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Antifungal Phytochemical Compounds of *Cynodon dactylon* and their effects on*Ganoderma boninense*

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ABSTRACT

Cynodon dactylonis a type of perennial grass that possesses great medicinal values.It is traditionally used as a rejuvenator, for wound healing and was believed to be able to cure many diseases and infections. Scientifically it has been reported to possess many pharmacological activity including antidiabetic, cardioprotective, antidiarrheal and antibacterial properties. However, the role of C. dactylon in combating plant fungal pathogen was scantly reported. In the present study, antifungal activityofC. dactylon ethanol Solid Phase Extraction (SPE) extract against Ganoderma boninense was investigated.The antifungal activity and Minimum inhibitory concentrations (MICs) were evaluated using agar diffusion bioassay. In this study, elute fraction of C. dactylon ethanol SPE extract was effectively suppressed the G. boninense growth after 14 days of incubation (MIC=20.00 mgmL-1). Based on Liquid Chromatography-Mass Spectrometry (LCMS) analysis, some possible antifungal compounds against G. boninense were identified as Tokoronin, Ophiopogonin C and Cyclopassiflosides (Saponins), Elemicin (Phenolics), 5-oxo-7-octenoic acid, Stearidonic acid and 17-Hydroxylinolenic acid (Fatty acids), Neocnidilide (carboxylic acid), Gingerglycolipid B and Apiole.

Keywords: Cynodon dactylon, Ganoderma boninense, antifungal,

INTRODUCTION

Antifungal activity from medicinal plants had been studied intensively by previous researchers [2,25,23]. Not only important in medicine, crude extracts of some well-known medicinal plants are also used in controlling some of the plants pathogens [24]. Despite the remarkable antimicrobial activity from medicinal plants against human fungal pathogens, it had been well acknowledged by scientific community and the agricultural practitioners that some medicinal plants also possess great potential in combating fungal plant pathogens. The uses of plant-derived products as disease control agents have been studied, since they tend to have low mammalian toxicity, less environmental effects and wide public acceptance [20]. The devastating Basal Stem Rot (BSR) disease in oil palm which caused by *Ganoderma boninense* is a fatal disease and considered the most serious disease affecting oil palm in South East Asia [4]. The losses caused by BSR can up to 80% after repeated planting cycles and it is also estimated about 90% of the estates in West Malaysia were reported with the presence of *G. boninense* [18]. *Cynodon dactylon* is a type of perennial grass that possesses great medicinal values. It is traditionally used as a rejuvenator, wound healers and was believed to be able to cure many diseases and infections [15]. Scientifically it has been reported to possess many pharmacological activity including antidiabetic, cardioprotective, antidiarrheal and antibacterial properties [19,3,1]. However, the role of *C. dactylon* in combating plant fungal pathogen was scantly reported. Therefore, the present study has been designed to screen the potential antifungal activity from some phytochemical compounds of *C. dactylon*against *G. boninense*.

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Objectives:

This study is aimed to investigate the antifungal activity of *C. dactylon*Solid Phase Extraction (SPE) extract against the most devastating pathogen in oil palm, *G. boninense* and to identify the possible antifungal compounds via Liquid Chromatography-Mass Spectrometry (LCMS) analysis.

Materials and Methods

3.1. Plant Collection:

Wild ecotype of the plant was collected in area of Kota Kinabalu (Lat: 6.034826, Long: 116.12316), Sabah, Malaysia. Voucher (jgobilik 1090/2011) was kept in School of Sustainable Agriculture (SPL), Universiti Malaysia Sabah (UMS) and a duplicate was submitted to BORH Herbarium, Institute of Tropical Biology and Conservation (ITBC), UMS for future reference.

3.2. Cynodon dactylon Ethanol Crude Extraction:

The whole plant of *C. dactylon* was thoroughly cleaned using distilled water to remove soil and dirt and then dried for 24-72 hours in a drying chamber at 40-50^{O}C to remove water content from the plant. To optimize and enhance the extraction yield, the dried plant was homogenized using a mechanical blender (Waring® Commercial Blender). Approximately 100g of the plant powder later was soaked into 200mL of ethanol and shaken on a platform shaker (LabCompanion^{TM}) at 150 rpm at temperature of 25 ^{O}C to obtain the plant extracts. The soaking process was repeated three times for each extraction to obtain a complete extraction. The extracts obtained were then evaporated and concentrated under reduced pressure (768mmhg to 7mmhg) using Rota Vapor^{TM} (BUCHI) to achieve final concentration of 1g of extract per mL of solvent. The Aliquot was then kept in -20^{O}C until further use.

