Activity of some natural products and their toxicity in controlling Colletotrichum gloeosporioides Penz.

Ghassan.F. Al.Samarrai and Rana Ibrahim Khaleel

Department of Biology, College of Education, University of Samarra, Samarra, Iraq
College of Engineering, University of Samarra
ghassan.f@uosamarra.edu.iq

Abstract
Three ethanol extracts (concentrations 500-5000 ppm) from Eucalyptus camaldulensis L., Nerium oleander L (Oleander) and Ricinus communis L (Castor) were evaluated for their anti-microbial activity on the fungus Colletotrichum gloeosporioides Penz. The toxicity (LC50) of these extracts was determined using the (BST) Brine Shrimp test. Crude extraction of Oleander and Castor plant showed a complete fungal inhibition zone in PDA medium at 3000ppm (100%) in the mango anthracnose in vitro, while Eucalyptus recorded more than 80% fungus inhibition. The LC50 value of those extracts was ranged from 5 mg/ml to 495mg/ml, levels that were for below the high toxic level of 0.2 mg/ml based on their safe levels fungal inhibition of Colletotrichum gloeosporioides. The botanicals can be considered as alternative to synthetic in future researches.

Keyword - Mango anthracnose, synthetic fungicides, botanicals, Environment pollution, eco-friendly.

1. INTRODUCTION
Fungicides are widely used in conventional agriculture to control mango anthracnose. Prolonged usage often poses health problems. Mango (Mangifera indica L.) is one of the popular seasonal fruits found mainly in the tropical and subtropical countries. It is widely grown in different countries of the world [1, 2]. Mango crop is attacked by a number of diseases of which anthracnose of mango caused by Colletotrichum gloeosporioides, is one of the most important diseases and loss (30%) due to this disease is substantial. The disease is currently managed with synthetic fungicides such as Benzimidazoles, Dithiocarbamates and Phthalimides but there is,
however, a growing global interest on their replacement with other alternatives such as with environment–friendly biopesticides like botanicals or biological control. In order to discover new biologically active compounds that can be used to control disease, many wild plants are still being studied for their anti-microbial properties [3, 4]. Some plant extracts (botanicals) have been reported to control on growth of fungal pathogen in plant [5]. The present study evaluated extracts of three plant species for their anti-fungal properties against anthracnose of mango in vitro, in addition to their toxicity levels based on the LC50 values.

2. EXPERIMENTAL

2.1. Pathogen

Parts of anthracnose-infected tissue from mango were removed by using sterile scalpel and grown in sterilized conditions in PDA media. Several aseptic single-spore isolations (under lamina chamber) were made from colonies propagated in PDA petri dishes under laboratory conditions at 25°C for 7 days. The fungal isolates from PDA were shifted separately for further isolation by culture method as recommended by [6] to get pure isolates. The identification of *Colletotrichum gloeosporioides* Penz., from the pure colonies was verified using microscopic study based on taxonomic and morphological references.

2.2. Preparation of plant extracts

The extraction technique used was a modification by [7] method. 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. The dark spongy materials were dried in an oven at 37°C for 2 days and the dried powder was stored in small sterilized 5ml screw-capped glass bottles that were stored in the refrigerator at 4°C until further use.
2.3 Preparation of plant extract-dilutions

Crude extract from each plant (0.5, 1, 2 and 3 gm) dissolved in 2ml organic solvent DMSO or Dimethyl sulfoxide. This was diluted further by mixing with water to obtain concentrations of 500, 1000, 2000 and 3000ppm.

2.4 In Vitro Screening of Plant Extracts

The in vitro screening technique used in the research was the modification from the study of [8]. Based on the specification from the PDA plastic bottle (Sisco Research Laboratories Pvt. Ltd.), 39gm dry powder of potato dextrose agar (PDA) were in cooperated in 1 litre distilled water and stirred continuously at 60°C until it was fully dissolved and attained homogeneity. Forty-five ml of the liquid PDA medium was in cooperated into 108 fifty-ml glass conical flasks (for the 6 treatments consisting of 500 ppm, 1000 ppm, 2000 ppm, 3000 ppm, fungicide Guazatine, and DMSO or Dimethyl sulfoxide as control) and autoclaved for 20 minutes at 121°C. DMSO, a colorless liquid, is a scavenger of free radicals that is miscible in water and a wide range of organic solvents. It is an important polar solvent that dissolves both polar and non-polar compounds [9].

