Journal Of Research Technology & Engineering
www.jrte.org

Identifying New Pathogen for Black Rot Disease of Tea and Incorporated *Wedelia trilobata* Extraction and Captan for Controlling Black Rot Disease.

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Received:27 Dec 2023; Revised: 28 Dec 2023; Accepted: 31 Dec 2023; Available online: 10 Jan 2024

Abstract: The black rot disease (causal fungus; *Cortcium theae* and *Cortcium invisium*) of tea (*Camellia sinensis)* is one of the foliar diseases in Sri Lanka. It is a serious concern for the tea industry due to the reduced tea yield and quality. Recently, synthetic fungicides have been used to control black rot disease, but they are more toxic substances and pollute the environment. Therefore, the present study was performed to identify the effective integrated method by using botanical extract of *Wedelia trilobata* (common name: Tharuka and Arunadevi) and Captan to control disease while reducing chemical fungicide requirement. The Black Rot pathogen was isolated on Potato Dextrose Agar from symptomatic mature tea leaves and identified the pathogenicity of the isolated pathogen using a detach leaf assay. Molecular analysis confirmed that the isolated pathogen as *Daldinia eschscholtzii* though causal pathogens are *Cortcium theae* and *Cortcium invisium.* Phytochemicals from the stems, leaves, and flowers of *Wedelia trilobata* were extracted with ethyl acetate and Soxhlet extraction. *In vitro,* antifungal assays were conducted using the poison food technique with treatment of 4000 ppm, 12000 ppm, and 24000 ppm of each of the stem, leaves, and flower extracts; 200 ppm, 600 ppm, and 1200 ppm of Captan. The Dunnet test showed that 4000 ppm, 12000 ppm, and 24000 ppm of stem and flower extract, and 24000 ppm of leaves extracts, and 200 ppm, 600 ppm, and 1200 ppm of Captan were significantly different. Stem extract showed minimum growth of the mycelium of the pathogen. At low concentration, 4000 ppm of stem extract and 200 ppm of Captan were incorporated with three different volume ratios, namely; 1:1, 1:2, and 2:1, 4000 ppm of stem and 200 ppm of Captan, respectively, and an antifungal assay was performed for each of the treatments. According to that, an incorporated 2:1 ratio of stem and Captan could mitigate 100 % mycelium growth of a pathogen.

Index Terms— Black rot, *Camellia sinensis*, Poison food method, *Wedelia trilobata, Daldinia eschscholtzii*

1 INTRODUCTION

Legends from China and India indicate that use of tea occurred as far back as 2,737 years BC when Shen Nung, “Divine Healer,” was the Chinese Emperor who found himself with a beverage harboring a pleasant aroma and refreshing taste after dried leaves accidentally blew into hot boiling water. In the first century of BCE, Sichuan Province of China began tea cultivation and after introducing to the Japan and other Asian countries. Presently, more than 60 nations around the world cultivate tea. Five million hectare of land area is covered tea plantation [1].

From this ancient practice, today also Tea (*Camellia sinensis*) is the one of the most common beverages in all over the world. Tea is used to reduce inflammation, improve blood flow, treat infectious diseases, purify the body, and maintain mental equilibrium [1].

Being a plantation crops, significantly different pests and diseases are damaged tea. In tea plants, disease is very problematic and can cause death of the bush. Algae, fungi, bacteria. Parasite and virus are caused healthy of plants.

Black Rot tea disease is another foliar disease in Asia, Africa and South America. *Corticium theae* and *Corticium invisum* are the causal pathogen of black rot disease [2]. Similar damage is given by both fungi who attack all tea plants from their seeding stage to upward. In early stage, reddish- brown damage similar to sun-scorch damage can be seen on the upper surface of the tea leaves and evenly brown or gray patches can be seen on the lower surface on the tea leaves. Later, mixture of brown, yellowish-brown and grey lesions can be seen on upper surface of the leaves and light brown or greyish-white color appear on the lower surface. Usually lower surface covered with a network or cream to brown mycelium [3]. Both fungi produce basidiospores in fructifications on the lower surfaces of leaves. These spores are transferred into the uninfected portions of the leaves through wind or water or tea pluckers. Directly, black rot is responsible for reduction of tea yield which can be seen as up to 50% on a bush attack by black rot when left untreated for four seasons consequently [2]. Both fungi attack the maintenance leaves and cause gradual deterioration in the health of the tea bush. Quality of the tea leaves and yield of tea is reduced [3]. Nagassa Dechassa et al. showed that, the causal pathogen of black rot is *Corticum koleroga* according to the macroscopic and microscopic characters of the culture [4].

