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RESEARCH ARTICLE

Purification of *Gymnema inodorum* Leaf Extract and Its Antifungal Potential Against *Colletotrichum gloeosporioides*

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Abstract Resistance of *Colletotrichum gloeosporioides*, the causal agent of mango anthracnose disease, to systemic fungicides is of concern. In this study, we evaluated a suitable extraction method for gymnemic acid content from *Gymnema inodorum* and its ability to control *C. gloeosporioides*, the causal agent of mango anthracnose disease. In vitro study of *G. inodorum* extract and gymnemic acid against the most virulent and carbendazim-resistant *C. gloeosporioides* isolate was evaluated. The Soxhlet extraction method gave the highest gymnemic acid yield, followed by the aqueous extraction method. The study of the effect of crude extracts from *G. inodorum*, SP4, MT4, and CR4 at a concentration of 1000 ppm showed significant ability to inhibit *C. gloeosporioides* mycelia growth by 30.20%, 28.30%, and 26.43%, respectively. The results of the 5000 ppm crude extract effect on spore germination showed that the extract of MT4 and MT6 showed a significant decrease in fungal spores during the test period. Therefore, from the results of this study, *G. inodorum* extract obtained from the aqueous extract could be used in postharvest disease control of mango. It can also serve as an alternative material for farmers who intend to reduce their use of chemicals.

Keywords Mango anthracnose · Antifungal · *Colletotrichum gloeosporioides* · *Gymnema inodorum* · Gymnemic acid

Significance statement: *Gymnema inodorum* has antifungal activity and is promising as an environmentally friendly alternative to conventional fungicides. Its mode of action and proper concentration must be deduced for it to have commercial potential.

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Introduction

Fungal diseases are the major problem in postharvest mangoes, the deterioration from fungal diseases such as stem-end rot (*Lasiodiplodia theobromae*), Sooty mold (*Capnodium* sp.) [1], and especially anthracnose disease caused by *Colletotrichum gloeosporioides* Penz [2]. The fruit's symptoms include dark brown to black decay spots, necrotic lesions, sunken tissue, and decay of mango pulp. During transportation or the ripening stage, the lesions will enlarge and spread out and can cause the whole fruit to rot. This disease is common in most mango varieties and affects mangoes' quality and yield. The disease causes damage to all stages of mango growth and development, especially the ripening stage, and this problem is detrimental to the exportation of mangoes. Infection of the pathogen may be latent in unripe mango fruit. The symptoms typically develop rapidly upon ripening. These are associated with adjacent cells being destroyed and lead to water-soaked lesions [3]. However, the penetration of the pathogen is limited to the epidermal cells, and cuticle layer during the immature stage, and disease develops during

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fruit ripening. The mechanism of cuticle penetration is based on two processes; mechanical force through melanin production or the degrading enzyme, cutinase, and/or a combination of both. During the ripening stage, the mango cells are weakened as a result of a gradual decline in the amount of resorcinol in the mango, a phenolic compound that can inhibit fungal pathogens [4].

The management programs to eradicate the mango anthracnose disease are not specific. Integrated management methods such as cultural control, biological control, chemical control, and intrinsic resistance are recommended [5]. Chemical fungicides were a practical and efficient method for disease control. Benzimidazole fungicides have been used to control numerous fungal plant diseases. Nevertheless, continuous and widespread use of fungicides can lead to the rapid development of resistant strains in the target fungi [6]. The benzimidazole fungicide, carbendazim, interferes with fungal β-tubulin polymerization and inhibits mitotic division. Resistant fungi have a reduced binding affinity with carbendazim to β-tubulin and mitotic division proceeds normally. The resistant fungi show evidence of amino acid substitutions in β-tubulin conferred at the protein expression level [7]. Because resistance to this systemic fungicide has been found in the fungal pathogen, an alternative solution or new environmentally safe substance should be studied for control of the disease. Over the past decade, much research has focused on the use of plant products for control of phytopathogens. These plant products are biodegradable, bio-efficacious, economical, and environmentally safe [8].

Gymnema inodorum (Lour.) Decne. (Family Apocynaceae) is a medicinal plant in South Asia and Southeast Asia. In Thailand, the young aerial parts of *G. inodorum* (GI) have been used as a food ingredient in Northern Thai cuisine and as a diabetes therapeutic agent [9]. *Gymnema inodorum* contains triterpene glycosides belonging to the oleanane and dammarane classes, a gymnemic acid. Triterpene glycosides, oleanane- and ursane-type, have been reported to control many pathogenic microorganisms [10]. A wide range of human and plant diseases are controlled including *Candida albicans*, *Fusarium* sp., *Magnaporthe grisea*, *Botrytis cinerea*, *Aspergillus* sp., *Bacillus pumilus*, *B. subtilis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, among others [11, 12]. In this study, we evaluated a suitable extraction method for gymnemic acid content from *G. inodorum* and its ability to control *C. gloeosporioides*, the causal agent of mango anthracnose disease.

Material and Methods

Plant Material and Preparation of *G. inodorum* Extracts

The fully mature leaves from at least 3 year old *G. inodorum* (GI) had been collected during May–July in 2020 from 4 different areas in 3 provinces, i.e., Thoeng in Chiang Rai province (CR), Mae-tang in Chiang Mai province (MT), Hang-chat (HC), and Sob-prab (SP) in Lampang province. Two varieties of GI No. 4 and No. 6 were used for the present study. The extraction methods were performed separately in triplicate: fresh filtrate method, Soxhlet extraction, and organic solvent extraction. The extract was concentrated in a rotary evaporator, followed by lyophilization, and a crude extract was obtained. Percentage of the crude extract yield was calculated and used for purification in the GA study.

Method 1: Fresh Filtrate Method

GI leaves were rinsed in clean water and dried at room temperature (30 ± 2 °C) for an hour. One kg of fresh leaves was cut into small pieces, and5 liters of distilled water were added (1:5 W/V). The mixture was filtered with a straining cloth and heated at 70 °C. The extracts were evaporated in a rotary evaporator to obtain the crude extract, dried in a fume hood, weighed again, and kept at 4 °C for bioassays.

Method 2: Soxhlet Extraction

The GI leaf samples were cleaned with water and dried at 60 °C for 24 h in a hot-air oven. The dried leaves were ground into a powder (< 0.825 mm size). One hundred grams of powdered GI was placed into a cellulose thimble, 250 ml of 95% ethanol was added (1:2.5 W/V), and the extraction method was performed for 90 min[13]. The extracts were evaporated in a rotary evaporator to obtain the crude extract and dried in a fume hood, weighed again, and kept at 4 °C for bioassays.

