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Identification of Causative Pathogen of Flower Bud Wilt Disease in *Dendrobium* sp. and *In-vitro* Growth Inhibition by Medicinal Plant Extracts

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Author's contribution

The sole author designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study was to isolate and identify the causal agent of wilting of flower buds of *Dendrobium* orchid in Sri Lanka and to search for environmental friendly control measures for flower bud wilt disease as a possible alternative to synthetic fungicides.
Study Design: The experiment was conducted using a completely randomized design.
Place and Duration of Study: Faculty of Agricultural Sciences, Sabaragamuwa University of Sri Lanka from June 2013 to May 2014.
Methodology: The causal fungus isolated from the diseases plant was identified based on their morphology, pathogenicity and ribosomal DNA spacer sequences. The media amended with methanol plant extracts of different concentrations (25%, 12.5%, 6.25%, 3.125%) of tested medicinal plants were inoculated with mycelia discs (4 mm diameter) taken from the advancing edges of 10 day-old pure cultures ofisolated fungus andincubated for 7 days. The fungicidal activities of MIC of methanol extracts were studied after immersing fungal blocks in solution for 1, 3, 6, 12 and 24 hours respectively. The media amended with methanol and recommended

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fungicide were considered as negative and positive control respectively.
Results: Based on morphological characteristics and rDNA spacer sequences, isolates were identified as *F. proliferatum.* This is the first record of flower bud wilt disease in *Dendrobium* sp. in Sri Lanka. Results showed that radial growth of fungus was significantly impaired ($P \leq 0.05$.) by the addition of the extracts in the culture medium used. The test fungus differed in their reaction to the different extracts but on the whole, growth inhibition increased with the concentration of each extract. The most active extracts, 25% methanol extracts from sweet flag shows a marked effect with inhibition values of 77% against *F. proliferatum* whereas those from wild basil inhibited the growth by 55%. Out of four plants extract screened, sweet flag showed more than 80% fungal inhibition after 12 hour immersion and other extracts could not exceed 60% inhibition after any exposure time.
Conclusion: The present study concludes disease found in *Dendrobium* orchid was caused by *F. proliferatum and* Methanol extracts of Sweet flag contain antifungal constituents for the control of *F. proliferatum.*

Keywords: Flower bud wilting; Dendrobium; Fusarium proliferatum; antifungal; plant extracts.

1. INTRODUCTION

Orchids, important ornamental plants, are valued worldwide for their attractive flowers [1]. Plants are either grown for cut flower or as ornamental potted plants [2]. Orchids are the most popular tropical cut flowers which are being grown commercially for exports as well as for the local market in Sri Lanka [3]. Floriculture industry in SL is relatively new compared to other agriculture commodities. Since early 2000 cut flowers have been identified as one of priority groups of commercial crops, as local and foreign demand of cut flowers have increased [3].

Since early 2013, previously unknown disease is adversely effects on orchid cultivation in Sri Lanka. Severe wilting of flower buds of *Dendrobium* orchids were observed in Royal botanic garden and some commercial cultivation of Sri Lanka. Infection is observed in young flower buds. At the beginning flower buds shows water stress condition and then it progress in to permanent wilting and ultimately infected buds shrivel and become dark brown in colour. This disease has huge impact on *Dendrobium* orchid production by decreasing marketable flowers. However there is no previous record on this disease of orchids in Sri Lanka. As a results no environmental friendly control measures has been established and growers are totally depend on heavy use of fungicides.

Although Flower imports are not inspecting for pesticide residues in cut flower or potted plants. Worker exposure is of particular concern in greenhouses, where different chemicals are used in enclosed spaces increasing risk of exposure through the skin and by inhalation [4]. The great public awareness of Environmental and health issues has stimulate an increasing demand to reduced pesticide use and safe agricultural products. Therefore, in recent years much attention has been given to non-chemical treatment to protect them against many plant pathogens.