Foliar fungal pathogens are attacked in tea plantation therefore, tea cultivation require the use of large number of chemical fungicides. Most frequently, synthetic fungicides are used to control both disease but those are mostly toxic and pollute the atmosphere by spreading out in the air and accumulating in the soil. Due to that, effective organic controller or bio agent or integrated method need for inhibiting growth of fungal pathogen to control black disease while reducing chemical fungicide requirement.

Botanical extracts are alternatively use as fungicide which have not side effects on environment [5]. So that, the present study was performed to identify integrated method to control mentioned fungal disease. Therefore, botanical plant extracts of the leaves, stem and flowers of the *Wedelia trilobata* incorporated with Synthetic, low toxic fungicide namely Captan to determine antifungal activity against the fungal pathogen of black rot disease.

2 PROCEDURE

2.1 Phytochemicals Extracts Preparation

Collection and Preparation of Plant Material

Flower, stem and leaves of the *W. trilobata* were collected from Kuruwita area in Ratnapura District. Flower, stem and leaves of the *W. trilobata* were separated and washed in tap water. Plant parts were shade dried separately for one week. Dried parts were ground into fine particles separately. Dried parts were put into polythene bag and sealed until needing [6].

Fig 1: Prepared plant material of leaves, flower and stem of W. trilobata

Preparation of Plant Extracts

Preparation of plant extract was prepared according to the process describe by Langhi et al and Wang et al. [7]–[9]. Fifteen grams of each plant parts was weighted and extracted using 350 mL of ethyl acetate in Soxhlet apparatus until changing colorless of the solvent in chamber. After extraction, crude was obtained evaporating excess solvent using rotary evaporator at 40 ℃ and 60 rpm. The obtained extracts were transferred into McCarthy glass bottles and covered with aluminum foil. Covered bottle was placed in refrigerator at 4 ℃ until need for analysis.

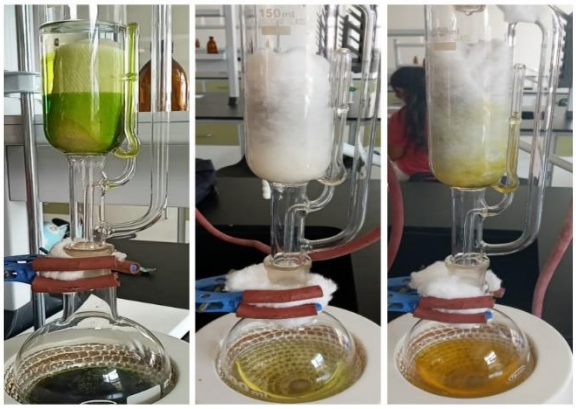

Fig 2: Soxhlet Extraction of Leaves, Stem and flower of *W. trilobata*

2.2 Isolation of Pathogen

Collection of Infected Leaves

Infected tea leaves were collected from Badulla tea state. Symptoms of Black rot disease of tea identified following Rana et al [3].

Isolation of Pathogen

Possible causative fungal pathogen of black rot was isolated from the Potato Dextrose Agar (PDA) according to the procedure described by Sarvya et al. [10].

Pathogenicity Testing

Detach leaf inoculation technique was performed to identify cultured causal pathogen among the isolated fungal plates according to the process of Sanjay Rana [3] (Fig. 3 and Fig. 4).

Fig 3: A: Shows the number of leaves of black rot spread out of ten leaves after Koch's postulate, B: shows the front side of the infected black rot disease, C: shows back side of the infected leave of black rot