Method 3: Organic Solvent Extraction

The GI sample was macerated separately with organic solvents (1:5 W/V), ethanol, ethyl acetate, and hexane (AR., Fisher Chemical™, UK) for 3 days at room temperature (30 °C). The extracts were filtered through Whatman No. 1 filter paper. The extracts were evaporated in a rotary evaporator to obtain the crude extract, dried in a fume hood, weighed again, and kept at 4 °C for bioassays.

Detection and Quantification of Gymnemic Acid

For chromatographic analysis, 5 mg of gymnemic acid (GI) crude extracts was dissolved in 10 ml of 50% (V/V) methanol. One ml of 11% KOH was added and heated on a boiling water bath under reflux for an hour and then cooled. Then, 0.9 ml of 12 N HCL was added and refluxed for an hour in the water bath. After cooling, the pH was adjusted to 7.5–8.5 with 11% KOH. This solution was dissolved with 10% (V/V) methanol. Then, the sample was transferred to a 10 ml volumetric flask and used for further analysis using HPLC. The percentage of GI was estimated using the area under the curve obtained from the sample by comparing the same with the standard. Chromatography was carried out using FLEXAR^{TM} LC Systems, PerkinElmer HPLC; Brownlee Analytical C18 column (250 × 4.60 mm i.d. × 5.0 µM) with Mobile phase consisting of (A) 0.5% phosphoric acid solution (B) methanol (12:88); flow rate was 0.6 ml/min; column temperature was 30 °C; photodiode detector array (PDA) wavelength 218 nm; HPLC Isocratic elution mode. The HPLC estimation was carried out by injecting 5 µl of the sample solution with the duplicate sample. Deacyl gymnemic acid at a concentration of 3.91–500 µg/ml was used for standardization.

Extraction and Purification of the Pure Compound for NMR

GI crude extract was dissolved in 200 ml ethyl acetate and then, filtered with a cotton ball. The precipitate was resuspended in 100 ml methanol and sonicated. The extracts were evaporated in a rotary evaporator, and hot methanol was added to obtain the supernatant. The sample was examined by column chromatography using ethyl acetate: methanol (90:10/30:70) as the mobile phase.

Pathogen Inoculum and Carbendazim-Resistant Phenotype Assay

A survey and collection of samples was conducted of Nam Dok Mai mangoes showing anthracnose symptoms from the market in Lampang Province. The symptoms and morphology of diseased mangoes were characterized in the laboratory using a microscope. The pathogen was identified morphologically using a microscope. The fruiting structures of *Colletotrichum* spp. were observed and identified following Sutton [14]. The pathogenicity test was conducted using a completely randomized design (CRD) with four replications. *Colletotrichum* spp. isolates were grouped into five categories [15]. The symptoms and severity of the disease on mangoes were recorded based on the size of lesions 7 days after inoculation at room temperature. Thus, the most virulent strain of the pathogenic isolate was obtained and used for further study. The carbendazim resistance of all isolates of *Colletotrichum* spp. was studied by phenotypic assay. The level of resistance to carbendazim was evaluated and grouped into four representative phenotype reactions [15].

Effect of *G. inodorum* Extracts on Pathogen Growth (In Vitro)

The GI extracts were obtained from the previous experiment to test for their antifungal activities to control *C. gloeosporioides*. The highly carbendazim-resistant and aggressive isolate of *C. gloeosporioides,* isolate 2.1, from the previous experiment was used in this experiment. In vitro antifungal activities of GI extracts were determined using a dual culture test. The experiment was done using a CRD with three replications. Means were compared by DMRT at $p = 0.05$. Twenty microliters of GI extracts were dropped on a paper disk and dried at room temperature (30 ± 2 °C). Thereafter, the highly carbendazim-resistant isolate of *C. gloeosporioides* growing on PDA for 7 days was cut using a sterilized cork-borer at the leading edge of the colony to get mycelial agar plugs, then transferred to the middle of the dual culture plate. Data were collected as colony diameter and transformed to percent inhibition of colony growth (PICG) [16]. The effective antagonist actinomycetes were selected according to PICG. Over 50% were taken to study in further experiments. The formula of percent inhibition of colony growth (PICG) was modified from Lokesha and Benagi[16]: $PICG = C - T/C \times 100$; where C = colony diameter of the pathogen in the control plate and T = colony diameter of pathogen in a dual culture plate. The GI extracts were tested for inhibition of the colony growth of *C. gloeosporioides*. The dual culture test was conducted using a completely randomized design (CRD) with five replications.

Poisoned Food Technique

Crude extract of 0.025, 0.05, 0.10, 0.15, and 0.2% was amended in PDA and sterilized at 121 °C, 15 lb/inch^2 pressure for 15 min. Fifteen milliliters of amended media were poured into Petri plates and allowed to cool and solidify. A culture disk of the 7-day-old culture of the *C. gloeosporioides* was then placed at the center of the Petri dish, and the cultured plates were incubated at 25 ± 2 °C for 7 days. The Petri dishes containing media devoid of the extract but with the same volume of DMSO served as the control. After incubation, the colony diameter was measured in mm[16]. The fungitoxicity of the crude extract in terms of percentage inhibition of mycelial growth was calculated using the formula: Percent inhibition = $C - T/$

$C \times 100$; where C = Average increase in mycelial growth in the control plate and T = Average increase in mycelial growth in the treatment plate. The experiment was done using a CRD with three replications.

Determination of Percent Inhibition of Spore Germination by a 96-Well Plate Method

A conidial suspension of *C. gloeosporioides* was prepared in sterile distilled water, and the spore concentration was adjusted to 1.0×10^5 conidia/ml. Twenty microliters of the conidial suspension and 20 µl of crude extracts (5000 µg/ml) were then placed in the 96-well plate and mixed well. DMSO and carbendazim at a volume of 20 µl served as the control. The setup was incubated for 12 h at 25 ± 2 °C. Spore germination was counted under a compound microscope[16]. The percent of inhibition was calculated using the formula: Percent inhibition = $C - T/C \times 100$; where C = Number of spores germinated in control and T = Number of treated spores germinated. The experiment was done using a CRD with three replications.

