Assessment of Antifungal Properties of Leaf Extracts of Eichhornia and Neem as Biopesticide on Fungal Pathogens of Cabbage *(Brassica oleracea var capitata L)*

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Abstract : The antifungal activity of Neem *(Azadirachta indica)* and Eichhornia *(Eichhornia crassipes)* extract against *Aspergillus*, *Fusarium*, *Candida* and *Mucor* strains respectively were studied. The samples were collected and laboratory analysis were carried out to identify, isolate and control the fungi associated with *Fusarium* wilts, Leaf spot and White mould of Cabbage. The plant extracts inhibited mycelial growth at various levels and the degree of suppression gradually increased with increasing concentration of 50mg, 100mg, 150mg and 200mg concentrations. The superior inhibition among them was found at 200mg methanolic extract of Neem and Eichhornia against *Aspergillus* and *Fusarium.* There were no significant differences between 150mg and 200mg concentration, but no inhibition was seen in case of *Candida* and *Mucor.* Analysis of discrepancy results on different concentrations of mycelial growth shows that the plant extracts were highly remarkable.

IndexTerms **- Neem, Eichhornia, Methanol extract, Inhibition percentage, Activity index.**

I. INTRODUCTION

Cabbage *(Brassica oleracea var capitata L)* a biennial crop for its dense leafy heads. It is grown on an area of 528,300 ha., at the productivity of 22.9 tons per ha. (Gajanana *et al.,* 2004). This being second largest producer Worldwide and accounted for 7.3% of India's total vegetable production (Kandpal, 2014). It belongs to family Cruciferous which is extensively grown in India for its good source of vitamins, minerals and fibre, low in calories and with remarkable high levels of calcium, phosphorous, iodine and potassium (Muhammad *et al.,* 2010). It is been cultivated in temperate and tropical regions of the World viz India, China, Germany, Indonesia, Russia, Poland, Korea, Japan, Taiwan, Turkey, Ukraine, USA, Uzbekistan and several other countries (Kamesh *et al.,* 2017).

There are currently a considerable number of plant-based crop protection products that are available commercially (Zarins *et al.,* 2009). Attributes like applications and less restrictive (sometimes non-existent) maximum residue limits are enticing growers to trade portions of their synthetic crop protection portfolios for Biocontrol options (Fitches *et al.,* 2004). Especially popular are the Integrated Pest Management (IPM) strategies that employ a combination of biological and synthetic

crop protection products in order to achieve synergies of action. In these strategies, properly timed applications of biological products can decrease a grower's total need for synthetic pesticides (Choudhary *et al.,* 2003). The success of India's agriculture is attributed to a series of steps that led to availability of farm technologies which brought about dramatic increases in productivity in 70's and 80's often described as the Green Revolution era (Nikhat *et al.,* 2010). The major sources of agricultural growth during this period were the spread of modern crop varieties, intensification of input use and investments leading to expansion in the irrigated area. In areas where 'Green Revolution Technologies' had major impact, growth has now slowed (Srijita *et al.,* 2001).

Bio-pesticides are ecofriendly pesticides which are obtained from naturally occurring substances, microbes and plants (biochemicals). Not all natural products are Biopesticides. Some are chemical pesticides if they act on nervous system of the pest (Fitches *et al.,* 2004). Through the use of Biopesticides in a wider way, agriculture and health programs can be beneficially affected. There are many disadvantages associated with the use of chemical pesticides like genetic variations in plant populations, reduction of beneficial species, damage to the environment or water bodies, poisoning of food and health problems such as cancer which makes Biopesticides to come into picture (Wanren *et al.,* 2018). Plant extracts have been less harmful to animals and humans and found to be very useful to the environment (Zarins *et al.,* 2009). Their usage reduces risk of exposure to chemicals, reduces water pollution through fertilizer runoff, reduces number of applications, causes less harm to beneficial pests, biodegradable, and provides better nutritional quality. Thus, this study is designed such that to identify, isolate and to control the fungi associated with *Fusarium* wilts, Leaf spot and White mould of Cabbage using Neem and Eichhornia as biopesticides in vitro.