3.3. Preparation of Cynodon dactylon Solid Phase Extraction (SPE) Extract:

Strata^{TM}-X 33um Polymeric Sorbent reverse phase (200mg/6mL) (Phenomenex) cartridges with 12-cartridges manifold system was used. Methanol absolute (1 mL) was used to activate the sorbent and further equilibrated with sterile deionized distilled water (1 mL). Ethanol extract of *C. dactylon*was then loaded into the cartridges and left inside the SPE sorbent matrix for few seconds up to a minute. The loaded sample was then washed with 1% methanol (1 mL). The resulted fraction yielded from wash procedure was collected and labelled as 'flush fraction'. Finally, the remaining samples inside the SPE sorbent matrix were eluted with 2mL of methanol:acetonitrile (1:1; v/v), collected and labelled as 'elute fraction'. The aliquots were taken to dryness using purified nitrogen gas. Dried aliquots were stored in -20^{O}C for further bioassays. Both flush and elute fractions were collected and tested for their respective antifungal activity.

3.4. Ganoderma boninense Culture:

Ganoderma boninense was isolated from infected oil palms in Kota Marudu Sabah and their identity was molecular identified [5]. The fungal cultures were maintained on Potato Dextrose Agar (PDA) for further use.

3.5. Agar diffusion bioassay:

Agar diffusion bioassay for elute and flush fractions of *C. dactylon* ethanol SPE extract was conducted on*G. boninense.* In this bioassay, 1mL from each fraction was incorporated into their respective media (PDA) to make a final concentration of 10mgmL^{-1}prior the hardening of media. The dried *C. dactylon* ethanol extact was first dissolved in acetone before incorporated into the media. Approximately 7-8 days old culture of *G. boninense* was plucked from the edge of the culture using sterile cork-boarer with0.8cm diameter and placed at the middle of the media containing *C. dactylon* SPE extracts. For determination of minimum inhibitory concentrations (MICs), a series of concentrations of *C. dactylon* SPE extract (0, 5, 10, 20 mgmL^{-1}) was incorporated into PDA. Agar without plant extract, but containing an identical concentration of acetone, served as controls. The growth of the pathogen was expressed as radial growth (cm).

3.6. Mass Analysis and Compound Identification:

The Liquid Chromatography-Mass Spectrometry (LCMS) analysis was carried out using an Agilent 1200 series coupled with Agilent 6200 series Quadrapole Time of Flight (Q-ToF) Mass Spectrometry (MS) Dual Electrospray Ionization (ESI) detector.Mass spectra analysis on elute fraction of *C.* dactylon SPE extract was done using the Agilent MassHunter Workstation-Qualitative analysis Software. In this software, few mass to charge ratio (m/z) peaks from a respective chromatogram were generated and the most abundant m/z was selected for generating the most probable mass for particular compound through Find by Molecular Feature algorithm. Each identified mass representing particular compounds was subjected to compound identification using online metabolites spectral database, METLIN (http://metlin.scripps.edu). The identity of compounds from SPE fractions was identified by matching their true molecular mass with existing chemical compound databases in METLIN. Besides, METLIN, other online databases including PubChem, KEGG and HMDB were also utilized to enhance the compounds identification.

Results:

4.1. Antifungal effects of C. dactylon against G. boninense:

Both elute and flush fractions of ethanol SPE extract are subjected to agar diffusion bioassay to determine the antifungal activity against oil palm pathogen, *G. boninense*. After 14 days of incubation along with continuous observation, the elute fraction of ethanol SPE extract showed greater antifungal activity via inhibition of radial growth of *G. boninense* in contrast to the flush fraction (Table 1). The elute fraction of *C. dactylon* ethanol SPE extract was able to suppress the fungal growth with more than 70% of radial growth (final growth of *G. boninense*=2.34±0.15cm) after 14 days of incubation in contrast to the control plate (final growth of *G. boninense*=8.00±0.00cm). Meanwhile, the flush fraction of the plant ethanol SPE extract was only able to suppress *G. boninense* growth up to approximately 38% of radial growth (final growth of *G. boninense*=4.92±0.11cm). Due to stronger antifungal activity exerts by the elute fraction, subsequent bioassay was done to determine the minimum inhibitory concentration (MIC) of the extract to fully suppress *G.boninense* growth. Figure 1 shows different concentration of the plant elute fraction of ethanol SPE extracts along with controls and their effect on *G. boninense* growth. Among four of the concentrations tested, approximately 20.00mgmL^{-1} of the plant extract was able to fully suppress the fungal growth up to 14 days of incubation (final growth of *G.boninense* = 0.74±0.05), although at days 14, little growth of *G. boninense* was observed from the plates, which might probably suggesting the overcome effect due to the degradation active compounds proposed by Gonzalez-Lamothe *et al*. [13]. No significant different ($P<0.05$) in growth of *G. boninense* on control media compared to media with no SPE-elute fraction extract showed no adverse effect exert by the solvent used while no growth of *G. boninense* on Nystatin-containing media after the 14 days of incubation showed the fungi is not antifungal resistant.

