

## Efficacy of methanol extract of *Morinda citrifolia* fruit against Sunflower Downy mildew Disease

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### Abstract

Downy mildew of sunflower is one of the most devastating seed and soil borne disease caused by *Plasmopara halstedii* which is very challenging to eradicate once established in the field. In the present study methanolic extract from *Morinda citrifolia* fruit was screened for their anti-mildew activity and phytotoxicity. Leaf disc assay revealed that, as the concentration of extract increases from 1% to 5% a significant decrease in sporulation was observed. Higher concentration leads to browning of leaf surface indicating phytotoxicity. Similarly, upon seed treatment lower concentrations (1% - 5%) were found improving the seed germination and plant growth, and higher concentration (above 7%) significantly reduced the seed quality variables. Phytochemical analysis of methanolic extract showed the presence of phenolics, alkaloids, steroids, flavonoids, carbohydrates, tannins, reducing sugars and saponins. Further, bioactive compounds from crude extract were partially purified through preparatory thin layer chromatography and evaluated for their anti-sporulant activity. Among the five compounds extracted, four were found suppressing the sporulation to various extents and compound with Rf value 0.87 was recorded significantly with higher anti-sporulant activity.

**KEYWORDS:** Sunflower, *Morinda citrifolia*, Downy Mildew, anti-mildew activity, Phytotoxicity.

### INTRODUCTION

The sunflower (*Helianthus annuus* L.) is an annual herbaceous plant which belongs to the family Asteraceae. It is one of the top three important edible oil yielding seed crop grown across the world. Sunflower is susceptible to many fungal, bacterial and viral diseases. More than 30 diseases have been identified in sunflower (Gulya *et al.*, 1994) with various etiologies. Among them downy mildew of sunflower caused by an oomycetes phytopathogen *Plasmopara halstedii* (Farl.) Berl. and de Toni, has been recognized as a potentially destructive disease in major sunflower producing countries due to its seed, soil borne, and systemic nature of infection. The disease is extremely challenging to eradicate once it is established (Kulkarni *et al.*, 2006). As high as 80 % loss in productivity was reported due to downy mildew disease in sunflower (Mayee and Patil, 1986). In India, the downy mildew disease of sunflower was reported around 1986 in Marathwada region of Maharashtra states followed by Andhra Pradesh, Karnataka, Maharashtra, Tamilnadu and Punjab in subsequent years.

In order to manage downy mildew diseases farmers are dependent on chemicals, especially metalaxyl as seed treatment. However, recent findings showed the adverse effect of these chemicals on environment and also possibilities of development of new resistant pathogenic strains. Hence it is important to search an alternative, ecofriendly way of alleviating downy mildew disease of sunflower. On the other hand, the antimicrobial activities of natural and plant-derived products are reported against a wide range of phytopathogens (Gulter 1988; Arya and Perello 2010). Uses of these natural products are considered as safe, ecofriendly and resource are locally available.

*Morinda citrifolia* L. also known as noni or Indian mulberry is a small evergreen tree, originating in tropical Asia. In the tropics, it has been greatly valued medicinally and the plant is normally cultivated for its roots, leaves and fruits. Fruit juice is used as an alternative medicine for its potential anti-microbial, anti-cancer, anti-inflammatory and antioxidant effects (Wang *et al.*, 2002). Various parts of the plants, crude and partially purified compounds from *M. citrifolia* was extensively used for several human and animal diseases (Kamiya *et al.*, 2004). Aree *et al.*, (2009) investigated antifungal activity of noni fruit juice extract on *Candida albicans*. But, its potential in managing the plant diseases is less reported. Hence, in the present study we have evaluated the potential of fruit extract of *M. citrifolia* for their anti-sporulant activity and phytotoxicity. Further purification and characterization of anti-sporulant compound and field studies are under progress.