Medicinal plants represent a rich source of antimicrobial agents [5,6]. Many of the plant materials used in traditional medicine are readily available in rural areas at relatively cheaper price. Medicinal plants extracts are promising as alternative or complementary control means because of their anti-microbial activity, nonphytotoxicity as well as biodegradability [7,8]. Our previous study [9] shows methanol extract of wild basil and sweet flag were highly effective against pathogenic fungi *Rhizoctonia solani, Colletotrichum musae* and *Fusarium oxysporum.* Thus, the aim of this study was to isolate and identify the causal agent of wilting of flower buds of *Dendrobium* orchid in Sri Lanka and to search for environmental friendly control measures for flower bud wilt disease as a possible alternative to synthetic fungicides.

2. MATERIAL AND METHODS

2.1 Symptoms, Isolation and Identification of Pathogen

Symptomatic flower buds were surface sterilized for 2–3 min in 0.5% NaOCl in distilled water, rinsed in 4 changes of sterile distilled water and air dried under aseptic conditions. Pieces

excised from wilted flower bud were transferred to potato dextrose agar (PDA). Cultures were incubated at 25℃ in the dark. 10 days later, single spore cultures were obtained from colonies emerging from the infected tissues and examined morphologically. The taxonomic criteria of [10,11] were followed to identify the isolate from wilted *Dendrobium* orchid.

The sequence of the internal transcribe spacer region of ribosomal DNA (rDNA ITS) were analyzed for confirm species of isolated fungal pathogen using ITS1 (5′-TCCGTAGGTGAACCTGCGG3′) and ITS4 (5′-CTCCGCTTATTGATATGC3′) primers [12]. Presence of single amplicon was confirmed and PCR products were purified using purification Kit and quantified. The nucleotide sequence was determined by Big Dye Terminator v3.1 cycle. The sequence was aligned with NCBI data base using BLAST to identify the isolated fungus.

2.2 Pathogenicity Test

Potted plants of *Dendrobium* orchids at flower bud initiation stage were sprayed with a conidial suspension (1x 10^6 conidia /ml) of the isolates, and then incubated in green house at 25℃-30℃ in humid condition for 14 days. Several health plants were sprayed with sterilized water only to serve as control.

2.3 Sample Collection and Extraction

Both Rhizome and Leaves of *Acorus calamus* (Sweet flag) and Leaves *Ocimum gratissimum* (wild basil), *Justicia adhatoda* (Adulsa) and *Vitex negundo (five-leaved chaste tree)* were collected from surrounding areas of Belihuloya, Sri Lanka. The dried plant materials were milled to a fine power using grinder and stored in the dark at room temperature (25±30℃) in airtight containers. The air dried plant materials were ground to a fine powder and 1 g of finely ground plant material was used for the extraction. Dried samples were defatted with hexane. Dried samples were defatted with hexane. 1g of defatted plant material was used for the extraction in 20 ml of methanol three times. The solution was kept overnight at room temperature (25±30℃) and filtered using filter papers (Whatman filter paper No. 1). Combined methanol extracts was vacuum dried and the resultant residue was used for further study.

2.4 Effect of Different Concentrations of Extracts on Radial Growth of test Organisms

Plant extracts were tested for their efficiency against the pathogen by using an agar dilution technique [13]. Different concentrations of the extracts; 25%, 12.5%, 6.25%, and 3.12% were obtained by amending PDA. The amended medium was dispensed into sterile Petri plates and allowed to solidify with streptomycin (100µg/ml). A 4-mm diameter mycelia disc of isolate from wilted *dendrobium* orchid was inoculated on each amended agar plate. Inoculated plates were incubated at 25±30℃ and growth measured along the perpendicular lines. Daily radial growth of each test organism in any of the test extracts was recorded for 7 days. Each treatment was replicated thrice with appropriate untreated controls. Then all the plates were incubated at 25±30℃ in dark condition. The mycelia growth of fungus was measured after 24, 48, 72 and 96 hours. The percent inhibition of the mycelia growth over control was calculated using the following formula [14].

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

C: the colony diameter of the mycelium on the control plate (mm)
T: the colony diameter of the mycelium on the treated plate (mm).

2.5 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was taken from the results of the fungistatic activity. The lowest bio extracts and chemical concentrations with highest inhibition percentage were taken as MIC.