After autoclaving, the flasks were cooled down to about 45°C. Five ml of each plant extract, was added in flasks by using pipette that were gently agitated by hand for 2 minutes in order to mix the extracts properly. Media cultures were amended into 9cm in Petri-dishes. Chloramphenicol (250 mg/l per Petri dish) was added to the medium to prevent bacterial growth [10]. The experiment was performed under aseptic lamina conditions and replicated thrice. One ml, of Colletotrichum gloeosporioides, spore suspension (conc.1 × 10^6 spores/ml^-1) was added by pipette on to the centre of the amended PDA extracts. Inoculated plates were incubated at 25 °C for 10 days. The Petri-dishes inoculated without the extract concentration [DMSO and Fungicide (Guazatine) 1000 ppm] served as controls. Colony diameter was determined by measuring the average radial growth using a common metric plastic ruler. The inhibition zone was measured using the formula as follows:

\[ \frac{(\text{Test}-\text{control})}{\text{control}} \times 100 \]

2.5 Bioassay of Artemia salina (Brine shrimp lethality)

Brine shrimp lethality bioassay was carried out to investigate the toxicity of plant extracts. 50mg of Artemia salina (Leach) eggs were added to a 100 ml- volumetric flask containing 75ml Ocean/Sea water. The volumetric flask was kept in clean and sterile chamber under room condition for 48 h for the eggs to hatch into shrimp larvae. The experiment was divided into ten groups [10 flasks: 8 extract concentrations + 2 controls (DMCO and fungicide)]. Forty-five (45ml) of sea water was added to each flask, and 20 hatched larvae of A. salina (Leach) taken after 48 of the initiation of hatching, were added
to each flask. One ml of each concentration (640, 320, 160, 80, 40, 20, 10 and 5 μg/μl) was added to each flask (1 to 8) respectively. Fungicide (Guazatine 1000ppm) and DMSO without plant extracts flask (9 and 10) served as controls. Each dosage and controls were tested in three replicates (Plate1) using Completely Randomized Design (CRD). Mortality data was recorded by counting the surviving (larva) with the aid of a magnifying glass. The percentage of lethality of brine shrimp larva was calculated at each concentration for each sample using \[ \text{% deaths} = \frac{(\text{Test}-\text{control})}{\text{control}} \times 100 \].

3. Results

3.1 Effect of plant extracts on mycelium growth

The study showed different significances between treatments and controls at \( p \leq 0.05 \). Generally, the results (Table 1) showed that complete inhibition of the growth of \textit{Colletotrichum gloeosporioid} reached (100 %) of crude plant concentration extracts at 3,000ppm \textit{Nerium oleander}. \textit{L} (Oleander) and \textit{Ricinus communis}. \textit{L} (Castor), while crude extract of \textit{Eucalyptus camaldulensis} \textit{L} showed more (80%), negative control performed with serial dilutions of DMSO, it was observed that there was no effect on fungal growth (0%).

Table 1 Effect plant extracts(ppm) of \textit{P. digitatum} growth inhibition (cm\(^2\))in petri dishes PDA inoculated and incubated at 27°C for 7 days \textit{in vitro}.

| Plants               | Concentrations(ppm) |
|----------------------|----------------------|
|                      | 500                  | 1000                  | 2000                  | 3000                  |
| \textit{Nerium oleander}. \textit{L} | 4.1c                  | 2.5b                  | 1a                    | 0.00a                  |
| Castor i             | 3.93b                 | 2.9c                  | 1.9c                  | 0.00a                  |
| Eucalyptus           | 2.76a                 | 2a                    | 1.55b                 | 1.1b                   |
| DOMS                 | 8d                    | 8.5d                  | 9.2d                  | 8.9c                   |

3.2 Brine shrimp lethality bioassay

The cytotoxic activities of all the extracts of \textit{Nerium oleander}. \textit{L} (Oleander) and \textit{Ricinus communis}. \textit{L} (Castor) and \textit{Eucalyptus camaldulensis} \textit{L}. were studied by brine shrimp lethality bioassay. The \( \text{LC}_{50} \) values of parts of plant extracts were 5, 20, 30 μg mL\(^{-1}\), respectively, compared with values of (Guazatine and DOMS) that reached to 326 and >1,000) (Tab.2).