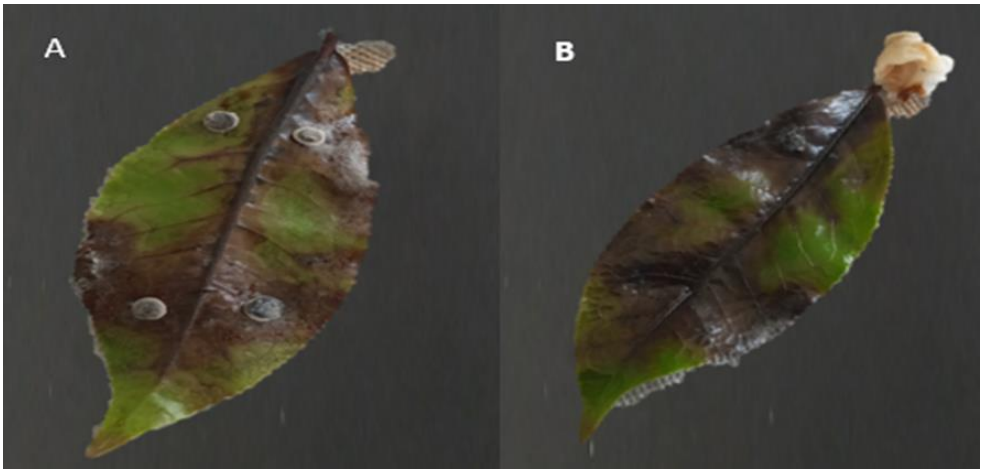

Fig 4: Shows the results after the 10 days after Koch's postulate (Second trial), A: shows the front side of the infected black rot disease, B: shows back side of the infected leaves of black rot.

Morphology Evaluation

Colony color, form, elevation and margin of macroscopic characteristics of the both fungal isolation was observed under naked eye [11]. Shape of the spores, diameter of the spores, hyphae and mycelium were

examined by microscopic evaluation preparing slides [12].

Molecular Analysis for identifying pathogen

Black rot fungal culture plate was sent to the Genetech Molecular Diagnostic and School of Gene Technology to identify fungi up to species level.

2.3 Antifungal Activity Testing

Preparation of Concentration Serious of Phytochemical Extracts and Captan

Concentration series of 4000 ppm, 12000 ppm and 24000 ppm were prepared each of the leaves, stem and flower extracts by dissolving ethyl acetate [13].

Weight of 4 mg, 12 mg, and 24 mg of each parts of the extract were dissolved in 1 mL of ethyl acetate to prepare 4000 ppm, 12000 ppm and 24000 ppm respectively.

Concentration serious of 200 ppm, 600 ppm and 1200 ppm of Captan were prepared from 2000 ppm of stock solution of Captan which is prepared dissolving 2 mg with 1 mL of ethyl acetate.

Antifungal Assay for Plant Extracts and Captan Separately

Antifungal activity of phytochemical extract was tested for pathogen in poison food technique according to process which described by S. Satish et al. [13]. Volume of 20 mL of PDA was pour into petri dish and 500 µL of each concentration of each parts extracts solution of each volume separately was amended before solidifying of PDA medium. Then it was spread using spreader. After solidifying the medium, 6 mm of mycelial plug of isolated pathogen was placed using cork borer separately each concentrated different parts extract. Cultured plates were put into polythene cover and placed in incubator at 28 ℃. Diameter of the mycelial growth of black rot pathogen was determined after five days. Six replicate of anti-fungal assay were performed for black rot pathogen.

Antifungal Assay for Integrated Plant Extract and Captan

Concertation level of 4000 ppm of stem and 200 ppm of Captan were incorporated with three different volume ratios for assaying black rot pathogen. Volume ratios of 1:1, 1:2, and 2:1 were incorporated for identifying antifungal assay.

2.4 Phytochemicals Identification

S. A. D. T. L. Wijesundara et al. and Gusthinnadura Oshadie De Silva et al. have used qualitative phytochemical analysis method [14], [15]. According to that process, phytochemicals were identified. Weight of 100 mg of leaves, stem, and flower extract of *W. trilobata* was dissolved in 10 mL of ethyl acetate in test tubes. Then, chemical tests were carried out.

2.5 Data Analysis

Data was analyzed using Minitab 21 statistical software. The experiment was conducted Complete Randomized Design, with Dunnett multiple comparison test and Tukey pairwise comparison was performed with 5% significance level.

3 RESULTS AND DISCUSSION

3.1 Collection of Plant Material and Phytochemical Extraction

The plant type and plant parts taken to the test were decided with available literature sources which interpret their antimicrobial activity and medicinal use of the *W. trilobata*. Plant material were shade dried to prevent the possible changes in composition of the extract as a result of moisture content. Plant parts are ground into tiny particles to increase effective extraction by increasing surface area of the plant material to contact with solvent.

Soxhlet extraction was performed for getting the highest amount of extraction from the material than the other conventional extraction method. According to the literature, the ethyl acetate solvent which used for the Soxhlet extraction to obtained both polar and non-polar secondary metabolites from the material.