2.6 Fungicidal Activity

Further study about fungicidal activity of each plant extract and Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile) were done for the minimum inhibitory concentration (MIC). Fungicidal activities of each treatment against *F. proliferatum* were done by immersing the fungal block in minimum inhibitory concentration of each solution separately for 1, 3, 6, 12 and 24 hours. PDA media plates were prepared and treated fungal blocks were inoculated aseptically in the center of the plate. The agar blocks were washed prior to inoculation on PDA plates to remove the extracts. Fungal blocks which were dipped in

methanol for the above time periods were used as control. Three replications were prepared for each treatment. All culture plates were incubated at room temperature in dark condition. The mycelia growth was measured by taking the colony diameter after 36, 72 and 96 hour. The comparison of the fungicidal activity of minimal inhibition concentrations of plant extracts and Chlorothalonil were done by calculating the present inhibition of the mycelia growth over control by using the formula [14].

2.7 Data Analysis

The experiment was conducted using a completely randomized design (CRD). Standard errors of means of three replicates were computed using computer software Microsoft Excel. ANOVA (analysis of variables) was used to analyze the recorded data. Statistical analyzing software (SAS) 9.0 was used to analyze the data. The mean separation was done by Duncan's multiple range test at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Symptoms, Isolation and Identification of Pathogen

At the beginning flower buds shows water stress condition (Fig. 1a) and then it progress in to permanent wilting (Fig. 1b) and ultimately infected buds shrivel and become dark brown in colour (Fig. 1c).

F. proliferatum colonies on PDA produced white mycelia initially and developed to purple-violet with age (Fig. 1d). Microconidia were produced from monophialides and polyphialides in chains in the aerial mycelium (Fig. 1e). All isolates of this species produced abundant microconidia in club shaped with a flattened base and no septate (Fig. 1f). Macroconidia were very long, slender, and straight with three to five septa (Fig. 1g). Apical cell was curved or tapered and chlamydospores were absent. In BLAST analysis, isolate had a 100% sequence identity with sequence of *F. proliferatum* (FJ040179.1 and EU151486.1). This sequence data have been deposited in GenBank NIH genetic sequence database under accession KM023784.

Based on these morphological characteristics and rDNA spacer sequences, isolates were identified as *F. proliferatum.* This is the first record of flower bud wilt disease in *dendrobium* orchid in Sri Lanka.

3.2 Pathogenicity

Symptoms were reproduced on all orchid plants inoculated with the isolates. Wilting and ultimate shriveling of flower bud were appeared after 10 days of inoculation. Wilted flower buds remarkably began one week after inoculation (Fig. 1h,i).Then the inoculated flower buds shriveled and dried off within 10 to 14 days after inoculation (Fig.1j). Control plants had no symptoms. The same fungus was consistently re-isolated from diseases plants, but not from health control, demonstrating that isolates were pathogenic to *Dendrobium* orchids.

3.3 Effect of Different Concentrations of Extracts on Radial Growth of test Organisms

Antifungal activity of four medicinal plants *Acorus calamus* (Sweet flag) *Cimum gratissimum* (wild basil), *Justicia adhatoda* (Adulsa) and *Vitex negundo (five-leaved chaste tree)* extracts was assayed and the effect of plant extracts on the growth of fungal strain *F. proliferatum* was observed. The data revealed that significant reduction in growth of *F. proliferatum*a gainst medicinal plants extract of Sweet flag. The plant extract of *Justicia adhatoda* (Adulsa) and *Vitex negundo (five-leaved chaste tree)* showed comparatively very low activity against *F. proliferatum* where as wild basil showed moderate level of growth inhibition Fig. 2.

All the plant extracts exhibited different degrees of antifungal activity against *F. proliferatum.* The growth of *F. proliferatum* was highly inhibited by all the tested concentrations (3.12% - 25%) of methanol extracts of sweet flag compared with control, the correspond inhibition ranging from 36% - 75% Fig 3. However, wild basil showed moderate level of antifungal activity (18%-55%) followed by Adulsa (21%-38%) and five-leaved chaste tree (12%-14%) (Fig. 3).