Minimal Inhibitory Concentration: MIC and Minimal Fungicidal Concentration: MFC

The lowest fungal growth inhibition concentration and the minimum fungicide concentration of the crude extract were determined. The causative agents were examined using the broth dilution method by dissolving crude extracts of GI. Dimethyl sulfoxide (negative control) and fungicide (carbendazim, prochloraz) were the control treatments. The extract was prepared by serial twofold dilution to 256, 128, 64, 32, 16, 8, 4, 2, and 0 µg/ml. Twenty microliters of the *Colletotrichum* sp. spore suspension (5×10^8 CFU/ml) were added into 96-well plates containing crude extract and incubated under a constant light condition at 30 ± 2 °C. The minimum of extracts that could inhibit the growth of fungi were determined by counting the number of spores remaining using a haemacytometer, at 0, 12, 24, and 48 h and considered an MIC; and the minimum fungicide concentrations (MFC) were likewise revealed. The experiment was done using a CRD with three replications.

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) using the Statistix 10 software package. Mean comparisons were made using the Least Significant Difference (LSD) test at a 5% probability level.

Results

Plant Material and Preparation of *G. inodorum* Extracts

The extractions of GI from different areas in Lampang, Chiang Mai, and Chiang rai province were conducted. The fresh filtrate extraction method gave the highest crude extract from the Hang Chat District, Lampang Province, and the average crude extract percentages of GI leaf No. 4 (HC4) and No. 6 (HC6) were 5.90 and 5.84, respectively. A total of 24 samples of GI leaves were extracted by the Soxhlet extraction method, and the percentages of the crude extracts were determined. The results in Table 1 reveal that GI from Hang Chat (HC) district gave the highest yield of crude extract, followed by GI from Thoeng district (CR). The organic solvent extraction of GI leaves, hexane, ethyl acetate, and ethanol, showed that the percentage trend of dried GI in ethanol was higher. Ethyl acetate and the hexane crude extracts yielded the least amount (Table 1).

Detection and Quantification of Gymnemic acid (GA)

Determination of GA in the crude extracts by the HPLC technique was conducted. The crude extract was dissolved in methanol solvent and then filtered and analyzed for GA content. By repeating the analysis of each sample twice, a chromatogram of the sample was developed. Quantification of GA in the crude extract of GI was determined by using the RP-HPLC technique. The standard Deacyl gymnamic acid (3.91–500 µg/ml) was found at a 7.30 min retention time. A standard graph was obtained with a linear coefficient $R^2 = 0.9997$ and a linear equation $y = 1653.9x + 6051.9$ (Table 2; Fig. 1).

Extraction and Purification of the Pure Compound for NMR

From the extraction of GI, it was found that nine fractions of the substance were obtained. Preliminary tests using thin layer chromatography (TLC) found that the third fraction, which is probably GI, weighed 0.0193 g. TLC using a mobile phase of methanol: ethyl acetate (10:90) was spotted with 1% anisaldehyde solution in acid and found that the Rf value matched the deacyl gymnemic acid standard. The third fraction was analyzed by high-performance liquid chromatography (HPLC). In HPLC, the chromatograms of the pure substance isolated from the third fraction had the same retention time as the standard Deacyl gymnemic acid at 4.281 min. From Nuclear

Table 1 Yield of crude extract from different methods

Treatment	Yield of crude extract (%)[1/]				
	Fresh filtrate extraction	Soxhlet extraction	Organic solvent extraction		
			Hexane	Ethyl acetate	Ethanol
CR4[2/]	5.08[a3/] ± 2.09	17.99[a] ± 1.48	0.91[f] ± 0.02	2.05[b] ± 0.05	4.16[b] ± 0.09
CR6	5.33[a] ± 1.65	18.35[a] ± 2.35	0.88[f] ± 0.01	2.73[a] ± 0.06	4.36[b] ± 0.12
HC4	5.90[a] ± 0.26	19.59[a] ± 2.67	1.62[c] ± 0.04	1.70[c] ± 0.02	1.26[g] ± 0.01
HC6	5.84[a] ± 0.71	18.43[a] ± 0.25	1.16[e] ± 0.01	1.71[c] ± 0.16	3.28[d] ± 0.25
MT4	3.98[a] ± 0.87	7.33[c] ± 1.85	6.01[a] ± 0.04	1.10[e] ± 0.10	6.79[a] ± 0.10
MT6	4.30[a] ± 1.03	8.70[c] ± 1.49	0.67[g] ± 0.03	1.36[d] ± 0.04	2.47[e] ± 0.01
SP4	3.98[a] ± 1.86	13.34[b] ± 1.66	1.36[d] ± 0.04	2.00[b] ± 0.07	3.49[c] ± 0.03
SP6	4.74[a] ± 1.89	13.90[b] ± 2.32	1.85[b] ± 0.06	2.05[b] ± 0.05	1.81[f] ± 0.09

[1/] Means from 3 replicates

[2/] Thoeng in Chiang Rai province (CR), Mae-tang in Chiang Mai province (MT), Hang-chat (HC), and Sob-prab (SP) in Lampang province

[3/] The different superscript letter in each column shows a significant difference among the treatments at $p < 0.05$

Table 2 Gymnemic acid content from different extraction methods

Treatment	Gymnemic acid content (mg/g)[1/]				
	Fresh filtrate extraction	Soxhlet extraction	Organic solvent extraction		
			Hexane	Ethyl acetate	Ethanol
CR4[2/]	30.20[c3/] ± 4.08	74.08[ab] ± 12.18	0.02[cd] ± 0.01	3.34[b] ± 0.14	2.80[cd] ± 0.19
CR6	34.84[bc] ± 2.60	79.68[ab] ± 3.27	0.04[b] ± 0.00	3.50[b] ± 0.04	4.33[b] ± 0.36
HC4	35.08[bc] ± 2.02	93.43[a] ± 10.25	0.02[cd] ± 0.01	0.99[e] ± 0.01	2.67[cde] ± 0.09
HC6	41.01[a] ± 2.48	77.11[ab] ± 15.43	0.01[de] ± 0.00	1.00[e] ± 0.01	1.61[e] ± 0.13
MT4	37.84[ab] ± 2.27	93.49[a] ± 22.65	0.18[a] ± 0.00	23.02[a] ± 0.22	36.53[a] ± 1.86
MT6	34.17[bc] ± 3.10	64.00[b] ± 4.44	0.01[e] ± 0.00	1.71[c] ± 0.01	2.43d[e] ± 0.13
SP4	39.45[ab] ± 4.08	87.15[a] ± 8.30	0.03[cd] ± 0.02	1.43[d] ± 0.10	2.58[cde] ± 0.23
SP6	37.67[ab] ± 4.03	91.03[a] ± 17.55	0.03[bc] ± 0.00	1.81[c] ± 0.27	3.63[bc] ± 0.16