4.2. Identification of Phytochemical compounds:

Some potential antifungal compounds were identified from the elute fraction through mass spectral analysis (Figure 2). Saponin is one of the antifungal compounds that presence in the elute fraction. Three types of saponin (Tokoronin, Ophiopogonin C and Cyclopassiflosides) were identified based on their mass spectral analysis. Meanwhile, Elemicin, one of the phenolic compounds that possess antifungal properties [29] was identified. Fatty acid and its derivatives were known to possess great antifungal activity [16,7]. In the present study, numbers of fatty acid compounds were identified. However, only some of them were reported to possess antifungal properties such as 5-oxo-7-octenoic acid [12], Stearidonic acid [30] and 17-Hydroxylinolenic acid [31]. Neocnidilide, a carboxylic acid was also the antifungal compound [27] that identified from the elute fraction. Other compounds that reported to exhibit antifungal properties such as Gingerglycolipid B [28] and Apiole [8] were also identified from the elute fraction.

Discussion:

The present study revealed the potential of *C. dactylon* as an alternative source for biocontrol agent against *G. boninense*. The use of plants as a natural source for developing fungicide and pesticide are now getting attention due to the pitfall possess by chemicals and pesticides towards human health and environment. The finding was remarkable as the elute fraction from the extract was able to suppress the fungal growth compare to the flush fraction. Phytochemical compounds identificationbased on mass spectra analysis revealed the presence of possible antifungal compounds in elute fractions. Saponins, which are believed to form the main constituents of many plant drugs and folk medicines, and are considered responsible for numerous pharmacological properties [11]. Although to date, no report on the antifungal activity from the identified saponin compounds, numerous studies have been done previously which prove the saponins' role as antifungal agent [10,6]. Sindambiwe *et al*. [26] had demonstrated the role of maesasaponin mixture which contains same class of saponins with Tokoronin, a steroidal saponin against *Epidermophyton floccosum, Microides interdigitalis* and *Trichophyton rubrum*. Li *et al.* [21] have shown a triterpenoidal saponin, jujubogenin saponin which also in the same class with Ophiopogonin C which found in the present study to possess antifungal activity against *Candida albicans, Crytococcus neoformans* and *Aspergillus fumigatus*. Meanwhile, triterpenoid saponins from the seeds of *Chenopodium quinoa* (Chenopodiaceae) have been reported to have antifungal activity against *Candida albican* [32]. In this study, two triterpenoidal saponins, Cyclopassifloside V and Cyclopassifloside VII were identified from the elute fraction, which might possess same antifungal activity as reported by the previous authors. In the present work, saponins were only detected in the elute fraction, which might possibly contribute to the antifungal activity of this fraction against *G. boninense*. Fatty acidis one of the most interesting compound that can exhibit great biological activities. In this study, several fatty acids were identified based on mass spectral analysis. Kabarra *et al*. [16] have intensively discussed the role of fatty acid as antimicrobial agents. In the present study, three fatty acids (5-oxo-7-octenoic acid, Stearidonic acid and 17-hydroxylinolenic acid) were

identified and might responsible for the inhibition on *Ganoderma boninense* growth. The finding in this study was in aggreement with the study done by Jananie *et al*. [14] and Kanimozhi *et al*. [17] which showed *C. dactylon* to contain fatty acid compounds such as Stearidonic acid and Hydroxylinolenic acid. From the previous works, Gershon and Shanks [12] had reported the role of 5-oxo-7-octenoic acid against *Aspergillus niger*, *Myrotheciumverrucaria* and *Trichoderma viride*. Thibane *et al.* [30] have demonstrated the antifungal activity of Stearidonic acid against *C. albicans* and *C. dubliniensis.* Meanwhile, 17-hydroxylinolenic acid had been studied for its antifungal effect against *C. albicans* [16], *Crinipellis pernicosa, Pyrenophora avanae, Pythium ultimum, Rhizoctonia solani* [31], *Alternaria solani* and *Fusarium oxysporum* [22]. It is suggested that these compounds might contribute to the antifungal activity against *G. boninense* and might be a significant component responsible for antifungal activity in elute fraction.In our study, the only antifungal carboxylic acid identified was Neocnidilide. Suzuki *et al*. [27] have demonstrated the role of Neocnidilide against mycotoxin-producing fungi. Neocnidilide might contribute to the antifungal effect in the elute fraction against *G. boninense* due to its antifungal effect. Other identified carboxylic acids were not reported to possess antifungal effect and they are most probably yielded from metabolic activity in the plant.Apart from the bioactive constituents discussed above, two compounds; Apiole and Gingerglycolipid B were identified from other classes of metabolites which might responsible for antifungal effect. Diego [9] have demonstrated the role of Apiole as an antifungal agent extracted from *Piper auritum* and *Piper holtonii* against *Colletotrichum acutatum* and *Botryodiplodia theobromae*. In another work, da Silva *et al*. [8] have found the abundance of Apiole in *Piper krukoffii* which might contribute to larvicidal and antifungal activity inthe plant methanol extract. Meanwhile, a glycolipid, Gingerglycolipid B was also reported by Tarawneh *et al.* [28] to exert exert significant antifungal effect against *Cryptococcus neoformans*. The present study suggests the potential of *C. dactylon* to be developed as a biocontrol agent against *G. boninense*.