## **MATERIALS AND METHODS**

### **Plant Material**

Sunflower seeds (Cv. Morden) susceptible to downy mildew disease was collected from National Seed Corporation, Mysore, Karnataka. The seed samples were surface sterilized with 0.2 % sodium hypochlorite for 2 min and subsequently washed with distilled water thrice. The surface sterilized seeds were used throughout the experiments. Mature fruits of *M. citrifolia* were collected from 'Indraprastha' Jeevadaraka Sasyavaata Kalalavadi village, Mysore.

### **Preparation of plant extract**

The mature fruits (1 kg) were washed with distilled water and chopped in to pieces and kept for drying at 45°C for 72 h. The dried fruit sample was coarse powdered by using mechanical blender and was extracted with methanol by using soxhlet apparatus. The extract was concentrated to dryness under reduced pressure and controlled temperature (50°C). Different concentrations (1, 2, 3, 5, 7 and 10 %) were prepared by dissolving it in minimum quantity of dimethyl sulfoxide (DMSO) and made up to desired volume with sterile distilled water.

### **Determination of anti-sporulant activity**

The downy mildew pathogen *P. halstedii* has been maintained in the sick plot of Department of Biotechnology, University of Mysore, Mysore since the last 10 years. In order to collect inoculum for the experiment, susceptible cv. Morden was grown in the sick plot. Thirty- day old infected seedlings showing profuse downy growth was selected in the late evening and inoculum was harvested early morning.

Anti-sporulant activity of methanolic extract against *P. halstedii* was investigated by leaf disc method (Haggag, 2002; Musetti, 2006 and Deepak, 2005). Leaves with disease symptoms were collected from infected plants grown in sick plot and washed in distilled water. The excess water was then removed by blot drying. Leaves were cut in to small discs of 10 mm diameter by using sterilized cork borers and subsequently immersed in plant extracts of different concentrations for 5 min. The treated discs were then incubated for 12-14 h in moist chambers by facing abaxial surface upside at  $22\pm 1^\circ\text{C}$  in the dark. After incubating in a moist chamber for 12-14 h, the leaf discs were analyzed for sporulation under stereo binocular microscope and categorized as complete inhibition (+++), moderate inhibition (++) , least inhibition (+) and no inhibition (-).

### **Phytochemical analysis of the extract**

The phytochemical analysis of methanolic extract of *M. citrifolia* was carried out following the standard procedures of Sofowora 1993 and Siddiqui *et al.*, 1997.

### **Sunflower seed quality assessment**

The crude methanolic fruit extract of *M. citrifolia* was dissolved in small amount of DMSO and was diluted to required concentrations with sterile distilled water. The highly susceptible sunflower seeds were treated with different concentrations of (1, 3, 5, 7 and 10%) fruit extracts for 6 h on a rotary shaker at 150 rpm (400 seeds in 100 ml extract). The treated seeds were blot dried and evaluated for their seed quality variables (root length, shoot length, % germination and seedling vigor) following roll towel method (International Seed Testing Association 2010). For each treatment four set of 100 seeds were used and the experiment was repeated thrice.

### **Thin layer chromatography (TLC) analysis**

The crude methanolic fruit extract was separated on precoated 20 x 20 cm TLC Silica gel 60 F 254 (Merck KGaA). 10  $\mu\text{l}$  of each extract was spotted on TLC plate at equal distance and the chromatogram was developed with standardized solvent system Ethyl acetate:Methanol:Water (100:6:4). The developed chromatogram was observed under visible and ultra violet (UV) light. Preparative TLC was conducted as explained above except the silica gel plates used were of 2 mm thickness. The spots at different Retention factor (Rf) values were scraped off and eluted using methanol. The eluted compound was tested for its anti-sporulant activity by leaf disc assay at 1 mg/ml concentration.

### **Statistical analysis**

All data of seed quality variables were expressed as mean  $\pm$  standard error and analyzed separately for each experiment and subjected to arcsine transformation and analysis of variance (ANOVA) (SPSS, version 16).

## **RESULTS**

### **Determination of anti-sporulant activity**

In the leaf disc assay, a gradual decrease in sporulation was observed with application of the different concentrations of methanolic extract in the increasing order from 1% - 5% (Table 1). Above 5 % concentration, sporulation was not observed and leaves turned brown in color which indicated the phytotoxicity of higher concentration.

