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TLC Bioautography Guided Detection and Biological Activity of Antifungal Compounds from Medicinal Plant *Acorus calamus* Linn

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ABSTRACT

Plant based botanical fungicides are one of the most important alternative for hazardous fungicides. Antifungal activity (AFA), identification and understanding the mechanism of action of predominant antifungal compounds present in medicinal plant (*Acorus calamus* L) were carried out in this study. The antifungal activity and both n-butanol extract and purified antifungal compound of *A. calamus* (L) was evaluated against five fungal strains (*Fusarium oxysporum*, *Fusarium solani*, *Colletotrichum gloesporioides*, *Botrytis cinerea* and *Alternaria solani*) by using agar overlay techniques and bioautography. Isolation and screen of antifungal compounds (AFC) was carried out by Thin Layer Chromatography (TLC) and bioautography. The AFC were elucidated and identified by using gas chromatography-mass spectrometry. The mode of action of purified AFC from *A. calamus* was studied by Evans blue staining and generation of Reactive Oxygen Species (ROS). Both n-butanol extracts and purified AFC exhibited different degrees of antifungal activity against all fungal pathogens tested. Both n-butanol extract from leaf and rhizome of *A. calamus* and purified AFC showed prominent one major spot on TLC after detection with p-anisaldehyde. In bioautographic technique, clear inhibition zones at same distance of TLC spot was observed against all fungal strains examined. The major purified antifungal compounds were identified as a β-asarone (94.3%) and α-asarone (3.4%). Fungal cells treated with different concentration of purified AFC showed a rapid production of ROS. The extent of dead cells stained with Evans blue dye seemed to correlate with the level of ROS production. These results suggested both n-butanol extracts and purified compounds from *A. calamus* show antifungal activity against plant pathogenic fungi and main compound attributed for antifungal activity is β-asarone. Furthermore, AFC caused damages to the cell membrane, leading to altering cell morphology and eventually cell death. Thus *A. calamus* extract has potential application as an alternative or integrated method to chemical control on plant diseases caused by five different plant pathogens examined.

Key words: Antifungal activity, *Acorus calamus* (L), asarone, antifungal compound, bioautography

INTRODUCTION

The intensive and frequent applications of fungicides are often required to control fungal pathogens causing devastated diseases of crops. The great public awareness of environmental and health issues as well as development of fungicide resistance in plant pathogens against synthetic fungicide has stimulated an increasing demands to shift away from the reliance on conventional fungicides. Plant based botanical extracts either as pure compounds or as standardized extracts provide an enormous bio resource of potential use as antifungal compounds due to their antimicrobial activities (Sasidharan *et al*., 2011; Cos *et al*., 2006; Fabricant and Farnsworth, 2001).

The common names of *Acorus calamus* Linn (family Araceae) are "Sweet flag" or "Waan-Nam" and native to central Asia and Eastern Europe (Gilani *et al*., 2006). It is a perennial, semi aquatic and smelly plant found both temperate and sub temperate and well known medicinal plant in traditional medicine in several countries (Mehrotra *et al*., 2003; Caius, 1986; Shin-Chen, 1973; Duke and Ayensu, 1985). *Acorus calamus* plants have been used in herbal medicine to cure fever, for asthmas, bronchitis and as an all-round sedative (Leung, 1980). Extract of rhizome and root and essential oil from this plant have been reported to possess several important biological activity including antifungal (Lee, 2005; Lee *et al*., 2004) antibacterial (McGaw *et al*., 2002; Phongpaichit *et al*., 2005) allelopathic (Nawamaki and Kuroyanagi, 1996), anticellular and immunosuppressive (Mehrotra *et al*., 2003) properties. However very few study has been done to investigate its potential use as antifungal activity against plant pathogenic fungi (Suvarna *et al*., 2011; Singh *et al*., 2010; Mungkornasawakul *et al*., 2002). Previous study (Dissanayake and Jayasinghe, 2013; Dissanayake, 2015) shows methanol extract of sweet flag were highly effective against pathogenic fungi *Rhizoctonia solani*, *Colletotrichum musae*, *Fusarium oxysporum* and *Fusarium proliferatum*.