II. MATERIALS AND METHODS

Study Area

The study area was selected on the basis of crop plantation and pesticidal effects, in the outskirts of Mysore City i.e. besides H. D. Kote Road (D. Salundi) which is nearly 12kms from the Mysore City, India. The cabbage seeds were planted on October, 2017 and were harvested in the month of February, 2018. The age of crop varied from 60 to 75 days, the field was estimated for about 12 acre and the cost of crop extent from labor till the harvest is Rs. 12,000 per year.

Sample Collection

The collection of samples involved identification and selection of suitable field. The samples showing the symptoms of white mould, black and yellow spots on the leaf lamina were taken into consideration and were brought in the department laboratory, DOS in Environmental Science, Manasagangothri, Mysuru, Karnataka, India.

Preparation of Fungal Media

Potato Dextrose Agar (PDA) is a general purpose basal medium for the identification, cultivation and enumeration of fungi. Potato Dextrose Agar contains dextrose as a carbohydrate source which serves as a growth stimulant, and potato infusion the provides a nutrient base for luxuriant growth of most of fungal species, 3.9 grams of PDA and 1 gram of Agar-Agar type-1 was weighed and mixed 250ml conical flask containing 100ml Distilled Water. The constituents were further mixed, autoclaved and poured into Petri plates for solidification. These plates were used to determine activity and antifungal percentage.

Pure Cultures

This involved the identification of morphologically dissimilar colonies from the incubated plates (old culture). At first, fresh Potato Dextrose Agar Media was prepared, autoclaved and solidified. To which the identified colonies were placed with the help of Inoculating loop and the plates were incubated at room temperature and daily observations were made and refrigerated for future use.

A fresh Potato Dextrose Broth (PDB Broth) was prepared by adding 2.4 grams of PDB media into 100ml of Distilled Water and was autoclaved. The pure culture strains were inoculated using inoculation loop into the PDB broth under aseptic condition in Laminar Airflow. The test tubes were incubated and maintained for future use.

Isolation and Identification of Pathogens

Diseased portion of the crop were scrapped with the aid of scissors and scalped into pieces aseptically and was put into the zip-lock polythene bag, which were made air tight (Banerjee *et al.,* 2009). The samples containing bag was stored in the refrigerator for about 24 hours at 1.6°C. The diseased portions were later introduced into the PDA media aseptically and incubated (Agrios *et al.,* 2005). Saline Solution of 100ml was prepared i.e. by dissolving 0.85 grams of Sodium Chloride (NaCl) in 100ml Distilled Water, the diseased portions were introduced into the Saline media with the help of clean forceps and was kept in Rotary Shaker for 24 hours.

A fresh Potato Dextrose Agar Media and Saline Solution in six test tubes were prepared and autoclaved. 30mg of antibiotic (Streptomycin) to prevent bacterial contamination was added. The media was poured into the Petri plates and named as 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} respectively. Further from the 24 hours Stock Saline Solution, a known value of 0.1 micro liter of the same was pipetted using sterilized Micro-pipette and was serially diluted through the six test tubes named 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} a Spread Plate was performed using L-shaped glass rod and the Petri plates were incubated at room temperature.

The fungi isolates were identified based on Morphological Characteristics by referring the Practical Microbiology Manual, R.C. Dubey and D.K. Maheshwari, (2008). These were further observed under the Light Microscope, using lactophenol in cotton blue as the staining agent (Altier and Theisis, 1995). The identified strains are *Aspergillus, Fusarium, Candida* and *Mucor*.

Preparation of Plant Extracts

Leaves of Neem (*Azadirachta indica*) and Eichhornia (*Eichhornia crassipes*) were collected from the surrounding areas. The Neem was collected from the Department of Environmental Science, Manasagangothri, and Eichhornia was collected from Kukkarahalli Lake, Mysuru. These were then washed thoroughly in running tap water and soaked in 2% Solution of Sodium hypochlorite for 20 minutes (Farzana *et al.*, 2014), rinsed thoroughly with sterilized Distilled Water and air dried at room temperature. The dried plant materials were milled and sieved to obtain minute size of 1mm, 200 grams of each finely ground plant materials were used for the extraction in Methanol respectively. The extraction process was carried out in Soxhlet Extractor. Here the source material containing the compound is placed inside the thimble, which is loaded into the main chamber of the Soxhlet Extractor and the Methanol which is taken as a solvent for the consideration is placed in Round Bottom flask (RB Flask). The extraction process takes place for a minimum of six hours until elute is colorless.