Soxhlet extraction was run 20 hours until colorless the leaves extraction. A phytochemicals which obtained by leaves are dark green color, semi solid extract. Dark yellow color, oily like extract was obtained by stem of the *W. trilobata* after 15 hours Soxhlet extraction. Sixteen hours were run the flower sample to obtain dark yellow color, semi solid extract. Extracts were stored in refrigerator condition because prevention of degradation of phytochemicals (*Rajeswara Rao, B.R.. (2015))* and growth of microbial growth and shelf life can be extended (5 to 10 years) (*Rajeswara Rao, B.R.. (2015)*). McCarthy botte covered with aluminum foil because avoid oxidation from light [17]. Highest yield percentage obtained from leaves of *W. trilobata* which showed as an 11.72%. Stem extract showed a minimum yield percentage (5.8%) (Table 01).

Table 1: Percentage of extraction yield obtained from each plant parts a W. trilobata

Plant Parts of *W. trilobata*	Weight of air-dried plant parts (g)	Weight of dried crude extract (g)	Percentages extraction yield (%)
Leaves	15.012	1.760	11.72
Flower	15.031	1.064	7.08
Stem	15.000	0.870	5.80

3.2 Isolation of Pathogen and Pathogenicity Testing

Five pure cultures of black rot isolates were obtained, after isolating and sub culturing of fungal pathogen. Colony appearance of the black rot cultures was difference in each other. One culture plate of causal

pathogen of black rot disease could be identified after the pathogenicity testing through the detached leaf assay. Leaves were taken and wound for the detach leaf assay because most of the fungal disease spread rapidly through the wound [18]. Two out of ten leaves had spread the black rot disease after 7 days. In second trial, one leaf showed black rot disease symptoms on lower surface detach leaf assay after 7 days (Figure 03 and 04).

3.3 Morphology Evaluation of Identifying Pathogen

Black rot pathogen showed white color mycelium on upper surface, white to brown color mycelium on lower surface, brown color septate hyphae, and brown color simple conidiophore. Colonies were circular in shape and entire margin (Fig. 05).