Despite the wide scale use of sophisticated HPLC and GC techniques, HPLC bioassays and hyphenated methods. TLC is still proving its worth as a simple, inexpensive and robust means for the chemical and biological screening of plant extracts, with subsequent activity-guided isolation of natural products (Hostettmann, 1991). In an effort to discover new antimicrobial compounds, many research studies have been screened plant extracts with different biological activities. In this regard, several bioassays were developed for screened antimicrobial properties of plant extracts (Hostettmann *et al*., 2001). Out of those techniques, bioautography is a highly efficacious assay for the detection of antimicrobial compounds because it allows localization of activity even in a complex matrix and therefore facilitates the target-directed isolation of the active constituents (Rahalison *et al*., 1991). Bioautography facilitate rapid and precise detection of new antimicrobial compounds from plants and other natural products. Especially this technique allows the localization of antimicrobial activity directly on a chromatographic plate where the organism is applied (Navarro *et al*., 1997). The method is fast, cheap and permits a better bioassay-directed fractionation of bioactive compounds (Hamburger and Cordell, 1987). Bioautography is particularly important to avoid the time-consuming isolation of inactive compounds.

Thus, the objectives of this study were to (1) Evaluate the antifungal activity of n-butanol extract of both rhizome and leaves of *A. calamus* (Sweet flag) against range of plant pathogenic fungi of significant importance in plant diseases, (2) Identify the antifungal compounds that contribute to the antifungal activity of both rhizome and leaves of *A. calamus* and (3) Understand the mechanism of action of the predominant antifungal compound present in *A. calamus* leaves and rhizomes.

MATERIALS AND METHODS

Sample collection and extraction: Both rhizome and leaves of *A. calamus* (Sweet flag) were collected from surrounding areas of Belihuloya, Sri Lanka. The dried plant materials were milled to a fine powder using grinder and stored in the dark at room temperature (25°C) in airtight containers until utilized at the Laboratory of Molecular Plant Pathology, Department of Biological and Environmental Sciences Faculty of Agriculture, Yamaguchi University, Japan. Dried samples were ground and defatted with hexane three times. The defatted sample was extracted with methanol three times. Combined methanol extracts were suspended into water and the suspension was then partitioned with n-butanol to solubilize antifungal compounds in the organic phase, leaving sugars, amino acids and salty compounds in the water phase. The n-butanol extract was vacuum dried and the resultant residue was used for further study.

Fungal isolates: Five fungal pathogens of significant important in plant diseases; *Fusarium oxysporum*, *Fusarium solani*, *Colletotrichum gloesporioides*, *Botrytis cinerea* and *Alternaria solani* were obtained from Laboratory previously mentioned fungi, those were cultured on Potato Dextrose Agar (PDA) (39 g of PDA, was suspended in 1 L of distilled water).

Assay of antifungal activity: Both rhizome and leaves of *A. calamus* extract were tested for their efficiency against the pathogen by using an agar dilution technique (Jorgensen *et al*., 1999). 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 (100 μg mL^{-1}). Four milliliter diameter mycelia discs of each fungus were inoculated on each amended agar plate. Inoculated plates were incubated at 25°C and radial growths of each fungal growth were recorded for 7 days. Each treatment was replicated three times with appropriate untreated controls. Then all the plates were incubated at 25°C in dark condition. The percentage of growth inhibition was calculated according to the following equation (Vincent, 1947):

$$\text{Inibition (\%)} = \frac{C - T}{C} \times 100$$

where, C is the colony diameter of the mycelium on the control plate (mm) and T is the colony diameter of the mycelium on the treated plate (mm).

Thin layer chromatography of *Acorus calamus*: Chemical constituents of the *A. calamus* extracts were separated on aluminium-backed Thin Layer Chromatography (TLC) plates (silica gel 60 F 254, 20×20 cm, layer thickness 0.25 mm, particle size of 10-20 μm; Merck, Darmstadt, Germany). The TLC plates were developed under saturated conditions with system developed in our laboratory, i.e., chloroform/methanol/water (6:3:0.5) and separated chemical compounds were detected using acidified p-Anisaldehyde (5.3 mL p-Anisaldehyde: 100 mL ethanol: 1 mL sulphuric acid) as a spray. After spraying, the chromatograms were heated at 110°C in an incubator to allow for optimal colour development.