Effect of the Extract Concentration on Radial Growth of Fungal Strain

Plant extracts which could suppress the fungal growth were tested for their efficiency against the identified pathogen. A known concentration of the Plant Extracts was measured and dissolved in Methanol. Potato Dextrose Agar Media was prepared in four Petri plates and autoclaved. After solidification of PDA Media, the fungal growth in PDB Media containing in the test tubes were taken and the plates were swabbed with the help of sterilized cotton buds. Later wells of equal size were made on the plates, to which the concentration of 50mg, 100mg, 150mg and 200mg were added.

The extracts used for the antifungal assay were added into the wells containing the Potato Dextrose Media and the Fungal Strain. The media amended with Methanol was considered as negative and Fluconazole (150mg) was considered as a positive control, triplicates of the same were performed.

Determination of Activity Index (Jayanthi *et al.*, 2013):

The activity index of the crude plant extract was calculated as:

$$\textbf{Activity Index (AI)} = \frac{\textbf{Zone of Inhibition of the Extract}}{\textbf{Zone of Inhibition obtained for Standard Antibiotic Drug}}$$

The efficiency of the plant products was evaluated and the radial mycelial growth over the control, which was calculated by using the following formula (Dissanayake *et al.*, 2014):

$$\textbf{Inhibition (\%)} = [(C-T)/C] \times 100$$

Where, C and T represent the diameter of controlled and treated colony. To avoid bacterial contamination 30mg Streptomycin was added in 100ml of PDA Media.

III. RESULTS AND DISCUSSION

Hydrophobicity is an important characteristic feature of plant extracts (Jayanthi *et al.*, 2013). The discrepancy of the plant extracts and their effectiveness against different microorganisms depend upon the chemical composition of the extracts and

membrane permeability of the microbes for the chemicals and their metabolism involved (Baral *et al.,* 2011). The antifungal activity of extracts fractionates and compound of *Eichhornia crassipes* and *Azadirachta indica* against *Brassica oleracea* var. *capitata* was carried with Methanol as solvent. For eco-friendly and sustainable management of Fungal diseases, the present study has tested the antifungal activity and their Respective Dilutions used against Fungal pathogens of Cabbage (Dissanayake, 2014). A clear zone of growth inhibition was noted due to diffusion of drug. The diameter of the inhibition zone denotes the relative susceptibility of the test microorganisms to a particular anti microbe (Arulpriya *et al.,* 2010).The extracts and the inhibition were evaluated against the standard drug Fluconazole (150mg) and Methanol. The concentration of the Plant Extracts ranging from 50mg, 100mg, 150mg and 200mg dissolved in 1ml of Methanol respectively. It was found that at 200mg concentration of almost all plant extracts were effective in reducing the mycelial growth of Fungal strains (Table 1, 2 and 3). From the investigation, considering the effects of Neem and Eichhornia (table 3), it was observed that leaf extract of Neem and Methanol at 200mg concentration effectively suppressed the mycelial growth of the *Aspergillus* Strain for about 85%. This is due to the presence of the essential oils which contain phenolic compounds (Phangthip *et al.,* 2005). There have been reports of Neem and its applications with regards to Pervaiz *et al.,* and Ogechi *et al.,* reported the control of fungal diseases on *Fusarium* Strains. Mondali *et al.,* studied the efficacy of different extracts of Neem leaf on seed borne fungi *Aspergillus*, chemical characterization of the Neem leaf extracts were studied in vitro on the culture medium. The growth of both the fungal species was inhibited significantly. The concentrations ranging from 150mg, 100mg and 50mg showed the radial mycelia growth of 70%, 66% and 57%.