The fungicidal activities of selected plant methanol extracts against *F. proliferatum* are shown in Table 1. This test was done to study the ability of different plant crude extract to kill the vegetative forms of *F. proliferatum* after immersed in solutions for different time periods .Inhibition ratios were calculated after the incubation period and data was analyzed with ANOVA by using statistical analyzing software

(SAS). Significant differences of two variables were determined by Duncan's Multiple Range test at ($P \leq 0.05$). According to results, sweet flag was shown the significant inhibition (more than 80%) of the vegetative growth of the fungus after 12 hours immersion time (Table 1). In addition, the fungicidal activity was varied with exposure time. Although all the other plant extracts showed inhibition of the fungal growth compared to the control, after 24 hours of immersing, they didn't show any significant fungicidal effect on *F. proliferatum.*

Prior to the report from Sri Lanka *F. proliferatum* was reported as orchid pathogen in Hawaii [15], Malaysia [16], Australia [17], Japan [18] and was associated with diseases of orchid in both Germany [13] and Korea [19,20]. The morphology and culture characters of isolates from *dendrobium* orchid agreed with description of *F. proliferatum* [10,11]. However, according to several authorities, this species is very difficult to identify accurately based on morphological characteristics alone [10,11]. Therefore, PCR amplification with sequence of the internal transcribe spacer region of ribosomal DNA (rDNA ITS) were conducted to confirm the identity of *F. proliferatum* isolates. DNA sequence data of isolates obtained in this study coincided with previous data of several other *F. proliferatum isolates (*FJ040179.1 and EU151486.1) with 100% identity. According to present study the disease found in *dendrobium* orchid was caused by *F. proliferatum.* This is the first report of a *F. proliferatum*in *Dendrobium* orchids in Sri Lanka.

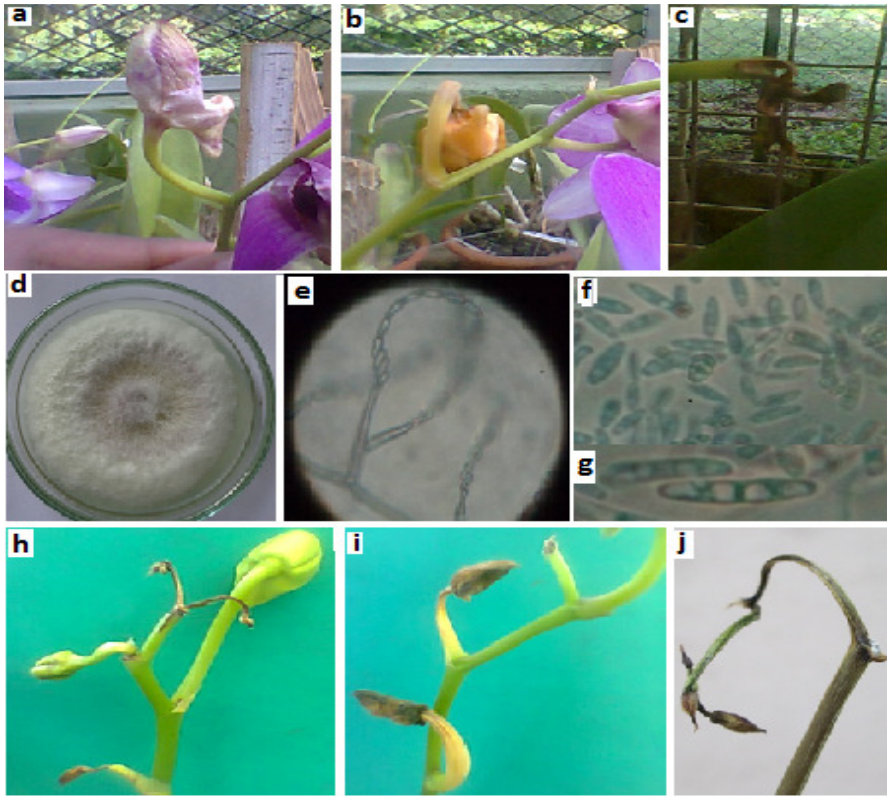

Fig. 1. Symptoms and pathogen morphology of flower bud wilt disease caused by *F. proliferatum* a-c Natural symptoms and signs. a. flower buds with water stress condition. b permanent wilting of flower buds c. shrivel and dark brown flower buds d-g morphology of the isolated pathogen cultured on PDA at 25±30℃ in the dark for 10 days. d. colony morphology e. microconidia produced in monophialides and polyphialides f. Microconidia g. Macroconidia h-j symptoms on flower buds after inoculation with isolated pathogen h-i. Wilting symptoms one week after inoculation j. shriveled and dried off flower bud 14 days after inoculation

