difference between the two treatments. Immersion for 3 min yielding moderate inhibition (23.47±3.06%), while treatment of 1 min resulted in the lowest inhibition (9.18±1.77%), with values significantly distinct from those of the remaining treatments. These findings suggested that immersion for at least 5 min is sufficient to achieve maximal antifungal activity of the coffee pulp extract against the pathogenic fungus.

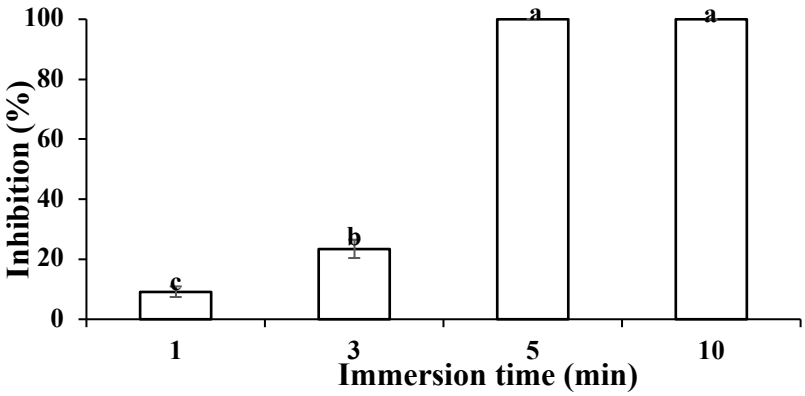

Figure 4. Impact of immersion duration in coffee pulp extract on suppression of *Penicillium digitatum*, the pathogen causing green mold in tangerines

Discussion

This study demonstrated that extracting phenolic compounds from coffee pulp using 70% ethanol acidified with 1% HCl was the most effective method for recovering bioactive compounds. This condition yielded highest extraction efficiency, including TPC, TFC, TTC, and antioxidant activity, compared to other ethanol concentrations. The present findings corroborate previous studies by Duangjai *et al.* (2016) and Alara *et al*. (2021), demonstrating that moderately concentrated ethanol under acidic condition significantly enhances phenolic extraction. Although 50% ethanol yielded the highest chlorogenic acid content (13.17±0.04 mg/g sample), the 70% ethanol extract contained the highest caffeine content (39.45±0.09 mg/g sample). Both compounds are known to play important roles in antifungal activity, particularly against *Penicillum* spp., as supported by Sangta *et al*. (2021) and Geremu *et al*. (2016). The antifungal assay revealed that immersion *P. digitatum* mycelial in coffee pulp extract at a concentration of 0.1 g/mL for at least 5 min completely inhibited mycelial growth (100%). This result aligned with finding from Nonthakaew *et al*. (2015) and Sangta *et al*. (2021), which reported strong antifungal activity of phenolic-rich coffee extract, especially against *Penicillium* species. These results supported the promising use of coffee pulp extract as a sustainable biological agent for postharvest disease management, particularly green mold in citrus fruits, a major concern in tangerine postharvest disease. Utilizing coffee waste extracts could reduce reliance on synthetic fungicides, promote environmentally friendly practices, and add value to agro-industrial by-products.

Nonetheless, additional studies are required to assess the stability of the active ingredients under practical conditions, conduct field trials, and develop commercial formulations to ensure praticial application in agriculture and food indrustries.

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Conflicts of interest

The authors declare no conflict of interest.

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