### Phytochemical analysis

The phytochemical analysis of crude methanolic extract of fruits revealed the presence of secondary metabolites (Table 2).

### Sunflower seed quality assessment

Sunflower seed treatment with different concentrations of the extract revealed significant ( $P \leq 0.05$ ) enhancement of germination and seedling vigor index to varying degrees over the control. Maximum germination of 83% and seedling vigor index of 2905 were noticed in 5 % seed treatment compared to the control 72 % germination and vigor index 1836. The higher concentrations were found to be phytotoxic which was evident by decrease in the seed quality variables (Table 3).

### Screening of anti-sporulant activity of partially purified fractions by TLC

The partially purified fractions showed five bands with Rf value ranging from 0.09 to 0.87. Among the five compounds extracted, four were found inhibiting the sporulation to various extents. The fraction with Rf value of 0.87 was significant in suppressing the sporulation of *P. halstedii* (Figure.1, Table 4).

## DISCUSSION AND CONCLUSION

The present study aimed to manage downy mildew pathogen using methanolic extracts of *M. citrifolia* fruits. The management of oomycetes phytopathogen is a challenge as it behaves differently from other fungi and is a seed and soil borne-systemically infecting pathogen. Deepak *et al.*, (2005), have evaluated the methanolic extract of 40 plant species for their anti-sporulating activity against downy mildew disease of pearl millet caused by *Sclerospora graminicola*. According to their observation, extracts of *Agave americana*, *Artemisia pallens*, *Citrus sinensis*, *Dalbergia latifolia*, *Helianthus annuus*, *Murraya koenigii*, *Ocimum basilicum*, *Parthenium hysterophorus*, *Tagetes erecta*, *Thuja occidentalis* and *Zingiber officinale* were endowed with anti-mildew metabolites. Similarly in our studies, the methanolic fruit extract of *M. citrifolia* has inhibited the sporulation of *P. halstedii* in the leaf disc method, which indicated the presence of anti-sporulant metabolites in the crude extract.

Rajeshwari *et al.*, (2008) evaluated 20 medicinal plant extracts against sporangial germination of grapevine downy mildew pathogen *Plasmopara viticola* *in vitro* and reported that neem seed kernel extract at 5% concentration significantly inhibited the sporangial germination of *P. viticola*. Antimicrobial activity of fruit extract of *M. citrifolia* against bacterial pathogens was reported in earlier studies (Natheer *et al.*, 2012). Rivera *et al.*, (2011) studied the antimicrobial activity of *M. citrifolia* fruit juice against different species of mycoplasma. They had reported that fruit juice showed highest antimicrobial activity against mycoplasmas tested. Also, they reported presence of saponins, tannins, alkaloids and triterpenoids in fruit juice which is in corroboration with our studies. In our studies, phytochemical analysis of methanolic fruit extracts of *M. citrifolia* with anti-sporulant activity revealed the presence of alkaloids, glycosides, tannins, terpenoids, steroids, flavonoids, reducing sugars, phenolics and saponins.

Seed treatment has emerged as an effective method for control of seed-borne pathogens and improve seed quality variables, as it requires less quantity of compounds and economical (Masum *et al.*, 2009). Hydro-priming of seeds has been effectively used to enhance the seedling emergence, uninformative and vigor of seedlings under both optimal and sub-optimal conditions (Demir and Venter 1999). In our studies as the seeds were treated with the extracts for 6 h the positive effect of hydro priming the seed quality could be ruled out. Afzal *et al.*, (2010) used aqueous extracts of neem, jimson weed, ginger and garlic for the management of seed-borne fungal pathogens in sunflower. According to their observations, neem and garlic extracts were best in suppressing the seed borne fungal pathogens. By suppressing the seed-borne fungal pathogens it is possible to decrease seed deterioration or decay under field conditions which results in increased seed germination and seedling vigor. Similarly in our studies we observed an increase in the seed germination and seedling vigor at lower concentration (up to 5%). Higher concentration of fruit extracts was found to be phytotoxic which was evidenced by decreased seed germination and seedling vigor (Table 3).

