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Evaluating eco-friendly botanicals (natural plant extracts) as alternatives to synthetic fungicides

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Abstract

Introduction. Fungicides are widely used in conventional agriculture to control plant diseases. Prolonged usage often poses health problems as modern society is becoming more health-conscious. *Penicillium digitatum,* the cause of citrus green mould, is an important postharvest pathogen which causes serious losses annually. The disease is currently managed with synthetic fungicides. There is, however, a growing concern globally about the continuous use of synthetic chemicals on food crops because of their potential effects on human health and the environment.

Materials and Methods. Different concentrations (500-5,000 ppm) of 5 ethanol extracts of Neem, Pong-pong, Chili, Lemon grass, and Ginger were compared with DMSO and fungicide (Guazatine,1,000 ppm) for their anti-fungal activity (inhibition zone) *in vitro* on PDA media and during storage conditions. Lethality test LC50 (BST) was followed to determine the lethal dose from plant extract compared with the lethal dose for synthetic chemicals (Guazatine).

Result. Crude extraction from Neem, Chili and, Pong-pong showed a complete inhibition zone at 3,000ppm (100%) in the green mould *in vitro.* At *in vivo,* concentrations (4,000 and 5,000ppm), Neem, Chili, and Pong-pong showed a high effect on the prevention of the development of mycelia growth *Penicillium digitatum* on the surface fruits in storage conditions at 25 °C±2. In addition, the lethal concentration (LC50) values of the crude extracts were investigated by using the Brine-shrimp (*Artemia salina Leach*) lethality test (BST). At 20.5 and 30 µg/ml-1, Neem, Pong-pong and hot Chili showed very high lethal toxicity on brine and effect. Lemon grass and Ginger killed 50% at 495 and 473 µg/ml^{-1}, respectively, compared with controls.

Conclusion. Pong-pong, Neem, and chili showed positive effects on the inhibition of postharvest fungi as alternatives to fungicides, while bearing in mind the increasing global pollution of the environmental. Extracts from Lemon grass and Ginger have interesting antifungal activity and they are also toxic in bioassay against shrimp. These extracts or botanicals have a bright future in modern plant protection to replace conventional synthetic pesticides.

Key words

eco-friendly botanicals, synthetic fungicides, natural plant extracts, environmental management

INTRODUCTION

The problems caused by synthetic pesticides and their residues have increased the need for effective biodegradable pesticides with greater selectivity. Alternative strategies have included the search for new types of pesticides, which are often effective against a limited number of specific target species, are biodegradable into non-toxic products, and suitable for use in integrated pest management programmes [1]. Applied chemical pesticides are one of the effective and fast means for reducing the loss of post-harvest diseases. Nevertheless, the excessive use of these chemicals for controlling mould fungi in fruit has been counterproductive, causing damage to the environment and humans, with increased demands to reduce the use of these chemicals that accumulate in fruits and vegetables [2].

This damage increased significantly with improper use and randomly left to grow in order to reduce the use of these chemicals that accumulate in fruits and vegetables. It is claimed that these fruits were major health problems after they became a crop of major commodities exported to different countries worldwide. As persistent hazardous chemicals, the use by governments and international organizations of many of these pollutants, such thiabendazole (TBZ) and imazalil [3], to gravity, previously used to control a wide range of fungi, led to an imbalance in the natural enemies in the environment, the low rate of which helps to maintain the pathogen [4].

The natural plant products derived from plants effectively meet this criterion and have enormous potential to influence modern agrochemical research. When extracted from plants, these chemicals are referred to as botanicals. The use of botanical pesticides is now emerging as one of the prime means to protect crops and their products and the environment from pesticides [5]. Botanicals degrade more rapidly than most chemical pesticides, and therefore are considered to be eco-friendly and less likely to kill beneficial pests than synthetic pesticides with longer environmental retention. Most of the botanical pesticides generally degrade within a few days, and sometimes even within a few hours [6]. The objective of the presented study was to evaluate the use of botanical pesticides of 5 plant extracts: Neem, Pong-pong, Chili, Lemon grass and Ginger as natural products, and examine their effectiveness as anti-fungal, eco-friendly, low-cost, and easy to prepare.