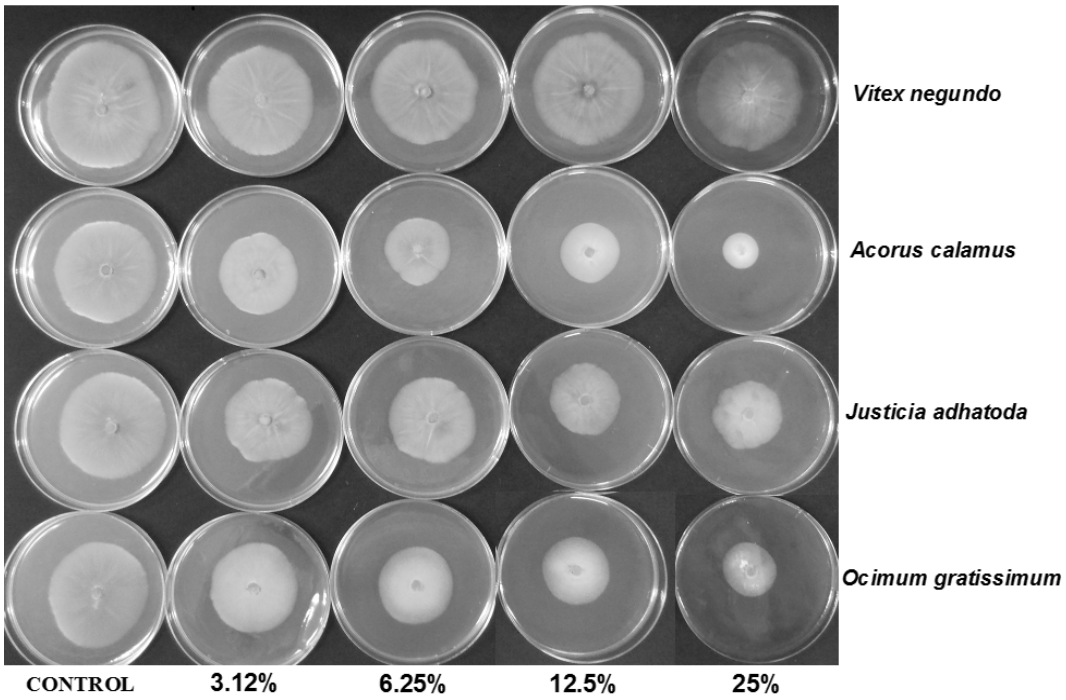

Fig. 2. Mycelium growth inhibition effect at different concentrations of medicinal plants extract *Acorus calamus* (Sweet flag), *Ocimum gratissimum* (wild basil), *Justicia adhatoda* (Adulsa) and *Vitex negundo (five-leaved chaste tree)*