Major bioactive compounds identified in *M. citrifolia* include scopoletin, octoanoic acid, terpenoids, alkaloids, anthraquinones, b-sitosterol, carotene, flavone glycosides, linoleic acid, alizarin, acubin, L-asperuloside, caproic acid, caprylic acid, ursolic acid, rutin, and peroxerone (Moorthy and Reddy, 1970 and Singh and Tiwari, 1976; Lavand and Larson, 1979; Farine *et al.*, 1996). The anti-sporulant activity of the methanolic extracts of *M. citrifolia* is mainly due to the presence of one or more bioactive compounds and their antimicrobial activity. Brett *et al.*, (2011) reported the two compounds, de-acetyl asperulosidic acid and asperulosidic acid, as major phytochemical constituents of *M. citrifolia* fruit, possess antibacterial activity. Satwadhari *et al.*, (2011) analyzed the chemical composition of *M. citrifolia* fruits and reported the presence of anthraquinones, saponins and scopoletin. The mobile phase, Rf value and the description given to scopoletin is very close to our results. The bioactive compound with higher anti-sporulant activity had Rf value of 0.87 and it appeared as blue band under UV. Hence, it is suspected that the anti-mildew compound obtained from the present study may be scopoletin. Further studies have to be done in order to conclude the results.

From the present study, it could be concluded that *M. citrifolia* fruit extract are endowed with anti-sporulant bioactive compounds which can be successfully used to manage sunflower downy mildew disease. Along with disease protecting potential properties, lower concentration of the extracts are also found to stimulate the early plant growth. But the higher concentrations of crude extracts are phytotoxic which was evidenced by browning of leaf discs and decreased seed quality variables.

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#### FIGURE CAPTION

Figure 1: a. Thin layer chromatography analysis of crude methanolic extract of *M. citrifolia* fruit extract and b. leaf disc assay for anti-sporulant activity against sunflower downy mildew with different bands of Rf values. Fraction e with Rf =0.87 showing inhibition of sporulation formation. a; Treated Sunflower leaf disc. b; Control leaf disc with sporulation.

**Table 1. Anti-mildew activity of methanol extract of *M. citrifolia* fruit extract against *P. halstedii*.**

| Plant species             | Family    | Part used | Leaf disc assay Concentrations (%) |    |     |     |     |
|---------------------------|-----------|-----------|------------------------------------|----|-----|-----|-----|
|                           |           |           | 1                                  | 3  | 5   | 7   | 10  |
| <i>Morinda citrifolia</i> | Rubiaceae | Fruit     | +                                  | ++ | +++ | +++ | +++ |

Anti-mildew activity was evaluated by following scale, Complete inhibition (+++), Moderate inhibition (++) , less inhibition (+) and no inhibition (-).

**Table 2. Phytochemical analysis of methanolic extracts of *M. citrifolia* fruit.**

| Phytochemical tests |                  | <i>M. citrifolia</i> |
|---------------------|------------------|----------------------|
| Tannins             |                  | +                    |
| Alkaloids           | Mayer's reagent  | +                    |
|                     | Wagner's reagent | +                    |
| Steroids            |                  | +                    |
| Phenolics           |                  | +                    |
| Flavonoids          |                  | +                    |
| Reducing sugar      |                  | +                    |
| Saponins            |                  | +                    |
| Carbohydrates       |                  | +                    |

**Table 3: Effect of seed treatment with methanolic fruit extract of *M. citrifolia* on germination and seedling vigor of sunflower.**