MATERIALS AND METHODS

Preparation of plants for extraction. Leaves from Neem and Pong-pong were collected from trees growing at the riverside park near Kangar, capital of Perlis State, Malaysia.

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The other plants, leaves and stems of lemon grass, chili fruits, and ginger tubers were purchased from the wet market in Kangar. The samples were brought to laboratory and washed under running water in order to get rid of dirt, insects and plankton. They were dried overnight in the laboratory electric oven at 40 °C. 100g of the materials were pulverized in an electric mixer, and preserved in labeled glass bottles that were sealed until use at a later date.

Preparation of plant extracts. The extraction technique used was a modification of Ruch and Worf method [7]. Up to 50g each of the oven-dried and power-pulverized material from plants were treated with 500 ml of 95% alcohol with constant stirring for 30 min. After stirring, the solutions were filtered through 2 layers of cheese-cloth gauze and Whitman's No. 2 filter paper before the filtrates were subjected to evaporation using a rotary evaporator at 60 °C for 60 min. to remove the ethanol. The dark spongy materials were dried in an oven at 37 °C for 2 days. The dried powder was stored in small sterilized 5ml screw-capped glass bottles that were refrigerated at 4 °C until further use.

Preparation of plant extract-dilutions. Powder from each plant (0.5, 1. 2, 3, 4 and gm) was dissolved in 2ml organic solvent DMSO (solvent dim ethyl sulfoxide, 99.5%) with concentrations mg/mL. This was diluted further by mixing with water to obtain concentrations of 500, 1,000, 2,000, 3,000, 4,000 and 5,000ppm.

Pathogen. Using taxonomic and morphological references, the identified pathogen was *Penicillium digitatum*. Highly aggressive, single-spore isolates of *P. digitatum*, originally isolated from citrus fruits, were grown on potato dextrose agar (PDA) at 25 °C for 7 days. Spores were harvested by flooding the media surface with sterile distilled water and gently agitating the plate to dislodge spores [8, 9]. Concentration spores were determined by using a hemacytometer and adjusted to $1x10^6$ spores mL^{-1}

***In vitro* screening – effect of plant extracts on mycelium growth of *Penicillium Digitatum*.** PDA media were incorporated into 45 glass flasks (50 ml) and autoclaved for 20 min. at 121 °C. After autoclaving, the flasks were cooled down to about 45 °C. 5 ml of each plant extract, (500, 1000, 2000, and 3000 ppm) was added to the flasks by using a pipette and then gently agitated by hand for 2 min. in order to mix the extracts properly. Media cultures were amended into 9cm in Petri-dishes. Chloramphenicol (250 mg^{-1} per Petri dish) was added to the medium to prevent bacterial growth [10]. The experiment was performed under aseptic lamina conditions and replicated 3 times. One ml, of *P. digitatum*, spore suspensions (conc.1×10^6 spores/ml^{-1}) was added by pipette onto the centre of the amended PDA extracts. Inoculated plates were incubated at 25 °C for 10 days. The Petri-dish inoculated without the extract concentration (water) used as negative control. Colony diameter was determined by measuring the average radial growth. The inhibition zone was measured using the formula of [11] as follows:

$$\% \text{ Mycelial inhibition} = \frac{\text{Mycelial growth (control/water)} - \text{Mycelial growth (treatment)}}{\text{Mycelial growth (control/water)}} \times 100$$

Storage conditions. Healthy, freshly citrus fruit was washed with tap water and then air dried and sterilized by immersion in 70% ethanol for 1 min. before spraying. The fruits randomly divided into 5 equal groups (5 fruits); all groups were stabbed to a depth of 5.0 mm with a 1.25 mm diameter needle. At the equator, all treatments were inoculated by a spray in suspension of *P.digitatum* (1×10^6 spores' mL^{-1}). 6 treatments were carried out after 1 hour as follows.