[1/] Means from 3 replicates

[2/] Thoeng in Chiang Rai province (CR), Mae-tang in Chiang Mai province (MT), Hang-chat (HC), and Sob-prab (SP) in Lampang province

[3/] The different superscript letter in each column shows a significant difference among the treatments at $p < 0.05$

Magnetic Resonance (NMR) and HPLC, it can be concluded that the pure substance isolated from the third fraction is GI. The isolated pure substance (third fraction) was then analyzed for its structure by NMR. It was found that the 1H NMR (400 MHz, MeOD) δ signal was found at (δ) 7.89 (s, 1H), 7.69. (*d*, J = 7.0 Hz, 1H), 6.52 (d, J = 6.9 Hz, 1H) of the benzyl group at 5.36 (s, 1H, C = CH-12) of the cycloalkene group at carbon-12 at 5.20 (s, 1H, 1′-Glu) of the glucose group at carbon-1′ at position 4.06 (s, 1H, CH2-28) of the CH2-O-CO group at carbon-28 [17]. From the results of the NMR analysis, it was determined that the GA group could be separated.

Pathogen Inoculum and Carbendazim-Resistant Phenotype Assay

A total of 30 isolates were isolated from the diseased mangoes. In culture, some of the fungi had fluffy white mycelium, while some of the isolates were gray to dark gray. Conidia were unicellular, smooth, colorless, cylindrical with a rounded head, size 2.5–4.1 × 5.5–16.5 µm (n = 30), some isolate produced orange to black spore masses, which is a characteristic of fungi in the genus *Colletotrichum*. The isolated fungi were tested for their pathogenicity, 16 isolates that were able to cause severe disease symptoms on plants were selected for further experiments. It was found that *C. gloeosporioides* isolate 2.1 induced significantly larger lesions on the mangoes tested than other isolates ($p < 0.05$). The size was

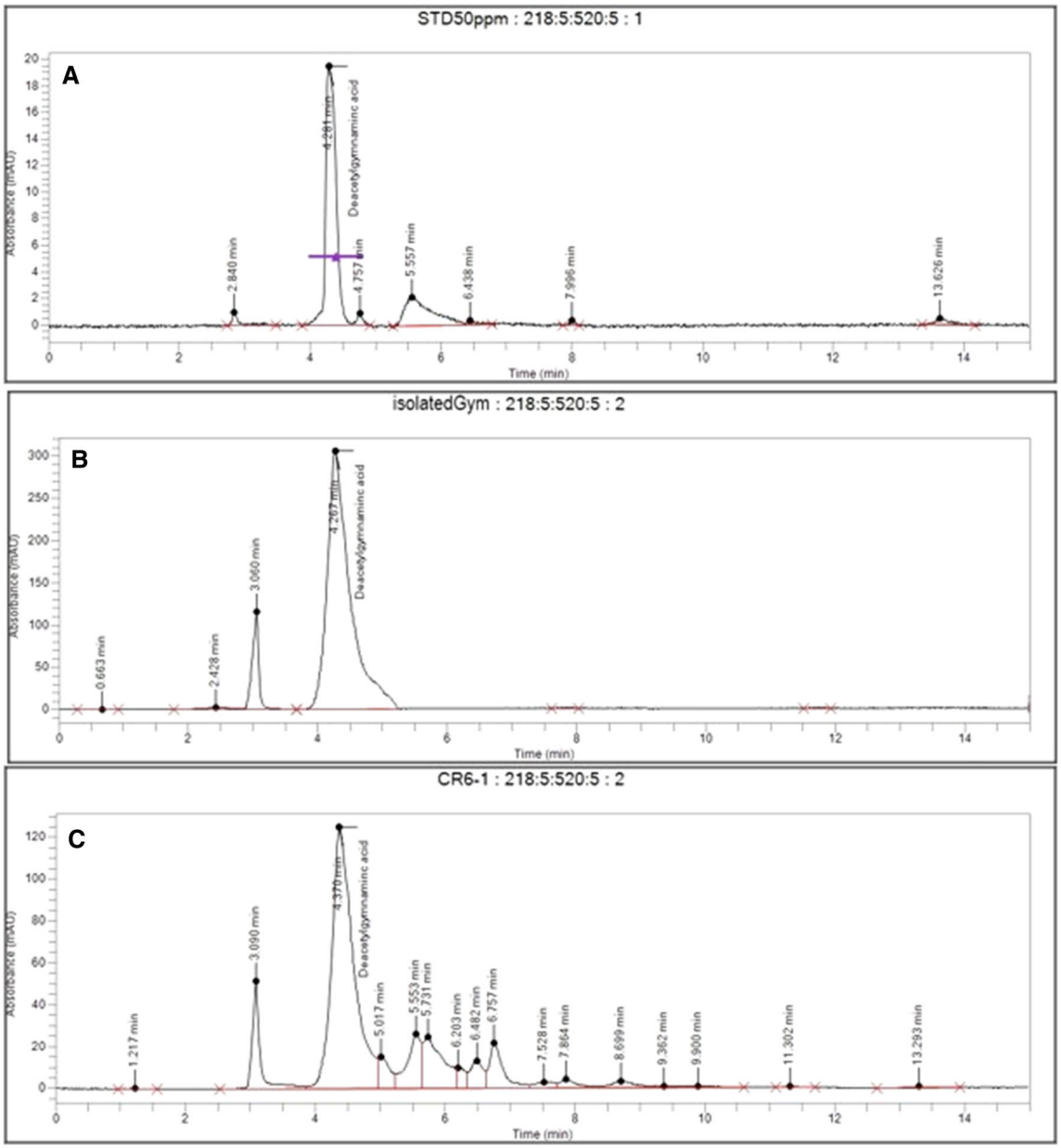

Fig. 1 **a–c** Chromatogram of HPLC. **a** third fraction, **b** standard deacyl gymnemic acid, **c** crude GI

78.08 mm., which classifies it as a highly virulent isolate (VH) level. When tested for resistance to carbendazim fungicide at 1000 ppm, it was found that it was able to grow, where non-genetically modified fungi normally cannot grow [18] (Table 3). Therefore, *C. gloeosporioides* isolate 2.1 was selected for further study.