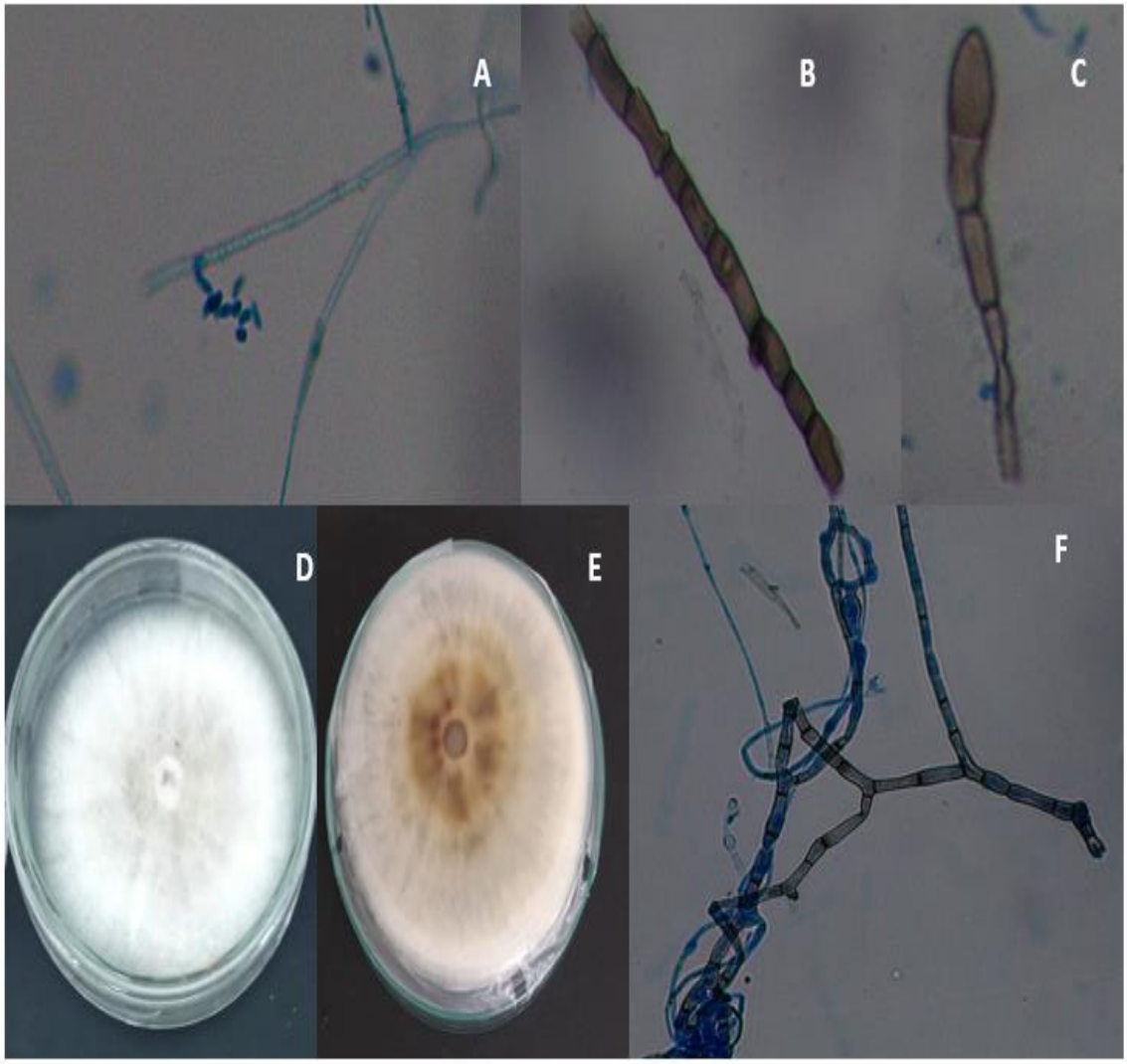

Fig 5: A: Spores, B: Septate hyphae, C: Conidiophore, E: Upper surface of isolated black rot pathogen after 7 days, F: Lower surface of isolated black rot pathogen after 7 days, F: mycelium structure of isolated black rot pathogen in 200 µm scale.

3.4 Molecular Identification

According to the molecular identification results of black rot pathogen, Genetech Molecular Diagnostic and School of Gene Technology proved isolated black rot pathogen as *Daldinia eschscholtzii* strain A0630. It is an entophytic fungus. Koch's postulated proved that, isolated pathogen could spread the black rot disease on the leaf. Therefore, isolated endophyte may be spread black rot disease. Endophyte has an ability to infect disease which is proved that Tersoo P Terna et al. [19].

3.5 Antifungal Assay and Data analysis of parts of the extract and Captan

The fungi-toxicity of the extracts in terms of percentage of mycelial growth was calculated using formula [13] (Figure 06 and 07).

$$Percentage\ of\ inhibition = \frac{dc-dt}{dc} \times 100 \quad (1)$$

dc = Average mycelial growth diameter in control (cm)
dt = Average mycelial growth diameter in treatment (cm)

Each concentration of each part extract and each concentration of Captan showed as a good fungicide for controlling black rot disease. Mycelial inhibition percentage can be shown in Table 02.

Table 2: Mycelial inhibition percentage of each concentration plant extract and Captan

Plant parts/ Fungicide	(Percentage of mycelial inhibition according with Concentration (%))		
	4000 ppm	12000 ppm	24000 ppm
Stem	58.26	66.07	71.43
Leaves	7.37	20.76	38.20
Flower	36.61	41.52	48.77
Fungicide	200 ppm	600 ppm	12000 ppm
	62.20	75	83.04

According to the lowest concentration, stem extract showed highest percentage of mycelial of inhibition of black rot pathogen. Lowest concentration of Captan showed highest ability of inhibiting mycelial growth than the lowest extract of stem.

Mycelial growth data of each plant extract and Captan tested with One-way Anova test to finding most suitable concentration of plant parts and Captan for incorporating. According to that result can be interpreted as this way. Mycelial growth diameter of each concentration of stem extract were compared Dunnet Multiple Comparisons with a control under 5 % significant level. It showed that, 4000 ppm, 12000 ppm and 24000 ppm of concentrations are significantly different with the controller. All the concentration of flower extract significantly different with the controller. Concentration of the 24000 ppm of leaves was significantly different with controller, but 4000 ppm and 12000 ppm of leaves concentrations were not

different with controller under 5 % significant level. Tukey Pairwise Comparisons method was performed to determine the difference between growths data each of the concentration level of each parts extract. Means of the growth data of each concentration of flower and stem were significantly different under the 5 % significant level. According to that result, leaves extract can be negotiated to prepare incorporated fungicide because, 24000 ppm is high concentration of this experiment which require high amount of extract so that only consider to incorporate minimum concentration of the extract. Therefore, 4000 ppm of growth data stem and flower were compared using Turkey Pairwise Comparison under 5 % significant level. According to that, both growth diameter mean of the plant extract are significantly different. Interaction was not happened with extraction part and concentration levels. But growth diameter mean of mycelium against the stem extract was minimum than the flower extract. So that, stem extract was selected to incorporate with Captan.