- Spraying with 2,000ppm
- Spraying with 3,000ppm
- Spraying with 4,000ppm
- Spraying with 5,000ppm
- DMSO (99.5%)
- Fungicides (Guazatine, 1,000ppm).

The treated fruits were packed in plastic boxes and incubated at 25 °C with >85% RH for 21 days. Evaluation of the treatment was carried out every week. Efficacy of treatment application was determined according to [12]. There were 3 replicate trials of 5 fruits. Representative samples of the 5 fruits per replicate were taken each week during the storage period until the percentage of decay reached 50%. The following equation calculates the number of decayed fruits divided by the total number of fruits:

$$\text{Undesirable fruits\%} = (\text{The number of undesirable fruits} / \text{Total number of fruit}) \times 100.$$

Cytotoxicity screening from crude fraction (LC50). Test Brine shrimp lethality is widely used in bioassay as a simple screening method to investigate the cytoxicity of plant extracts [13]. The crudes from plants extracts were used for the test (LC50). The eggs of the brine shrimp were collected from a fish shop in Kangar. 5mg of *Artemia salina* (Leach) eggs were added to flasks containing ocean/sea water (50ml). A flask was kept under an inflorescent bulb for 48 hours for the eggs to hatch into shrimp larvae following the method [14]. Concentrations for the test were prepared by dissolving them in DMSO to attain concentrations (5, 10, 20, 40, 130, 260, 390, and 520 μg ml^{-1}). DMSO and fungicide (Guazatine) were used as negative and positive controls. Solutions were transferred to all flasks and dosage tested in triplicate for all flasks (9 per test fraction). After 24 hours, the number of dead larvae was counted. The data were collected and analyzed by using Excel 2007.

RESULTS

Effect of plant extracts on mycelium growth of *Penicillium Digitatum in vitro*. The study showed different significances between treatments and controls at ($p \leq 0.05$). Generally, the results (Tab. 2, Fig. 2) showed that complete inhibition of the growth of *P. digitatum* reached 100 (93%) of crude plant concentration extracts at 3,000ppm from Chili and Pong-pong. In addition, crude extract from Neem, Lemon grass, and Ginger were highly effective at same the concentration, which amounted to 88.3, 88.3, and 81.5%, respectively. With negative control performed with serial dilutions of DMSO, it was observed that there was no effect on fungal growth (0%).

In storage conditions – effect of plant extracts on development of *Penicillium Digitatum* in citrus fruits. The treatments from Neem, Pong-pong, and Chili at

Table 1. 'Plant species' parts and concentration used in experiment *in vitro* and in storage

Common name	Plant species	Family name	Nature of the extract	Conc:in vitro (ppm) in vitro	Conc:in (ppm) in vivo
Neem	*Azadirachta indica* L.	Meliaceae	Leaf powder	500, 1000.2000,3000	2000.3000,4000,5000
Chili	*Capsicum frutescence* L.	Solanaceae	Fruit powder	500, 1000.2000,3000	2000.3000,4000,5000
Pong-Pong	*Cerbera odollam* L.	Apocynaceae	Leaf powder	500, 1000.2000,3000	2000.3000,4000,5000
Lemon grass	*Cymbopogon nardus* L.	Poaceae	Leaf powder	500, 1000.2000,3000	2000.3000,4000,5000
Ginger	*Zingiber officinale* L	Zingiberaceae	Tuber powder	500, 1000.2000,3000	2000.3000,4000,5000