Thin-layer chromatography-direct bioautography: Ten microliter (0.1 mg mL^{-1}) of *A. calamus* extract were loaded onto TLC plates in a narrow band and eluted using the chloroform/ methanol/water (6:3:0.5) solvent systems. The developed plates were dried under a stream of fast moving air for 3 days to remove traces of solvent on the plates.

All fungal strains were shake-incubated separately in potato dextrose broth (Becton Dickinson, Sparks, MD, USA) at 25°C for 7 days. The culture was filtered through three layers of gauze cloth to remove mycelia. Spores of the each fungus were obtained by centrifugation (3000×g, 10 min). Dehydrated Potato Dextrose Agar (PDA) was used to prepare an immersion of spores to spray on TLC plates. Fifteen milliliter of PDA solution was allowed to cool down. As soon as the temperature reached 48°C, spores were added to the PDA to give a concentration of approx. About 1×10^6 spores/mL for a 20/20 cm plate for each fungal strains *F. oxysporum*, *F. solani*, *C. gloesporioides*, *B. cinerea* and *A. solani*. The PDA media amended with spores of respective fungi was sprayed on TLC plates rapidly to avoid immediate solidification.

Isolation of antifungal compounds: The *A. calamus* extract were applied to Thin Layer Chromatography (TLC) plates (silica gel 60 F254, 20×20 cm, layer thickness 0.25 mm, particle size of 10-20 μm; Merck, Darmstadt, Germany), developed in chloroform/methanol/water (6:3:0.5) as the mobile phase and dried. After development, TLC plates were examined under ultraviolet light and spots marked on the plate were scraped off separately. Compound was extracted from the silica gel with 80% ethanol and the tube containing AFC was centrifuged at 12000×g for 10 min. The resulting supernatant was vacuum-dried to obtain the dried AFC having a white appearance. The dried AFC were examined for purity by TLC and subjected to antifungal activity tests as described above with three different concentrations (100, 300 and 500 μg mL^{-1}).

Identification of bioactive compound: The spot on the TLC plate, which showed high antifungal activity, was subjected to structural analysis. The structure of the compound with antifungal activity was elucidated and confirmed using Nuclear Magnetic Resonance spectroscopy (NMR). The isolated compounds were identified using a Shimadzu QP-5050 GC-MS (Shimadzu, Kyoto, Japan) equipped with a capillary column of DB-WAX (0.25 mm i.d.×60 m; film thickness, 0.25 mm). The oven temperature was programmed to increase from 50-230°C at a rate of 2°C min^{-1}. The injection port and ionizing source were kept at 240 and 230°C, respectively. Helium was the carrier gas, at a flow rate of 1.5 mL min^{-1} and ionization voltage was 70 eV.

Detection of reactive oxygen species in fungal cells: *Fusarium oxysporum* was shake-incubated in Potato Dextrose Broth (PDB) (Becton Dickinson, Sparks, MD, USA) at 25°C for 7 days. The culture was filtered through three layers of gauze cloth to remove mycelia. Conidial cells of the fungus were obtained by centrifugation (3000 g, 10 min) of the supernatant. Conidial cells suspended in PDB (1×10^6 cells mL^{-1}) were shake-incubated at 25°C for 24 h. A 500 μL aliquot of the *F. oxysporum* cell suspension was mixed with different concentration of AFC (100, 300 and 500 ppm) dissolved in 80% (v/v) ethanol and the mixture was incubated for 3 h. Dead cells were detected by Evans blue staining under a light microscope. Generation of ROS in the fungal cells exposed to AFC was determined by monitoring the conversion of nonfluorescent dihydrorhodamine 123 (DHR123; Sigma-Aldrich) to fluorescent rhodamine 123 using a fluorescent microscope (BZ-9000, Keyence, Osaka, Japan).

Statistical analysis: The experiment was conducted using a completely randomized design. Standard errors of means of three replicates were computed using computer software Microsoft Excel. Analysis of variables (ANOVA) 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 $p<0.05$.