Mycelial growth data of 200 ppm, 600 ppm and 1200 ppm of Captan were analyzed Dunnet multiple comparison with a Control. In that, all the treatments were significantly different with control treatment under 5 % significant level. Treatment means were different in each other according to the Turkey Pairwise Comparisons under 5 % significant level. Therefore, 200 ppm concentration was chosen to incorporation because considering of lower concentration.

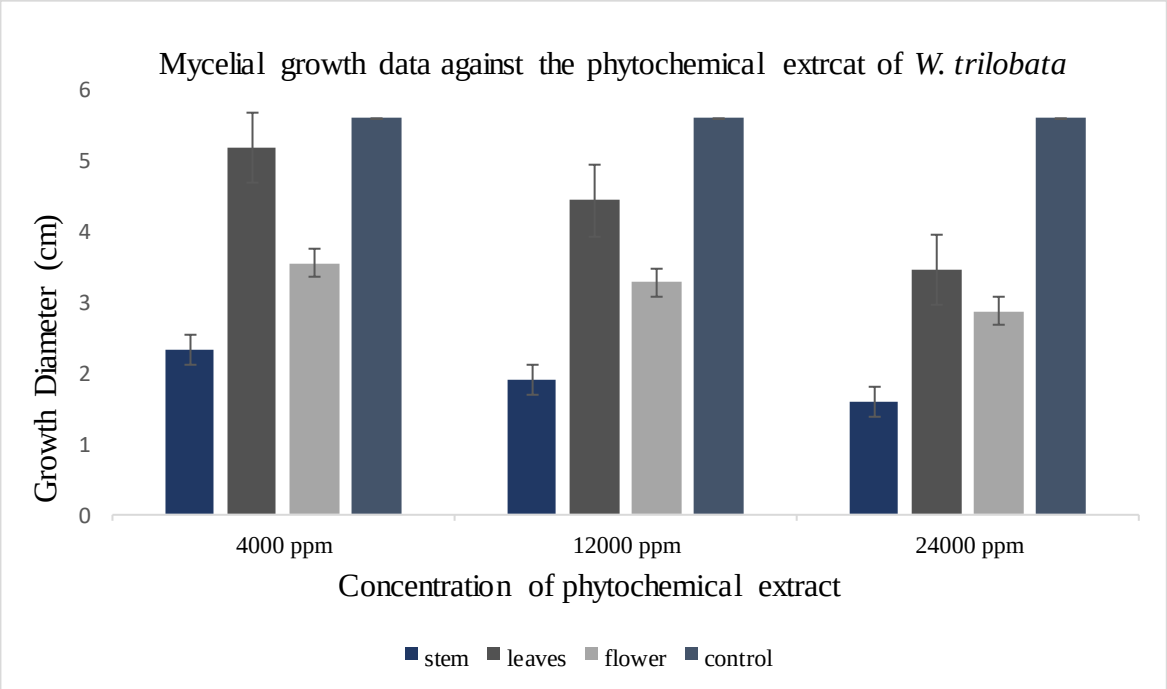

Fig 6: Growth diameter Vs concentration of extract and each concentration levels have been compared with average growth of controller.