In Vitro Effect of G. inodorum Extracts on Pathogen Growth

Tests of the efficacy of crude extracts were carried out using a paper disk diffusion test. Crude GI extracts were obtained through fresh filtrate extraction. The results of the study on inhibition of mycelium growth of C. gloeosporioides by the GI crude extract at 1000 ppm were compared with DMSO as a control. All samples of GI crude extracts were able to inhibit the growth of C. gloeosporioides isolate 2.1 mycelia and were significantly different from the control ($p \leq 0.05$). The SP4, MT4, and CR4 crude extract had the highest percentage of inhibition, with 30.20, 28.30, and 26.43 percent, respectively (Table 4). All samples of GI crude extracts inhibited mycelium growth significantly better than the control.

Table 3 Pathogenicity test of *C. gloeosporioides*

Isolate no	Wounding	Diameter of lesion (mm.)[1/]	Virulence level[2/]
1.1	No	$1.00^{no} \pm 0.00$[3/]	L
	Yes	$75.58^{b} \pm 1.37$	VH
2.1	No	$2.00^{mn} \pm 0.16$	L
	Yes	$78.08^{a} \pm 0.94$	VH
3.2	No	$0.00^{o} \pm 0.00$	N
	Yes	$35.95^{d} \pm 1.20$	VH
4.1	No	$0.00^{o} \pm 0.00$	N
	Yes	$3.23^{jkl} \pm 0.59$	L
5.1	No	$0.00^{o} \pm 0.00$	N
	Yes	$64.15^{c} \pm 1.50$	VH
6.1	No	$0.00^{o} \pm 0.00$	N
	Yes	$3.93^{jk} \pm 1.20$	L
7.1	No	$0.00^{o} \pm 0.00$	N
	Yes	$9.30^{h} \pm 2.46$	M
8.2	No	$0.00^{o} \pm 0.00$	N
	Yes	$1.00^{no} \pm 0.16$	L
9.2	No	$0.00^{o} \pm 0.00$	N
	Yes	$4.20^{j} \pm 0.22$	L
10.1	No	$2.98^{klm} \pm 0.34$	L
	Yes	$19.50^{e} \pm 2.08$	VH
10.2	No	$2.33^{lm} \pm 0.75$	L
	Yes	$20.00^{e} \pm 0.82$	VH
1.11	No	$0.25^{o} \pm 0.50$	L
	Yes	$5.43^{i} \pm 0.56$	M
2.11	No	$0.88^{no} \pm 0.63$	L
	Yes	$3.85^{jk} \pm 0.58$	L
8.11	No	$0.00^{o} \pm 0.00$	N
	Yes	$9.35^{h} \pm 1.01$	M
8.12	No	$0.00^{o} \pm 0.00$	N
	Yes	$12.23^{g} \pm 0.56$	H
8.2	No	$0.00^{o} \pm 0.00$	N
	Yes	$13.95^{f} \pm 1.01$	H

[1/]Means from 4 replicates

[2/]Virulence level: 0 mm = non-pathogenic isolates (N), 0.1–5.0 mm. = low virulence isolates (L), 5.1–10.0 mm = moderately virulent isolates (M), 10.1–15.0 mm = highly virulent isolates (H) and ≥ 15.1 mm = very highly virulent isolates (VH) (Suwan and Na Lampang, 2013)

[3/]The different superscript letter in each column shows a significant difference among the treatments at $p < 0.05$

Poisoned Food Technique

The concentration of 0.025% crude HC6 had a low mycelia inhibiting ability, at 87.97% in the poison-food bioassay. The higher concentrations (0.05–0.20%) of all the crudes extracts of GI showed full inhibition of *C. gloeosporioides* isolate 2.1 mycelial growth (Table 5).

Determination of Percent Inhibition of Spore Germination by a 96-Well Plate Method

The 5000 ppm crude extract was tested against the germination of *C. gloeosporioides* isolate 2.1 spores after 0, 12, 24, and 48 h; DMSO (negative control), and carbendazim (positive control) were used. The results revealed that aqueous crude extracts of MT4 and MT6 showed the most significant spore reduction, throughout the test period and significantly more than the control ($p < 0.05$), with the number of spores found at hours 0, 12, 24, and 48 after

Table 4 Effect of *G. inodorum* extracts on *C. gloeosporioides* isolate 2.1 by using a paper disk diffusion method

Treatment	Percentage of growth inhibition[1/]
Control	$0.00^{c} \pm 0.00$[2/]
CR4[3/]	$26.43^{a} \pm 6.52$
CR6	$25.13^{a} \pm 2.19$
HC4	$22.63^{ab} \pm 3.75$
HC6	$15.73^{b} \pm 3.96$
MT4	$28.30^{a} \pm 5.03$
MT6	$23.27^{ab} \pm 6.63$
SP4	$30.20^{a} \pm 3.80$
SP6	$23.27^{ab} \pm 4.74$

[1/]Means from 3 replicates; [2/]The different superscript letters in each column show significant difference at $p < 0.05$ among treatment; [3/]Thoeng in Chiang Rai province (CR), Mae-tang in Chiang Mai province (MT), Hang-chat (HC), and Sob-prab (SP) in Lampang province

treatment with the MT4 crude extract was 8.00×10^5, 2.89×10^5, 1.55×10^5 and 1.22×10^5 conidial/ml, respectively, while the MT6 crude extract resulted in 8.00×10^5, 3.11×10^5, 2.11×10^5 and 1.22×10^5 conidial/ml, respectively, during the same time periods (Table 6).

Minimal Inhibitory Concentrations: MIC and Minimal Fungicidal Concentration

The minimum inhibitory concentrations of *C. gloeosporioides* were as follows: when incubated for 12 h, Gymnemic acid (CR4, HC4, HC6, MT4, MT6, SP4, and SP6) and the crude extract of GI were 256 μg/ml, but the CR6 crude extract was > 256 μg/ml, while carbendazim was 1000 μg/ml. The MFC of gymnemic acid and GI crude extract was > 256 μg/ml after incubation for up to 24 h, gymnemic acid could not inhibit the growth of the causative fungus over a prolonged period. The results revealed that GA was unable to inhibit the growth of the causative fungi as the duration increased. However, the GI crude extracts CR4, CR6, HC4, HC6, MT4, MT6, SP4, and SP6 had an MIC of 10,000 μg/ml in inhibiting the growth of *C. gloeosporioides*. The MFC of Gymnemic acid was > 256 μg/ml, and the MFC of the GI crude extract was > 10,000 μg/ml. After incubation for 48 h, the crude extract of the GI lost the ability to suppress the growth of *C. gloeosporioides* (Table 7).