RESULTS

Assay of antifungal activity: The n-butanol extracts from the both leaves and rhizomes of *A. calamus* showed growth inhibition activity against all fungal isolates examined, although the extents of growth inhibition were different among the fungal stains. The different concentrations of the purified AFC (100, 300, 500 μg mL^{-1}) were evaluated by *in vitro* inhibition assay of mycelia growth on petri dishes. The purified AFC showed 100% mycelium growth inhibition for *B. cinerea*, *C. gloesporioides* and *A. solani* in the culture media amended with plant extract at 500 μg mL^{-1}. Whereas purified AFC from *A. calamus* showed moderate inhibition of mycelia growth of *F. solani* (67%) and *F. oxysporum* (55%) (Fig. 1).

Thin layer chromatography: Thin Layer Chromatography (TLC) separation of n-butanol extracts from leaves showed an eight spots (1-8). Whereas n-butanol extracts from rhizome of *A. calamus* showed four spots (3, 6, 7 and 8) (Fig. 2a). However TLC of n-butanol extracts prepared from rhizome and leaves from *A. calamus* consistently showed one main discrete spot (R_f = 0.7) in gross chemical composition and activity. Chemical compounds relevant to spots (1-8) were recovered from the TLC plates under UV illumination and subjected to antifungal activity test. The antifungal activity test revealed that only compound recovered from spots 8 (R_f = 0.7) had the predominant antifungal activity against fungal stains examined. The TLC separation of purified AFC consistently showed one main discrete spot with same R_f value (Fig. 2b). During development of TLC methodology a number of solvent systems were trialed and a mixture of chloroform/methanol/water (6:3:0.5) was eventually found to produce significant degree of separation of active compounds. The anisaldehyde-acid spray reagent was chosen as it was seen to detect a wider range of compounds.

Thin-Layer Chromatography (Direct bioautography): Inhibitory activity following TLC separation of n-butanol extracts was found to be attributed by the presence of only one major constituents spots 8 (R_f = 0.7) and gave clear zones of inhibition of fungal growth corresponding to the positions producing large inhibitory zones at R_f 0.7 for each fungal strains *F. oxysporum*, *F. solani*, *C. gloesporioides*, *B. cinerea* and *A. solani* (Fig. 3).

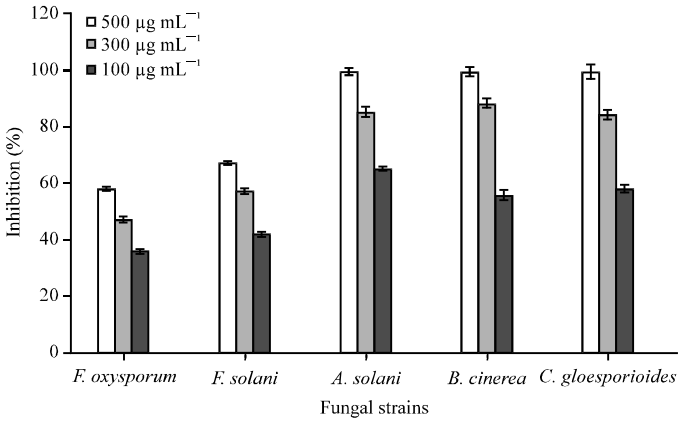

Fig. 1: Mycelium growth inhibition of plant pathogenic fungi *Fusarium oxysporum*, *Fusarium solani*, *Alternaria solani*, *Botrytis cinerea* and *Colletotrichum gloesporioides* with different concentration of purified AFC