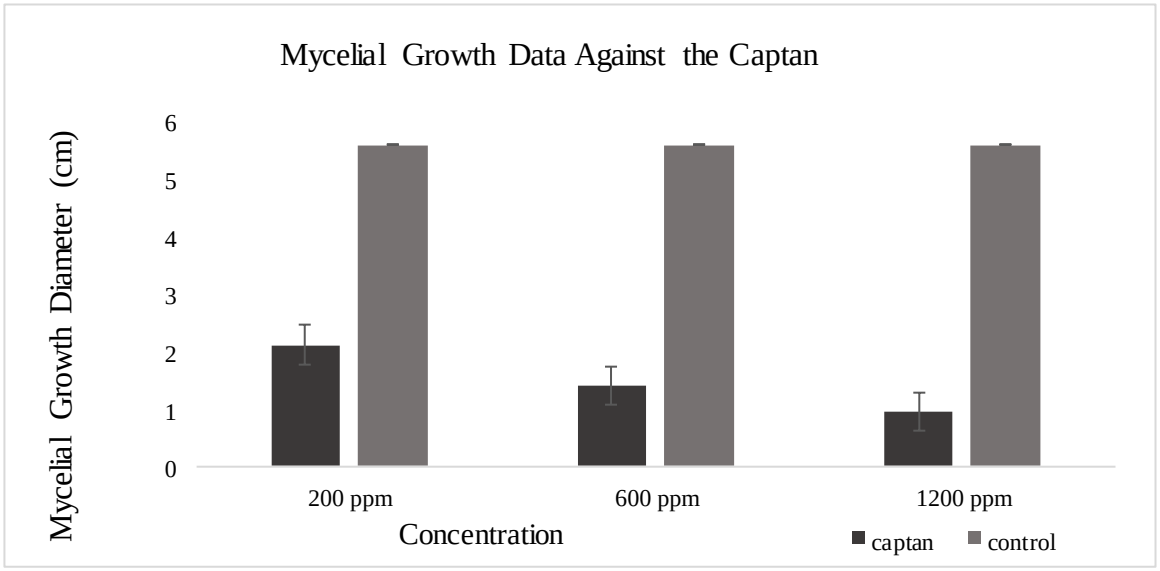

Fig 7: Growth diameter Vs concentration of Captan concentration levels have been compared with average growth of controller

3.6 Antifungal Assay and Data analysis of incorporated stem extract and Captan

Incorporated volume of 1:1, 1:2 and 2:1 of Stem (S) extract and Captan (C) were given excellent ability to mitigate growth of pathogen. S1: C2 ratio showed no growth of the mycelium of pathogen. Another both ratios showed same growth of mycelium of isolated pathogen (Fig. 08 and 09).

Dunnet Multiple Comparison test was performed with each treatment with growth diameter of 4000 ppm stem and 200 ppm of Captan as control separately under 5 % significant level. According to that result, all the treatment are significantly different from both of the control. All the treatment were significantly different from each other, it was interpreted performing Tukey Pairwise Comparison under 5 % significant level. But all treatment is in the same group, so that any of the volume ratio can be chose. So that, S1: C1 can be selected because considering minimum amount of Stem extract and Captan.

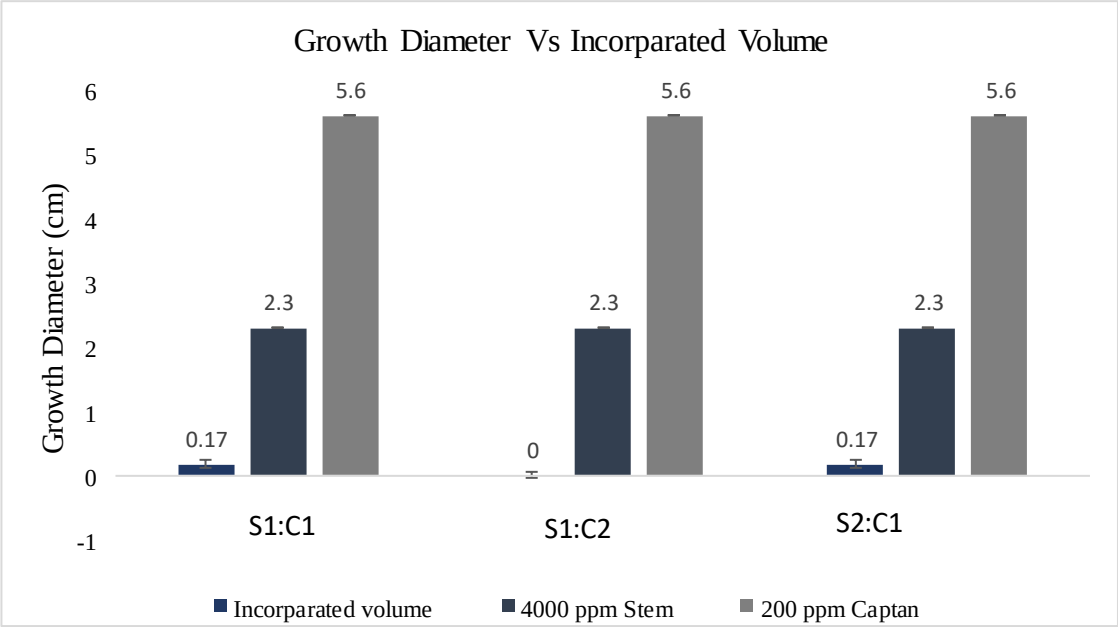

Fig 8: Growth diameter of the different volume ratio, comparing with initial growth of stem and Captan