Discussion

Gymnemic acid (GA) was detected by all extraction methods. In this experiment, 0.04 g/g dry weight of GA was produced. The content of GA in a plant can be determined by monitoring by chromatographic techniques (HPLC, TLC) or non-chromatographic techniques (immunoassay, phytochemical screening assay, and Fourier Transform InfraRed) [19].

The quantification of the active ingredient from crude extracts can use gymnemic acid as a marker to control the concentration of active ingredients in solution. In addition, Puratchimani and Jha [20] reported that gymnemagenin (GA without sugar) was used as a marker for gymnemic acid content in a sample and April–July, is the appropriate months for harvest *G. sylvestre* to obtain the highest content of gymnemic acid. Soxhlet extraction gave the highest amount of GA from all samples and sources, compared to fresh filtrate extraction and the organic solvents extraction methods. Soxhlet extraction is a high-efficiency extraction method suitable for small sample volumes but not practical when used for large-scale production. Therefore, the best method that should be conveyed for utilization is extraction

Table 5 Effect of poisoned food bioassay on spore germination of *C. gloeosporioides*

Treatment	Crude extract of *G. inodorum*					
	0.000%	0.025%	0.050%	0.100%	0.150%	0.200%
CR4	$0.00^{d} \pm 0.00$	$96.29^{ab} \pm 6.42$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$
CR6	$0.00^{d} \pm 0.00$	$96.60^{ab} \pm 5.88$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$
HC4	$0.00^{d} \pm 0.00$	$91.17^{bc} \pm 15.32$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$
HC6	$0.00^{d} \pm 0.00$	$87.97^{c} \pm 20.85$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$
MT4	$0.00^{d} \pm 0.00$	$93.21^{abc} \pm 5.89$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$
MT6	$0.00^{d} \pm 0.00$	$94.63^{abc} \pm 5.11$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$
SP4	$0.00^{d} \pm 0.00$	$95.22^{ab} \pm 8.28$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$
SP6	$0.00^{d} \pm 0.00$	$90.2^{bc} \pm 8.49$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$	$100.00^{a} \pm 0.00$

[1/]Means from 3 replicates; [2/]Different superscript letters in each column show the significant difference at $p < 0.05$ among treatment; [3/]Thoeng in Chiang Rai province (CR), Mae-tang in Chiang Mai province (MT), Hang-chat (HC), and Sob-prab (SP) in Lampang province

Table 6 Effect of *Gymnema inodorum* crude extract on *C. gloeosporioides* spore germination after incubation

Treatment	Number of conidial in suspension ($\times 10^5$ conidial/ml.)			
	0 h	12 h	24 h	48 h
Distilled H_2O	$8.00^{a1/} \pm 0.00$	$10.33^{a2/} \pm 4.04$	$17.00^{a} \pm 3.60$	$14.00^{a} \pm 4.00$
DMSO	$8.00^{a} \pm 0.00$	$10.00^{ab} \pm 3.00$	$7.00^{b} \pm 1.00$	$8.33^{b} \pm 1.00$
CR4[3/]5000 ppm	$8.00^{a} \pm 0.00$	$8.44^{abc} \pm 1.02$	$3.56^{cd} \pm 0.69$	$1.44^{c} \pm 1.02$
CR6 5000 ppm	$8.00^{a} \pm 0.00$	$5.89^{cd} \pm 1.54$	$2.78^{cd} \pm 1.39$	$2.11^{c} \pm 1.64$
HC4 5000 ppm	$8.00^{a} \pm 0.00$	$6.11^{bcd} \pm 3.08$	$2.00^{cd} \pm 1.17$	$1.89^{c} \pm 1.02$
HC6 5000 ppm	$8.00^{a} \pm 0.00$	$4.78^{cd} \pm 0.77$	$3.56^{cd} \pm 0.77$	$1.67^{c} \pm 1.45$
MT4 5000 ppm	$8.00^{a} \pm 0.00$	$2.89^{d} \pm 1.17$	$1.55^{d} \pm 1.71$	$1.22^{c} \pm 0.69$
MT6 5000 ppm	$8.00^{a} \pm 0.00$	$3.11^{d} \pm 0.84$	$2.11^{cd} \pm 0.84$	$1.22^{c} \pm 1.07$
SP4 5000 ppm	$8.00^{a} \pm 0.00$	$5.22^{cd} \pm 2.04$	$3.78^{cd} \pm 0.84$	$2.22^{c} \pm 0.51$
SP6 5000 ppm	$8.00^{a} \pm 0.00$	$7.22^{abc} \pm 4.14$	$4.11^{cd} \pm 1.95$	$1.78^{c} \pm 1.71$
Carbendazim[4/]	$8.00^{a} \pm 0.00$	$5.67^{cd} \pm 0.58$	$4.44^{bc} \pm 2.00$	$1.00^{c} \pm 2.52$

[1/]Means from 3 replicates; [2/]The different superscript letters in each column show the significant differences at $p < 0.05$ among treatments; [3/]Thoeng in Chiang Rai province (CR), Mae-tang in Chiang Mai province (MT), Hang-chat (HC), and Sob-prab (SP) in Lampang province; [4/]1000 ppm (recommended concentration)

Table 7 MIC and MFC of GI crude extract against *C. gloeosporioides*

Treatment	MIC/MFC (μg/ml)[1/]		
	12 h	24 h	48 h
Gymnemic acid	256	> 256	> 256
Crude CR4[2/]	256	10,000	> 10,000
Crude CR6	> 256	10,000	> 10,000
Crude HC4	256	10,000	> 10,000
Crude HC6	256	10,000	> 10,000
Crude MT4	256	10,000	> 10,000
Crude MT6	256	10,000	> 10,000
Crude SP4	256	10,000	> 10,000
Crude SP6	256	10,000	> 10,000
Carbendazim[3/]	1000	> 1000	> 1000