Table 1: Agar diffusion bioassay of *C. dactylon* ethanol SPE elute and flush fractions against *G.boninense*. The efficacy of the extracts was measured by the ability of the extracts to inhibit the fungal radial growth.

Day	Growth* of *G.boninense* in different ethanol SPE extract fractions		Control
	A	B	
1	0.70±0.00	0.70±0.00	0.7±0.00
2	0.70±0.00	0.70±0.00	0.7±0.00
3	0.70±0.00	0.70±0.00	0.7±0.00
4	0.84±0.05	0.70±0.00	1.00±0.11
5	1.18±0.04	0.70±0.00	1.80±0.12
6	1.56±0.05	0.70±0.00	3.50±0.16
7	1.96±0.04	0.70±0.00	5.10±0.23
8	2.68±0.08	0.70±0.00	7.30±0.05
9	3.77±0.09	0.70±0.00	7.90±0.04
10	4.61±0.09	0.76±0.09	8.00±0.00
11	4.78±0.09	1.00±0.07	8.00±0.00
12	4.83±0.11	1.48±0.08	8.00±0.00
13	4.90±0.11	1.96±0.11	8.00±0.00
14	4.92±0.11	2.34±0.15	8.00±0.00

*After 14 days of incubation, elute fraction of *C. dactylon* ethanol SPE extract showed stronger antifungal activity against *G.boninense*. Growth of *G.boninense* was measured in cm base on radial growth; the concentration of both elute and flush fractions of EtOH SPE extracts are 10mgmL^{-1}; Values presented are means of five replicates, ± stand. dev.
A=Ethanol SPE flush fraction; B= Ethanol SPE elute fraction

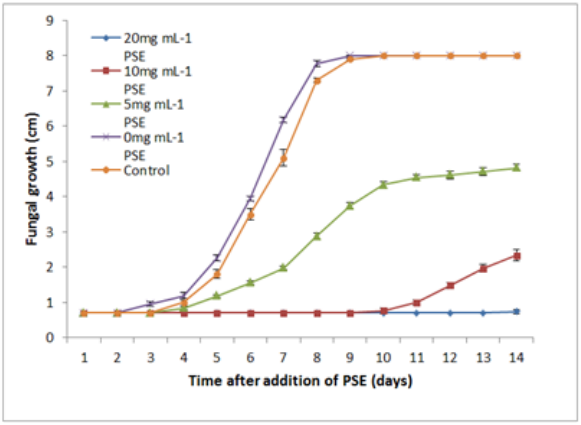

Fig. 1: Radial growth curves of *Ganoderma boninene* on PDA with different concentrations of *C. dactylon* ethanol SPE-based extract (PSE) incorporated into the agar after two weeks of incubation. The MIC value for the elute fraction is 20.00mgmL^{-1}

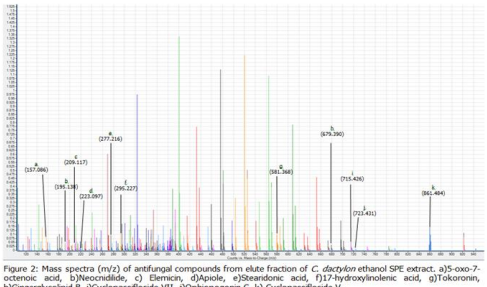

Figure 2: Mass spectra (m/z) of antifungal compounds from elute fraction of *C. dactylon* ethanol SPE extract. a)5-oxo-7-octenoic acid, b)Neocnidilide, c) Elemicin, d)Apiole, e)Steareidonic acid, f)12-hydroxylinolenic acid, g)Tokoronin, h)Gingerglycolipid B, i)Cyclopassifloside VII, j)Ophiopogonin C, k) Cyclopassifloside V.

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