The growth of *Fusarium* on the Neem leaf extract was observed. Pavela *et al.,* reported longevity of the cabbage aphids decreased with increasing *Azadirachta* in concentration. Joseph *et al.,* determined *Fusarium* wilt is an important disease of Brinjal crop causing significant reduction in yield. Kamesh *et al.,* studied the pathogen *Sclerotinia sclerotiorum* infects cabbage leading to a diseased condition called head rot. A significant 80% increase in the radial growth was observed at 200mg concentration, 150mg showed 61%, 100mg showed 57% and least inhibition was at 50mg i.e. 28%. Similarly the inhibition concerned with the Eichhornia leaf extract was performed. The *Aspergillus* Strains showed significant results. Jayanthi *et al.,* worked on *Eichhornia crassipes (Mart.) Solms*, showed the various antimicrobial effects of *Mucor* and *Aspergillus* strains. Bawai *et al.,* reported *Fusarium* wilt, caused by *Fusarium oxysporum* which is characterized by wilted plants, yellowed leaves and minimal/reduced or even total loss/absent crop yield. This review highlights the various documented methods employed in the management of the disease, which is highly destructive in both greenhouse and field grown tomatoes. Pawar *et al.,* analysed the anti-*Fusarium oxysporum f. spcicer* (FOC) and anti-*Alternaria porri* (*A. porri*) effects were evaluated for 75 different essential oils. The most active essential oils found were those of lemongrass, clove, cinnamon bark, cinnamon leaf, cassia, fennel, basil and evening primrose. At the highest concentration 200mg, an increase in radial growth for about 90% was recorded and followed by 150mg, 100mg and 50mg resulted 80%, 70% and 55%. Whereas for *Fusarium* strain, 90% inhibition was recorded at 200mg concentration and followed by 150mg, 100mg and 50mg resulted 85%, 68% and 57% respectively. No Zone of Inhibition was observed in case of *Candida* and *Mucor* Strains against Neem and Eichhornia leaf extracts. It is not surprising because Neem and

Eichhornia have been used since earliest times as an herbal medicine. Hence the fungal strains are said to be resistant and tolerant to the Biopesticides.

Table 1: Antifungal Activity (Zone of Inhibition-ZOI) and Activity Index (AI) Of the Neem extract

Sl. No	Conc.(mg)	ZOI and AI	*Aspergillus*	*Fusarium*	*Candida*	*Mucor*
01	50	ZOI	1.2	0.6	-	-
		AI	0.57	0.28		
02	100	ZOI	1.4	1.2	-	-
		AI	0.66	0.57		
03	150	ZOI	1.5	1.3	-	-
		AI	0.714	0.61		
04	200	ZOI	1.8	1.7	-	-
		AI	0.857	0.80		

The Activity Index of the test substance above 0.5 is considered as Significant Activity (Eliopoulos *et al.*, 2002)

Table 2: Antifungal Activity (Zone of Inhibition-ZOI) and Activity Index (AI) of the Eichhornia extract

Sl. No	Conc.(mg)	ZOI and AI	*Aspergillus*	*Fusarium*	*Candida*	*Mucor*
01	50	ZOI	1.1	1.2	-	-
		AI	0.55	0.57		
02	100	ZOI	1.4	1.44	-	-
		AI	0.7	0.68		
03	150	ZOI	1.6	1.8	-	-
		AI	0.8	0.85		
04	200	ZOI	1.8	1.9	-	-
		AI	0.9	0.90		

The Activity Index of the test substance above 0.5 is considered as Significant Activity (Eliopoulos *et al.*, 2002)

Table 3: Effect of various concentrations of Neem and Eichhornia Extracts

Sl. No	Fungal Strain	Conc.(mg)	Inhibition Percentage (%)	
			Neem	Eichhornia
01	*Aspergillus*	50	57	55
		100	66	70
		150	71	80
		200	85	90
02	*Fusarium*	50	28	57
		100	57	68
		150	61	85
		200	80	90
03	*Candida*	50	-	-
		100		
		150		
		200		
04	*Mucor*	50	-	-
		100		
		150		
		200		