Table 2. Brine Shrimp test toxicity of plant extracts under study

| plants | Part used | LC50 (μg/ml\(^{-1}\)) | Class |
|--------|-----------|----------------------|-------|

The toxicity from pong-pong at different concentration (5, 10, 20, 40, 80, 160, 320, 640 mg/L) are presented in Fig. 1. LC50 of crude extracts *Nerium oleander*.L was 5mg/L. Toxicity studies revealed that the ethanol extracts exhibit low toxicity (LC50 of 5 mg/L) against *Artemia salina* with percentage of the dead larvae was 50% at this toxicity level. The results on brine shrimps assay indicate that the extract has LC50 value greater than 2-20mg/L, which signified that *Nerium oleander*.L might not be toxic to human and could be a potential source of novel antibacterial compound to control infection.

The table below shows the toxicity levels of various plant extracts.

| Plant Name          | Part            | LC50 (mg/L) | Toxicity Reference |
|---------------------|-----------------|-------------|--------------------|
| *Nerium oleander*.L | Leaf            | 4           | Low toxicity       |
| *Ricinus communis* .L | Leaf          | 20          | Safe               |
| *Eucalyptus camaldulensis* L | Fruits     | 30          | Safe               |
| Guazatine           |                 | 326         | Safe               |
| DOMS                |                 | >1000       | Safe               |

*Toxicity level reference – high = 0.2mg/ml, medium=0.2-2mg/ml, low= 2-20 mg/ml, safe = >20 mg/ml [Anon.,12]*

After 48 hours, Brine shrimp lethality bioassay of crude extract *Ricinus communis*.L showed low cytotoxic activity against brine shrimp Larvae and LC50 Fig. 2. LC50 values of 5, 10, 20, 40, 80, 160, 320 and 640mg/L gave mortality percentages of 30, 38, 48, 62, 68, 70, 80 and 90% respectively. LC50 value at which 50% of *Artemia salina* (shrimp larvae) was found to be 20mg/L.

**Figure 1** Brine shrimp test (LC50) of the ethanol extract of *Ricinus communis*.L in brine shrimp lethality bioassay
Figure 2. Brine shrimp test (LC50) of the ethanol extract of Nerium oleander L. in brine shrimp lethality bioassay.

Toxicity result of Eucalyptus camaldulensis L. Fig 3. showed that the concentration of 20 and 40 mg/L gave mortality percentages of 42 and 58% respectively. The data analysis of the Larvae mortality of the brine shrimp gave a LC50 value of 30 mg/L (50% of the shrimp larvae were killed) after 48 hours. This indicates that Eucalyptus exhibits positive toxicity against the brine shrimp. Toxicity from other concentrations (5, 10, 80, 160, 320 and 640 mg/L) recorded mortality percentages of 24, 58, 70, 66, 76 and 86% consecutively.
Figure 3 Brine shrimp test (LC50) of the ethanol extract of *Eucalyptus camaldulensis* L in brine shrimp lethality bioassay.

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 [13]. These diseases could cause a 10%-30% decrease in crop yield and marketing quality [14]. 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 [15]. 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 [16]. 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 [17], 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 [18] has reported that lime fruit peel essential oil components inhibit linear growth on spore germination of *P. italicum*, *P. digitatum* and *Geotrichum caninum* [19]. In study [20], 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 Antifungal activities of essential oils from
Thymus and Mentha species have been also reported in other studies [21]. The presented study is in general agreement with the results of earlier investigations [22]. Some chemical compounds have been isolated from the seed [23] and bark [24] 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 [24] 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.

4. CONCLUSION

*Nerium oleander*, L (Oleander), *Ricinus communis*, L (Castor), and *Eucalyptus camaldulensis* L showed positive effects on the inhibition of postharvest fungi as alternatives to fungicides, while bearing in mind the increasing global pollution of the environmental. These extracts or botanicals have a bright future in modern plant protection to replace conventional synthetic pesticides.

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