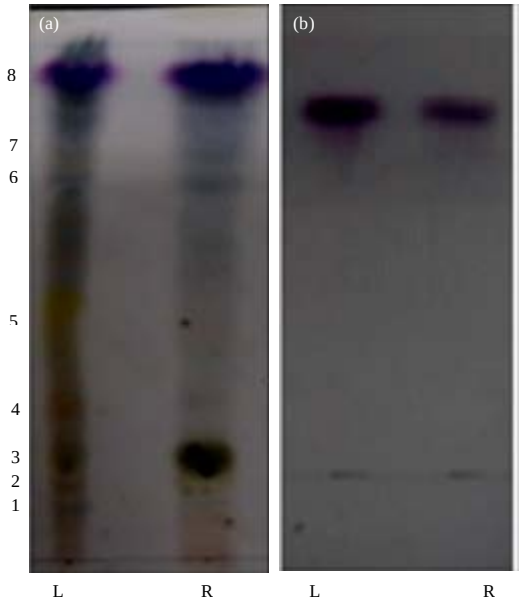

Fig. 2(a-b): (a) TLC profile of n-butanol extracts from the both Leaves (L) and Rhizomes (R) of *Acorus calamus* and (b) TLC profile of purified antifungal compound

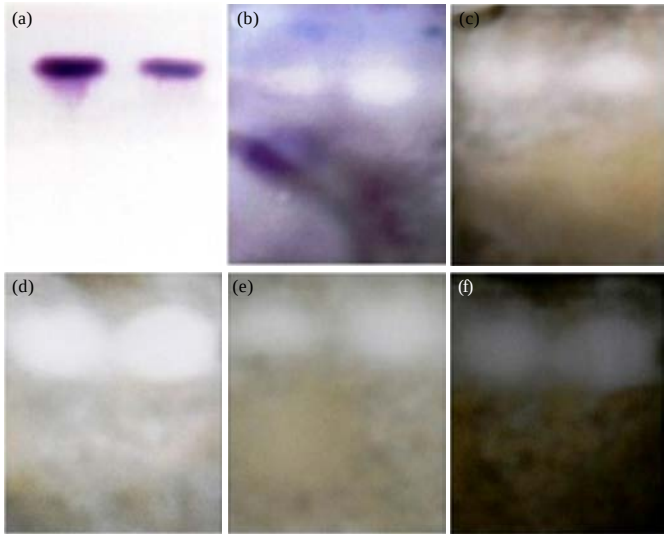

Fig. 3(a-f): (a) TLC profile of purified antifungal compound and Bioautography of purified AFC from *Acorus calamus* for fungal strains of, (b) *F. oxysporum*, (c) *F. solani*, (d) *C. gloesporioides*, (e) *B. cinerea* and (f) *A. solani*. White areas indicate occurrence of zones of inhibition of fungal growth

Identification of bioactive compound: The antifungal compound corresponding to inhibition zone of spots 2 were further purified by TLC. The structure of the compound with antifungal

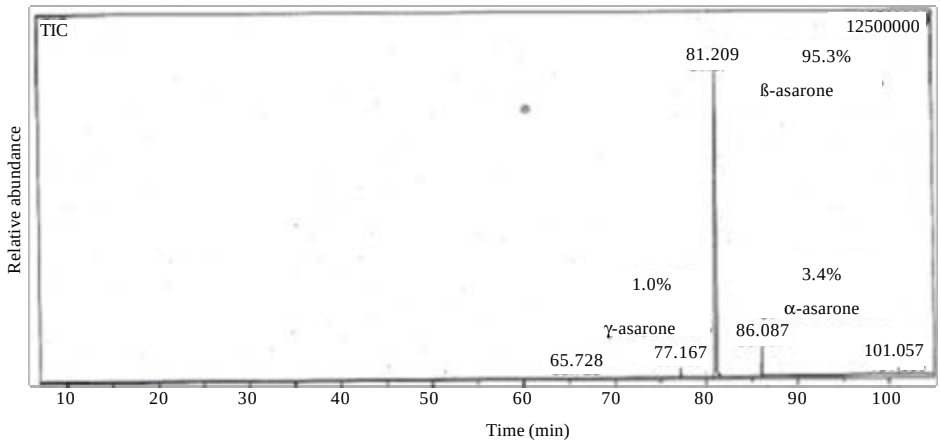

Fig. 4: Gas chromatography-mass spectrometry data of purified AFC from *A. calamus*