[1/]Means from 3 replicates; [2/]Thoeng in Chiang Rai province (CR), Mae-tang in Chiang Mai province (MT), Hang-chat (HC), and Sob-prab (SP) in Lampang province; [3/]1000 ppm (recommended concentration)

of fresh filtrate of GI from leaves, and it is practical and easy to apply. In addition, the solvent used is water, which is safer than other solvents used in this experiment [21]. It was found that all aqueous crude extracts at a concentration of 1000 ppm from GI were able to inhibit the growth of *C. gloeosporioides (*very virulent isolate*)* and significantly different from the control. In the literature, studies have been undertaken on the bioactive compounds from various medicinal plants with antifungal potential for plant pathogens. Saardluan and Sruamsiri [22] revealed that a *Piper retrofractum* ethanol extract at a concentration of 500 ppm and higher concentrations completely inhibited the growth of *C. gloeosporioides* equal to benomyl at 500 ppm. Antifungal activity of *Cinnamomum camphora* extracts against *Colletotrichum lagenarium,* the causal agent of anthracnose in cucumber, was also studied. The results showed that the extracts of cinnamon demonstrated 95% control of *C. lagenarium* at a concentration of 16 mg/ml; and the MIC_{50} was 2.596 mg/ml against *C. lagenarium* [23]. Likewise, the study of *Zingiber officinale* rhizome extract, *Polyalthia longifolia*, and *Clerodendrum inerme* leaf extracts exhibited more than 80 percent inhibition of mycelial growth and total spore germination inhibition of *C. musae* [24]. Although plant extracts in plant disease control are beneficial, sometimes the results may differ depending on the type of plant used to extract the active ingredient. A study of the effects of tobacco (*Nicotiana tabacum*) and castor plant (*Ricinus communis*) extracts showed that at concentrations up to 100,000 ppm, tobacco extracts were able to inhibit the growth of *C. destructivum* mycelium; acting as an "accelerator" of fungal germination better than controls [25]. GA inhibited the growth of *C. gloeosporioides* after being incubated for 12 h; the MIC was 256 μg/ml, and the MFC was > 256 μg/ml. Consistent with the report that gymnemic acid at a concentration of 400 μg/ml upward, inhibited the adhesive nanofibrillar activity of *Streptococcus gordonii* and *Candida albicans* mono-species and dual-species biofilms [26]. GA inhibited biofilm nanofibrils and reduce mycelial growth by inhibiting glyceraldehyde-3-phosphate dehydrogenase activity of *S. gordonii*. The suppression of GAPDH enzyme consequently stimulates the expression of biofilm

formation in *C. albicans*. The inhibition affected the germination of *C. albicans* and diminished the pathogenic ability.

Moreover, the aqueous extract of *Gymnema sylvestre* and gymnemic acid showed a large zone of inhibition against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. Consequently, the extracts showed a better antimicrobial activity than of the commercial antibiotics fluoroquinolone and ciprofloxacin [27]. Terpenes and their derivatives are secondary metabolites commonly found in essential oils and have antimicrobial activities against susceptible and resistant pathogens. Wagner and Merzenich [28] reported that the potential of antimicrobial agents was triggered via several mechanisms in integration, so this provides a multi-targeted pharmacokinetic effect, allowing concurrent elimination of existing resistance mechanisms in a specific pathogen. Crude extracts of GI and GA were able to inhibit the growth of the pathogen. However, the use of gymnemic acid should be in high concentrations. In contrast, a high concentration of crude extract might have an adverse effect on plant cell walls and cell membranes. *Gymnema* spp. contains a high content of phenolic compounds, including eugenol, methyl eugenol, 4-ethyl guaiacol, and 4-vinyl guaiacol, which will cause dissociation of the cell membrane causing cell wall degradation and cytoplasm coagulation [29, 30].

Conclusion

The extraction by the Soxhlet method produced the highest percentage of GI crude extract, followed by fresh filtrate extraction. Gymnemic acid was detected from the extraction methods by using the RP-HPLC and NMR. The pathogen used in this experiment was *C. gloeosporioides* isolate 2.1, highly pathogenic and resistant to carbendazim. One-thousand ppm of GI crude extract strongly inhibited the growth of *C. gloeosporioides* isolate 2.1 mycelia, with the highest percentage of inhibition from the SP4 extract, followed by MT4 and CR4. The effects of crude extracts on spore germination, after 0, 12, 24, and 48 h incubation, showed that MT4 and MT6 at 5000 ppm significantly reduced fungal spore number. The MIC and MFC of the crude extract and GI at 12 h after incubation were 256 µg/ml and > 256 µg/ml, respectively. After incubation for 24 h, the crude extract exhibited MIC and MFC of 10,000 µg/ml and > 256 µg/ml, respectively. While GA lost the ability to inhibit the causal fungus.

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Declarations

Conflict of interest The authors declare that we have no conflict of interest.

References

1. Muhammad AK, Abdul ML, Shahzad S (2004) Pathogenicity of *Lasiodiplodia theobromae* and *Fusarium solani* on mango. Pak J Bot 36:181–189
2. Zhafarina S, Wibowo A, Widiastuti A (2021) Multi-genetic analysis of *Colletotrichum* spp. associated with postharvest disease of fruits anthracnose in special region of Yogyakarta, Indonesia. Pak J Biol Sci 24:53–65. https://doi.org/10.3923/pjbs.2021.53.65
3. Prusky D, Mcevoy JL, Leverentz B, Conway LS (2001) Local modulation of host pH by *Colletotrichum* species as a mechanism to increase virulence. Mol Plant-Microbe Interact 14:1105–1113. https://doi.org/10.1094/mpmi.2001.14.9.1105
4. Hassan MK, Irving DE, Dann EK, Coates LM, Hofman PJ (2009) Sap properties and alk(en)ylresorcinol concentrations in Australian-grown mangoes. Ann Appl Biol 154:419–427. https://doi.org/10.1111/j.1744-7348.2008.00313.x
5. Wharton P, Diéguez-Uribeondo J (2004) The biology of *Colletotrichum acutatum*. An Jard Bot Madr 61:3–22. https://doi.org/10.3989/ajbm.2004.v61.i1.61
6. Nakaune R, Nakano M (2007) Benomyl resistance of *Colletotrichum acutatum* is caused by enhanced expression of β-tubulin 1 gene regulated by putative leucine zipper protein CaBEN1. Fungal Genet Biol 44:1324–1335. https://doi.org/10.1016/j.fgb.2007.03.007
7. Albertini C, Gredt M, Leroux P (1999) Mutations of the *β*-tubulin gene associated with different phenotypes of benzimidazole resistance in the cereal eyespot fungi *Tapesia yallundae* and *Tapesia acuformis*. Pestic Biochem Physiol 64:17–31. https://doi.org/10.1006/pest.1999.2406
8. Zakaria A, Baka M, Mousa MMA (2020) *In vitro* and *in vivo*, biocontrol activity of extracts prepared from Egyptian indigenous medicinal plants for the management of anthracnose of mango fruits. Arch Phytopathol 53:715–730. https://doi.org/10.1080/03235408.2020.1794308
9. Srinuanchai W, Nooin R, Pitchakarn P, Karinchai J, Suttisansanee U, Chansriniyom C, Nuchuchua O (2020) Inhibitory effects of Gymnema inodorum (Lour) Decne leaf extracts and its triterpene saponin on carbohydrate digestion and intestinal glucose absorption. J Ethnopharmacol 266:113398. https://doi.org/10.1016/j.jep.2020.113398
10. Kim B, Han JW, Thi Ngo M, Le Dang Q, Kim JC, Kim H, Choi GJ (2018) Identification of novel compounds, oleanane- and ursane-type triterpene glycosides, from *Trevesia palmata*: their biocontrol activity against phytopathogenic fungi. Sci Rep 8:14522. https://doi.org/10.1038/s41598-018-32956-4
11. Soberón JR, Sgariglia MA, Pastoriza AC, Soruco EM, Jäger SN, Labadie GR, Sampietro DA, Vattuone MA (2017) Antifungal activity and cytotoxicity of extracts and triterpenoid saponins obtained from the aerial parts of *Anagallis arvensis* L. J Ethnopharmacol 203:233–240. https://doi.org/10.1016/j.jep.2017.03.056
12. Satdive RK, Abhilash P, Fulzele DP (2003) Antimicrobial activity of *Gymnema sylvestre* leaf extract. Fitoterapia 74:699–701. https://doi.org/10.1016/s0367-326x(03)00154-0
13. Pandey AK, Yadav S (2010) Variation in gymnemic acid content and non-destructive harvesting of *Gymnema sylvestre* (Gudmar).