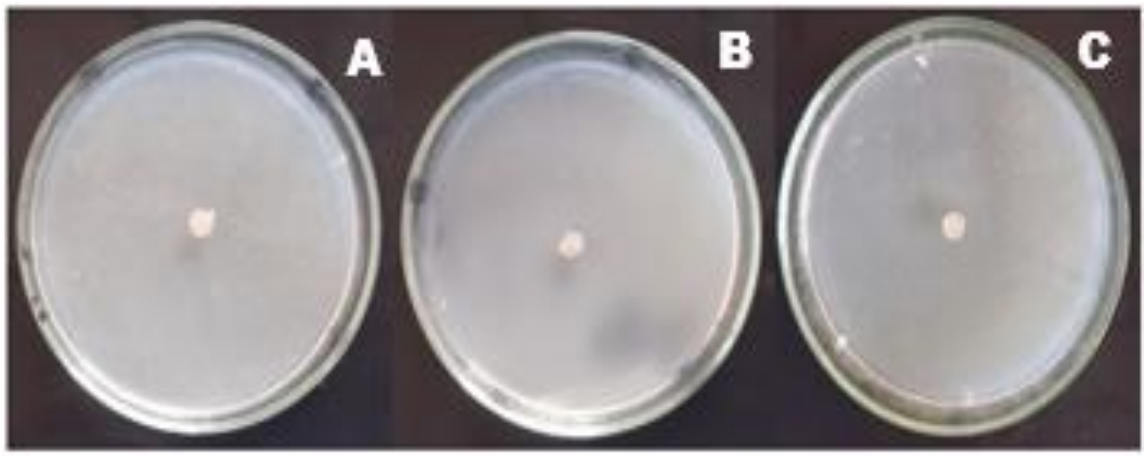

Fig 9: Incorporated Stem(S) and Captan(C) antifungal assay for black rot pathogen; A: S1:C1 ratio, B: S2:C1 ratio, C: S1:C2 ratio

3.7 Phytochemical Identification

Mayer's test for testing alkaloid

Stem, Leaves, and Flowers shown white creamy precipitate bottom of the test tube. It was interpreted presence of alkaloid.

Phenol testing

Leaves extract formed bluish black color which prove the presence of phenol. Stem and flower extract formed yellow color solution which prove that, not consist of phenol.

Terpenoid testing

Reddish brown coloration of the interface indicate presence of terpenoid. Leaves extract shown that coloration which interpreted, presence of terpenoid, yellow coloration of the interface was shown by the stem and flower extract.

Quinones testing

Stem and flower extract shown formation of yellow coloration with Quinones test which indicate having of Quinones. Leaves shown a dark greenish coloration.

Saponin

All the plant extract formed persistent foam that confirm the presence of saponin in the extract.

Lead acetate test for testing flavonoids

Formation of yellow color solution indicate the presence of flavonoid. Both parts of the extract showed yellow color solution.

According to the qualitative phytochemical identification techniques, alkaloid, flavonoid and saponin have all of the parts of the *W. trilobata*. Phenols and terpenoid have only in the leaves. According to the Sutapa et al. phenolic acid is responsible for inhibiting mycelium growth of *C. acutatum* [20]. Therefore, leaves extract has the ability to migrate growth of the pathogen of brown blight than the other parts of the *W. trilobata*. Alkaloid, flavonoid, quiononones, and saponin were presence in stem and flower extract of W. trilobata. Both stem and flower extract were not presence terpenoid and phenols (Table 03).

Table 3: Qualitative analysis of ethyl acetate extracts of stem, leaves and flower of W. trilobata

Tested phytochemicals	Extracts		
	Stem	Leaves	Flower
Alkaloid	+	+	+
Flavonoid	+	+	+
Phenols	-	+	-
Terpenoids	-	+	-
Quinones	+	-	+
Saponin	+	+	+

+ Equal to the positive & - equal to the negative

4 CONCLUSION

Molecular identification of black rot pathogen proved as an endophyte namely *Daldinia eschscholtzii* strain A0630. Daldinia eschscholtzii has an ability to spread black rot disease as Corticium theae and Corticium invisum.

Ethyl acetate extract of stem, flower and leaves of Wedelia trilobata and Captan had antimicrobial activity against the Daldinia eschscholtzii. Stem extract was the effective extract for inhibiting growth of black rot pathogen. Volume 1: 2 respectively Stem and Captan ratio could mitigate 100 % growth of black rot disease as an integrated method

5 ACKNOWLEDGEMENT

The authors acknowledge to Uva Wellassa University of Sri Lanka and Genetech Molecular Diagnostic and School of Gene Technology, Sri Lanka.

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