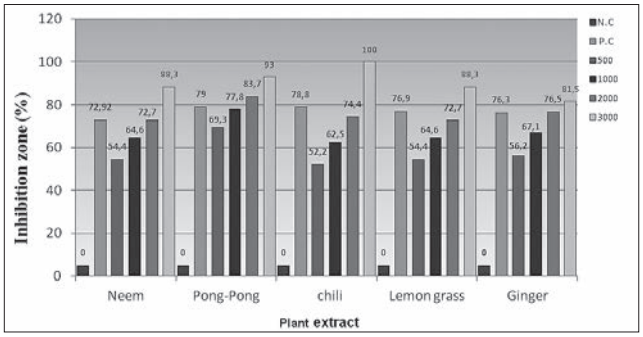

Figure 1. Effect plant extracts at different concentrations(ppm) in inhibition colony diameter *Penicillium digitatum* (%) in PDA after 10 days at 25°C compared with controls [fungicides (Guazatine/P.C) and DOMS /NC]

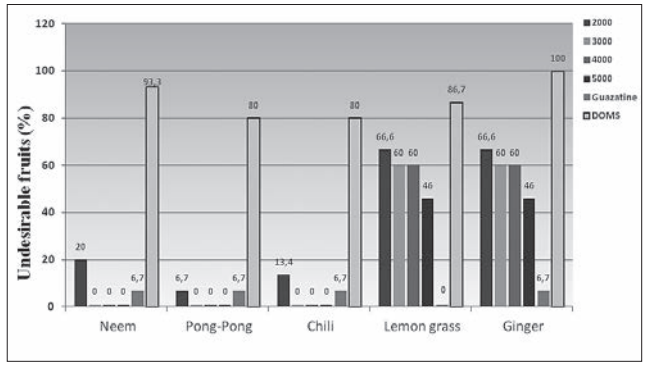

Figure 2. Effect of spraying plant extracts at different concentrations (ppm) on mean number orange undesirable fruits (%) after 21 days during storage condition (25±2°C) compared with controls [fungicides (Guazatine) and DOMS]

Table 2. Impacts concentration of extracts of *Neem,* Pong-pong, Chili, Lemon grass and Ginger (ppm) on the inhibition of colony growth (cm^2) of *Penicillium digitatum,* raised on PDA media incubated at 25°C for 10 days *in vitro* compared with controls [DOMS + fungicides (Guazatine)]

Plants	Comparisons					
	500	1000	2000	3000	Guazatine	DOMS
	Colony diameter (cm)					
Neem	3.9	2.9	2.33	1.33	2.2	8.1
Pong-Pong	2.76	1.99	1.46	0.63	1.9	9.2
Chili	3.9	2.96	2.1	0.00	1.86	9.
Lemon grass	3.9	2.96	2.33	1.33	2	8.
Ginger	4.16	2.67	2.23	1.38	2	8.1

*Positive control [fungicide (Guazatine)], * Negative control (DOSM).

concentrations of 4,000 and 5,000ppm prevented the full growth of green mould on the surface fruits stored at 25±2 °C for 21 days (Tab. 3, Fig. 2). In addition, a number of undesirable fruits (0%) with concentrations of 4,000 and 5000 ppm resulted in the curbing of the development of fungal growth. Spraying extracts from Lemon grass and Ginger did not show a considerable statistical significance when applied on fruits, compared with controls. Percentage of damaged fruits was between 66.6-46% for all concentrations from extracts of Lemon grass and Ginger.

Table 3. Effect of spraying plant extracts at different concentrations (ppm) on undesirable fruits (%) result in development of mycelium growth *Penicillium digitatum* on the surface orange fruits under storage condition at 25°C±2, compared with controls

Plants	Comparisons					
	2000	3000	4000	5000	Guazatine	DOMS
	Undesirable fruits (%)					
Neem	20	0	0	0	6.7	93.3
Pong-Pong	6.7	0	0	0	6.7	80
Chili	13.4	0	0	0	6.7	80
Lemon Grass	66.6	60	60	46	0	86.7
Ginger	66.6	60	60	46	6.7	100

*Disease index (decay reached 50 %) = Total fruit – undesirable fruits/ Total fruit x 100.