Effect of Zone of Inhibition for Neem Biopesticide

Effect of Zone of Inhibition for Neem Biopesticide

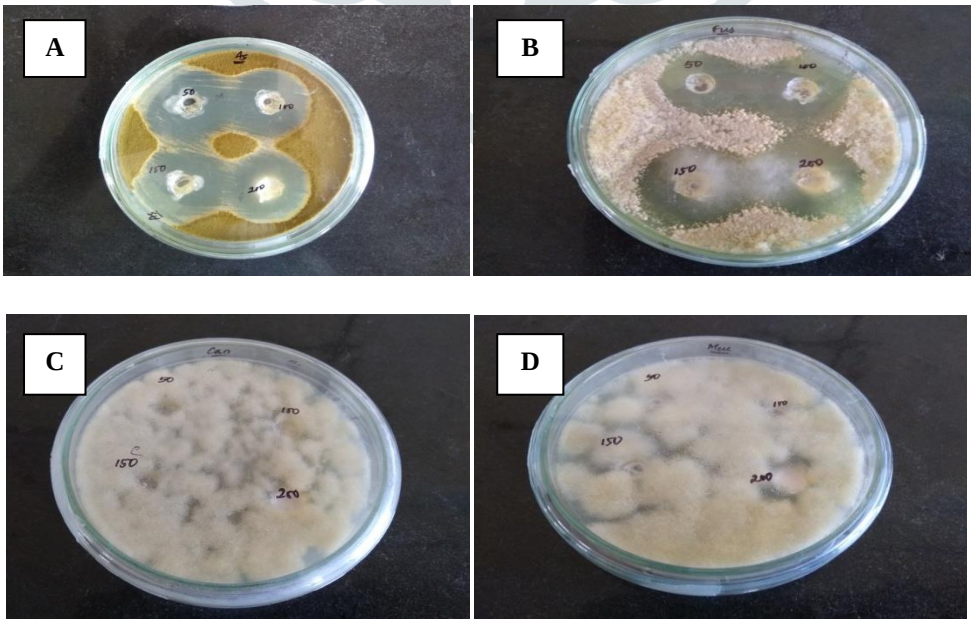

Figure 1. A - *Aspergillus*, **B -** *Fusarium*
C – *Candida*, **D -** *Mucor*

Effect of Zone of Inhibition for Eichhornia Biopesticide

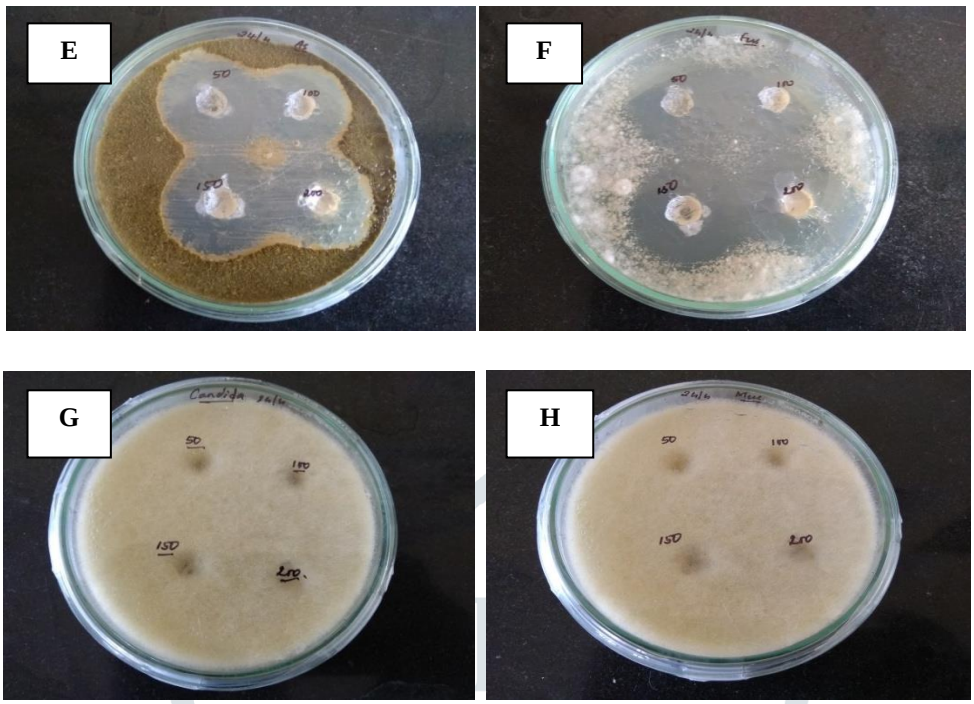

Figure 2. E - *Aspergillus*, **F -** *Fusarium*
G – *Candida*, **H –** *Mucor*

IV. CONCLUSION

Based on the laboratory studies carried out with regard to the antifungal properties of leaf extracts of Eichhornia and Neem on fungal pathogens of Cabbage, has yielded encouraging results in determining the activity index. The methanolic plant extracts exhibits phenomenal activity against the test organisms. Further field trials of these extracts would lead to strong antifungal activity compared to synthetic fungicides is recommended.

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