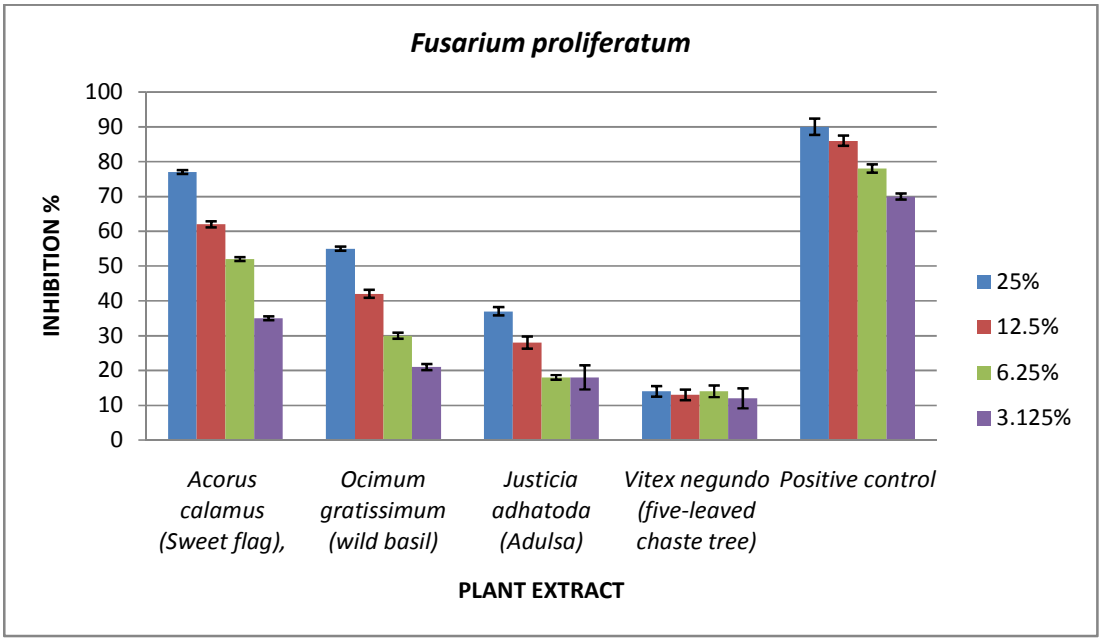

Fig. 3. Mycelium growth inhibition effect at various concentrations of medicinal plants extract *Acorus calamus* (Sweet flag), *Ocimum gratissimum* (wild basil), *Justicia adhatoda* (Adulsa) and *Vitex negundo (five-leaved chaste tree)* against *Fusarium proliferatum*

Table. 1. Fungicidal activity of various concentrations of medicinal plants extract, *Acorus calamus* (Sweet flag), *Ocimum gratissimum* (wild basil), *Justicia adhatoda* (Adulsa) and *Vitex negundo (five-leaved chaste tree)* against *Fusarium proliferatum*

Time hours	Inhibition of fungal growth %			
	Methanol extracts of			
	Acorus calamus **(Sweet flag)**	***Ocimum gratissimum*** **(wild basil)**	***Justici adhatoda*** **(Adulsa)**	***Vitex negundo*** **(five-leaved chaste tree)**
1	24^{x}	18^{x}	12^{x}	10^{x}
3	39^{x}	20^{x}	14^{x}	18^{x}
6	65^{y}	32^{x}	18^{x}	22^{x}
12	80^{Z}	48^{y}	28^{x}	30^{x}
24	92^{Z}	62^{y}	42^{y}	34^{x}

Numbers followed by the same letters in each row were not significantly different according to Duncan's Multiple Range test at ($P \leq 0.05$). Minimum inhibitory concentration of all plant extracts were considered as 25%

Biological control had attained importance in modern agriculture to diminish the hazards of intensive use of chemicals for pest and disease control [21]. Accordingly, the efficacy of different plant extracts *Acorus calamus* (Sweet flag), *Ocimum gratissimum* (wild basil), *Justicia adhatoda* (Adulsa) and *Vitex negundo (five-leaved chaste tree)* against *F. proliferatum* was studied *in vitro.* These all types of extracts showed different levels of antifungal activity and the relative differences were found to vary within the tested extracts and with increasing concentration of the extracts, a gradual increase in the inhibition potential of the *F. proliferatum* was recorded. Methanol extracts of Sweet flag were found highly effective in suppressing the growth of *F. proliferatum*. All the concentrations of the extracts significantly reduced the *in vitro* growth of the target fungal pathogen. There was 35-77% reduction in fungal growth due to different concentrations of the extracts. Comparing leaf and rhizome extracts of sweet flag with methanol, the inhibition percentage in each increased gradually with the extract concentration and completely suppressed the growth of *F. proliferatum* at 50%. Sweet flag has fungicidal properties due to the presence of beta-asarone in its tissues [22,23,24]. Our previous studies reported that Methanol extract of Sweet flag and wild basil was highly effective in controlling the growth of the plant pathogenic fungi *Rhizoctonia solani, Colletotrichum musae* and *Fusarium oxysporum* [9]. However, according to present study wild basil not shows significant growth reduction for *F. proliferatum*.

4. CONCLUSION

The present study concludes that Methanol extracts of Sweet flag contain antifungal constituents for the control of *F. proliferatum*. The finding of the present investigation could be an important step towards the possibilities of using natural plant products as bio pesticides in the control of plant diseases caused by *F. proliferatum*. Further green house and field experiments are suggested to investigate the *in vivo* effects of these extracts as compared to some commercial chemical fungicides for the management of flower bud wilt disease in *dendrobium* orchid.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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