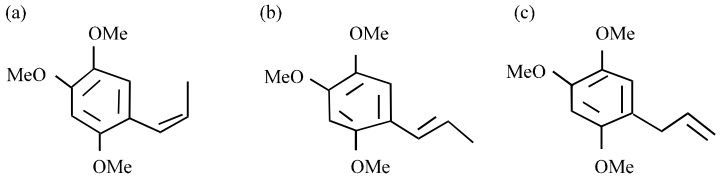

Fig. 5(a-c): Structure of (a) β-asarone (cis-2, 4, 5-trimethoxy-1-propenylbenzene), (b) α-asarone (trans-2,4,5-trimethoxy-1-propenylbenzene) and (c) γ-asarone (1-allyl-2,4,5-trimethoxybenzene) as deduced from NMR spectroscopy

activity was elucidated and confirmed using Nuclear Magnetic Resonance spectroscopy (NMR). According to Gas Chromatography-Mass Spectrometry analysis spot was identical to those of β-asarone (94.3%), α-asarone (3.4%) and γ-asarone (1%) (Fig. 4). The chemical structure of β-asarone (cis-2, 4, 5-trimethoxy-1-propenylbenzene), α-asarone (trans-2, 4, 5-trimethoxy-1-propenylbenzene) and γ-asarone (1-allyl-2, 4, 5-trimethoxybenzene) as deduced from NMR spectroscopy (Fig. 5).

Antifungal mode of action of β-asarone: β-asarone showed fungicidal activity in a dose-dependent manner (Fig. 6a) of the plant pathogenic fungi in PDB. *Fusarium oxysporum* cells treated with β-asarone were stained with DHR 123. Fungal cells treated with 500 ppm AFC, at which most fungal cells were killed by the compound, showed a rapid production of ROS (Fig. 6b). The extent of dead cells stained with Evans blue dye (Fig. 6a) seemed to correlate with the level of ROS production (Fig. 6b).

DISCUSSION

Although many studies (Ganjewala and Srivastava, 2011; McGaw *et al*., 2002; Rani *et al*., 2003; Phongpaichit *et al*., 2005) have investigated importance of biological activity, especially antimicrobial properties of *A. calamus* against human pathogens, very few studies (Suvarna *et al*., 2011; Singh *et al*., 2010; Mungkornasawakul *et al*., 2002) have been carried out

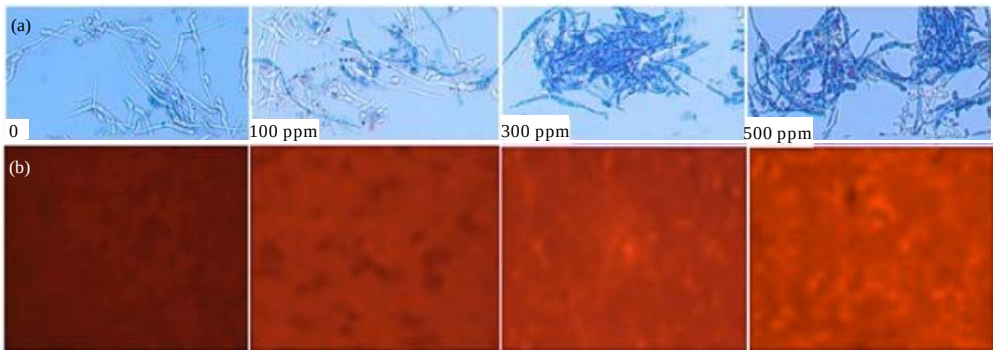

Fig. 6(a-b): Fungicidal action of purified AFC from *Acorus calamus* at different concentrations (a) Evans blue staining of *F. oxysporum* exposed to different concentrations (0, 100, 300 and 500 ppm) of purified AFC and (b) Epifluorescence microscopy *F. oxysporum* cells exposed to different concentrations (0, 100, 300 and 500 ppm) of purified AFC for 60 min

to find its potential as antifungal agents against plant pathogens. In this study, the antifungal activity of n-butanol extracts was tested from both leaves and rhizomes of *A. calamus* against phytopathogenic fungal strains of *F. oxysporum*, *F. solani*, *C. gloesporioides*, *B. cinerea* and *A. solani*. Both *Fusarium* fungal strains need higher concentration to inhibition of mycelium growth in *in vitro*. Similar results have been found in previous studies (Suvarna *et al*., 2011). Present study revealed n-butanol extracts from the both leaves and rhizomes of *A. calamus* has ability to impair the mycelia growth of all tested fungal stains confirming its fungicidal properties.