Pharmacogn Res 2:309–312. https://doi.org/10.4103/0974-8490.72330
14. Sutton BC (1992) The genus *Glomerella* and its anamorph *Colletotrichum*. In: Bailey JA, Jeger MJ (eds) *Colletotrichum:* biology, pathology and control. Commonwealth Mycological Institute, Wallingford, pp 1–26
15. Suwan N, Na-Lampang S (2013) Characterization and evaluation of carbendazim-resistance response of *Colletotrichum* species. Int J Agric Technol 9:1883–1894
16. Singh J, Tripathi NN (1999) Inhibition of storage fungi of black gram (*Vigna mungo* L.) by some essential oils. Flavour Fragr J 14:1–4. https://doi.org/10.1002/(sici)1099-1026(199901/02)14:1%3c1::aid-ffj735%3e3.0.co;2-r
17. Alkefaia NH, Ahamada J, Aminb S, Sharmac M, Mir SR (2018) Arylated gymnemic acids from *Gymnema sylvestre* R.Br. as potential α-glucosidase inhibitors. Phytochem Lett 25:196–202. https://doi.org/10.1016/j.phytol.2018.04.021
18. Kongtragoul P, Nalumpang S, Miyamoto Y, Izumi Y, Akimitsu K (2011) Mutation at Codon 198 of TUB2 gene for carbendazim resistance in *Colletotrichum gloeosporioides* causing mango anthracnose in Thailand. J Plant Prot Res 51:377–384. https://doi.org/10.2478/v10045-011-0061-5
19. Sasidharan S, Chen Y, Saravanan D, Sundram KM, Yoga Latha L (2011) Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr J Tradit Complement Altern Med 8:1–10. https://doi.org/10.4314/ajtcam.v8i1.60483
20. Puratchimani V, Jha S (2004) Standardization of *Gymnema sylvestre* R.Br. with reference to gymnemagenin by high-performance thin-layer chromatography. Phytochem Anal 15:164–166. https://doi.org/10.1002/pca.763
21. Dheeba B, Niranjana R, Sampathkumar P (2015) Efficacy of Neem (*Azadirachta Indica*) and Tulsi (*Ocimum Sanctum*) Leaf Extracts Against Early Blight of Tomato. Proc Natl Acad Sci India Sect B Biol Sci 85:327–336. https://doi.org/10.1007/s40011-014-0340-9
22. Sa-ardluan W, Sruamsiri P (2005) Inhibitory effect of java long pepper fruit extracts on *Colletotrichum gloeosporioides* causing anthracnose disease of mango fruit. J Agric 3(21):251–257
23. Chen Y, Dai G (2012) Antifungal activity of plant extracts against *Colletotrichum lagenarium,* the causal agent of anthracnose in cucumber. J Sci Food Agric 92:1937–1943. https://doi.org/10.1002/jsfa.5565
24. Bhutia DD, Zhimo Y, Kole R, Saha J (2016) Antifungal activity of plant extracts against *Colletotrichum musae*, the postharvest anthracnose pathogen of banana cv, Martaman. Nutr Food Sci 46:2–15. https://doi.org/10.1108/nfs-06-2015-0068
25. Akinbode OA, Ikotun T (2008) Evaluation of some bioagents and botanicals in in vitro control of *Colletotrichum destructivum*. Afr J Biotechnol 7:868–872. https://doi.org/10.5897/ajb07.676
26. Veerapandian R, Vediyappan G (2019) Gymnemic acids inhibit adhesive nanofibrillar mediated *Streptococcus gordonii–Candida albicans* mono-species and dual-species biofilms. Front Microbiol 10:2328. https://doi.org/10.3389/fmicb.2019.02328
27. Gupta RBP, Singh P (2014) Antimicrobial activity of Gymnemic acid on pathogens: *Gymnema sylvestre*. Int J Curr Microbiol App Sci 3:40–45. https://doi.org/10.15373/22778179/may2014/10
28. Nazzaro F, Fratianni F, De Martino L, Coppola R, De Feo V (2013) Effect of essential oils on pathogenic bacteria. Pharmaceuticals 6:1451–1474. https://doi.org/10.3390/ph6121451
29. Wagner H, Ulrich-Merzenich G (2009) Synergy research: approaching a new generation of phytopharmaceuticals. Phytomedicine 16:97–110. https://doi.org/10.1016/j.phymed.2008.12.018
30. Naik DG, Dandge CN, Rupanar SV (2011) Chemical examination and evaluation of antioxidant and antimicrobial activities of essential oil from *Gymnema sylvestre* R. Br Leaves J Essent Oil Res 23:12–19. https://doi.org/10.1080/10412905.2011.9700451