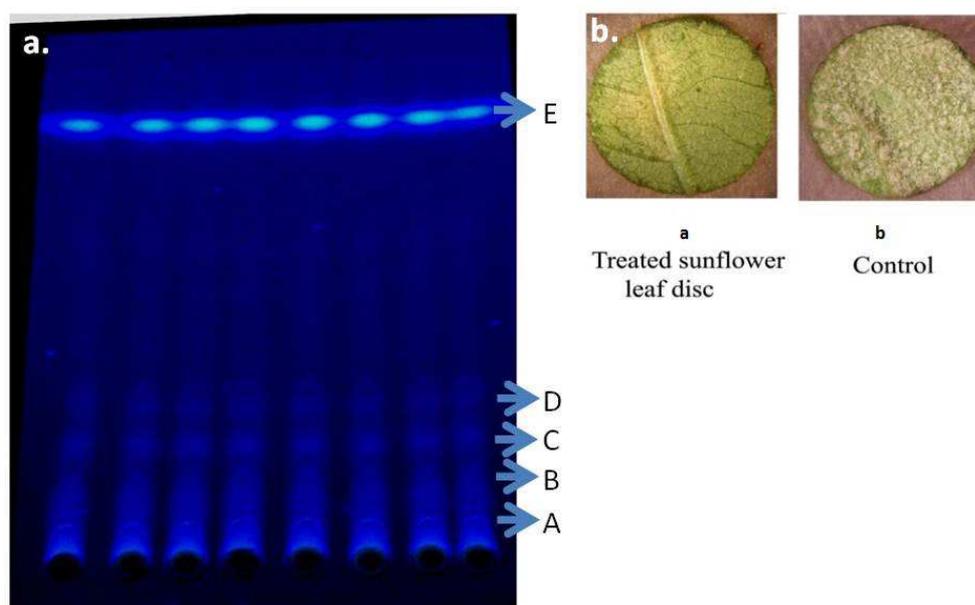
| Methanolic extract (%) | Germination (%)     | MRL                    | MSL                    | Vigor index          |
|------------------------|---------------------|------------------------|------------------------|----------------------|
| 1                      | 74±0.5 <sup>c</sup> | 15±0.5 <sup>cd</sup>   | 11.5±0.2 <sup>ab</sup> | 1961±25 <sup>c</sup> |
| 3                      | 77±0.5 <sup>b</sup> | 17.5±0.5 <sup>b</sup>  | 13.5±1.0 <sup>ab</sup> | 2387±91 <sup>b</sup> |
| 5                      | 83±0.5 <sup>a</sup> | 20.5±0.5 <sup>a</sup>  | 14.5±0.7 <sup>a</sup>  | 2905±22 <sup>a</sup> |
| 7                      | 62±0.5 <sup>d</sup> | 16.5±0.5 <sup>bc</sup> | 12±0.5 <sup>ab</sup>   | 1767±77 <sup>c</sup> |
| 10                     | 54±0.5 <sup>e</sup> | 13.5±0.5 <sup>d</sup>  | 11±0.5 <sup>b</sup>    | 1323±42 <sup>d</sup> |
| Control                | 72±0.5 <sup>c</sup> | 14±0.5 <sup>d</sup>    | 11.5±0.5 <sup>ab</sup> | 1836±91 <sup>c</sup> |

Values are the means ± SE from three separate experiments. Mean followed by the same letters in the column are not significantly different according to Tukey's b at P≤0.05. MRL: mean root length, MSL: mean shoot length, VI: vigor index.

**Table 4: Anti-mildew activity of partially purified fractions of methanol extracts of *M. citrifolia* fruit.**

| Fractions | Inhibition | Rf value |
|-----------|------------|----------|
| a         | -          | 0.09     |
| b         | +          | 0.18     |
| c         | +          | 0.25     |
| d         | +          | 0.36     |
| e         | +++        | 0.87     |

Anti-mildew activity was evaluated by following scale, Complete inhibition (+++), Moderate inhibition (++) , less inhibition (+) and no inhibition (-).



**Figure 1: a. Thin layer Chromatography analysis of crude methanolic extract of *M. citrifolia* fruit extract and b. leaf disc assay for anti-sporulant activity against sunflower downy mildew with different bands of Rf values. Fraction e with Rf =0.87 showing inhibition of sporulation formation. a; Treated Sunflower leaf disc. b; Control leaf disc with sporulation.**