Brine shrimp lethality bioassay. The cytotoxic activities of all the extracts of *Azadirachta indica* L., Cerbera *odollam* L., *Capsicum frutescence* L., *Zingiber officinale* L., and *Cymbopogon nardus* L. were studied by brine shrimp lethality bioassay .The LC_{50} values of parts of plant extracts were 20 (32-6), 5(15-0), 30(37-9), 473 (1,818-212) 495 (858-306) µg mL^{-1}, respectively, compared with values of controls (Guazatine and DOMS) that reached to 326 and >1,000) (Tab. 4).

Table 4. Brine Shrimp test toxicity of plant extracts under study

Plants	Part used	LC50 (µg/ml^{-1})
Azadirachta indica L (Neem)	Leafs	20 (32-6)
Capsicum frutescence L(Pong-pong)	Leafs	5 (15-0)
Capsicum frutescence L. (hot Chilly)	Fruits	30 (37-9)
Zingiber officinale L.(Lemon grass)	Leafs	473 (1818-212)
Cymbopogon nardus L.(Ginger)	Tubers	495 (858-306)
controls	Fungicide (Guazatine)	326 (540-198)
	DMSO	>1000

DISCUSSION

Many plant and plant products have been reported as having antimicrobial activities against plant pathogenic fungi [15]. The objective of the presented study was to investigate the effect of plant extracts as alternative synthetic fungicides in controlling mycelium growth of *Penicillium digitatum*, a pathogen for post- harvest diseases of citrus fruits [1]. These diseases could cause a 10%-30% decrease in crop yield and marketing quality [2, 16]. The use of biocontrol agents

in plant disease control with plant extracts, such as like lemon, citronella, clove, mint, thyme, and oregano oils, has been employed as alternative control measures to replace the conventional synthetic pesticides [17]. The plant extracts reported to be effective on the fungi *Penicillium digitatum* include garlic, *Withania somnifera, Acacia seyal* and *mustard horseradish,* used for the same purpose as natural alternatives [9, 15].

The action of the plant extracts may be due to the action of their bioactive compounds against fungi growth through preventing the growth of spores, such as *Penicillium italicum* and *P. digitatum,* which could replace synthetic chemicals in future to reduce their increase in the environment. These results are in agreement with M. A. Agrios [14], who reported that the fungicidal of oil obtained from thymus against several post-harvest pathogens may reveal the marked fungicidal activity of carvacrol in thyme. Moreover, Bashar MA, *et al*., [18] has reported that lime fruit peel essential oil components inhibit linear growth on spore germination of *P. italicum*, *P. digitatum* and *Geotrichum canium* [19]. In study by Ogawa JM [12], for evaluation of the effect of lime, thyme, and comphore oils for their inhibitory effect *in vitro,* different concentrations of each essential oil at 1.5 and 10% (v/v) were tested on the growth of *P. digitatum*. The best concentration at 10% showed the highest inhibition growth of *P. digitatum* for all tested oils, and significantly reduced the severity of disease in fruits, compared with controls. Previous reports have shown that spearmint essential oil possesses antifungal properties [20]. These oils have a wide range of bioactivity. Antifungal activities of essential oils from Thymus and Mentha species have been also reported in other studies [16, 21]. The presented study is in general agreement with the results of earlier investigations [5, 22].

Some chemical compounds have been isolated from the seed [20] and bark [23] of S. mahogany, a result that should encourage other researchers to undertake further studies, such as finding crude extracts from natural products as safer and less risky alternatives to agricultural pesticides. The earlier reports of antimicrobial activities [6] support the findings of the presented study. The inhibitory effects of crude extracts indicate that they can be selected for more applications in managing the environment, because there is a correlation between the effectiveness of the extracts and activity against the brine shrimp nauplii using crude extracts.

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