In this study, for first time we attempted to identify main active ingredient responsible for antifungal activity of *A. calamus* plant parts using TLC-Bioautography followed by gas chromatography-mass spectrometry analysis. According to present study, TLC bioautography-guided strategy confirmed that one major compound (spot 8) is significant contribution to the overall antifungal activity. According to Gas chromatography-mass spectrometry analysis the major antifungal compound was identified as asarone with their different isomeric forms β-asarone (94.3%) α-asarone (3.4%) and γ-asarone (1%) from *A. calamus*. β-asarone was seem to comprise a large proportion of extractable materials and was found to be principally responsible for antifungal activity in extracts of *A. calamus*. Many studies (Devi and Ganjewala, 2009; Venskutonis and Dagilyte, 2003; Rani *et al*., 2003; Perrett and Whitfield, 1995) reported that the phenylpropanoids β-asarone and α-asarone found in leaf, rhizomes and root are responsible for entire antimicrobial activity *Acorus calamus*. It has also been reported occurring in *A. gramineus* (Lee *et al*., 2004). However, no simple method is available for separation and isolation of asarones (alpha, beta and gamma) in single isomeric form due to their similar physical properties. So far, HPLC and GC-MS are two most frequently used techniques for the analysis of asarone. According to our findings showed TLC bioautography followed by gas chromatography-mass spectrometry analysis was highly applicable for identification of target-detected isolates of antifungal compound even up to isomeric forms. Results presented in this study about TLC fractions separation and contain of β-asarone and α-asarone in purified AFC is completely matches with those reported by Devi *et al*. (2013). TLC-bioautography method is very useful in isolating compounds with antimicrobial

activity because the R_f of the active compound can be used in bioassay guided fractionation instead of requiring labour intensive determination of activity of fractions.

The proportion of each chemical compound in the essential oil varies among the varieties of *A. calamus*, corresponding to the ploidy level of the taxon (Mazza, 1985; Rost and Bos, 1999). β-asarone concentrations vary markedly among the oils from the three varieties. The tetraploid plant oil is high in β-asarone (90-96%) and also contains α-asarone. The triploid plants contain a small portion of β-asarone (5%) in their oil and the diploid plants lack β-asarone but contain high amounts of geranyl acetate. Therefore according to our findings, *A. calamus* existing in Sri Lanka could be tetraploid.

Fusarium oxysporum cell suspension treated with purified AFC showed rapid cell death with increases of concentration and correlate with the level of ROS production. In the present study, that ROS was also produced in *F. oxysporum* cells treated with purified AFC. Interestingly, ROS production levels coincided with the extent of cell death, suggesting involvement of ROS production in the fungicidal action of purified AFC. Loss of membrane integrity in the fungal cells exposed to purified AFC was suggested by the increased uptake of the fluorescent dye, propidium iodide. ROS production occurs in fungal cells treated with α-tomatine, a spirostanol saponin of tomato (Ito *et al*., 2007). In the present study it was found that ROS was also produced in *F. oxysporum* cells treated with purified AFC from *A. calamus*. Interestingly, ROS production levels coincided with the extent of cell death, suggesting involvement of ROS production in the fungicidal action of AFC from *A. calamus*. These results suggest that purified AFC causes damage to the cell membrane, leading to altering cell morphology and eventually cell death. Based on Scanning Electron Microscopy (SEM) study (Phongpaichit *et al*., 2005) also reported β-asarones altered the fungal hyphal morphology with collapsed form.

The n-butanol extracts of *A. calamus* as well as purified compounds show antifungal activity and main compound attributed for antifungal activity is β-asarone. AFC causes damage to the cell membrane, leading to altering cell morphology and eventually cell death. These indicate potential application of *A. calamus* extract as an alternative or integrated method to chemical control of plant pathogens examined. However, prior to their use as botanical fungicide, toxicity tests against humans and other animals need to be conducted.

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