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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 12 (16): 1119-1126, 2009
ISSN 1028-8880

Implementation of Bio-Fungicides and Seed Treatment in Organic Rice cv. KDML 105 Farming

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Abstract: This study was aimed to evaluate the several chemical compounds of relatively composite structure with antifungal activity from Thai local medical plants. The antifungal activity of *Stemona curtisii* HK. f., *Stemona tuberose* L., *Acorus calamus* L., *Eugenia caryophyllus*, *Memmea siamensis* Kost. and an eugenol active compound were studied *in vitro*. Four pathogenic seed borne fungi, *Alternaria solani*, *Colletotrichum* sp., *Fusarium moniliforme* and *Rhizoctonia solani* were used as target organisms. The agar overlay technique and spore inhibition techniques were applied for the determination of their essential oil and active compound antifungal activity at various concentration; 0.10, 0.25, 0.50 and 1.00% (v/v) and untreated as control (0% v/v). Eugenol active compound showed the strongest antifungal activity on all species of tested fungal species. On the other hand, the antifungal activity of those bio-fungicides was lined up into a series from strong to low, as follows: *Eugenia caryophyllus*>*Acorus calamus* Linn.>*Stemona tuberosa* L.>*Stemona curtisii* Hk.f, while *Mammea siamensis* Kost. could not control any fungal species. Moreover, after eugenol application, lysis of spore and inhibition of mycelium growth were detected. Microscopic analysis exhibited complete lysis of spores after 24 h at a concentration of 1.00% v/v. Moreover, at the same concentration and 96 h incubation the mycelia growth was completely inhibited.

Key words: Bio-control, bi-fungicide, eugenol, plant extract, seed borne fungi, antifungal activity, mode of action, bio-farming

INTRODUCTION

Rice production is known to be attacked by many pathogenic fungi, are *Alternaria* sp., *Colletotrichum* sp., *Fusarium* sp. and *Rhizoctonia* sp. These microorganisms cause a serious deterioration when they occur in/on rice grain. Moreover, they can colonize to diverse substrates, because of their powerful arsenal of hydrolytic enzymes and they can be responsible for considerable economic losses (Zhu, 1998). Furthermore, some of the above-mentioned fungi can act as potential producer of mycotoxins, which potentially damage consumers' health (Evandro *et al*., 2005).

Traditionally, chemical treatments are used widely to protect the germinating seedling, during vegetative and reproductive growth and after harvest from pathogenic fungi infection (Aleieri *et al*., 1984). Currently, the use and expectations of chemical treatments are greatly concerned due to the impact of the chemical to the environment, which can be primary or secondary influences on human or other living organisms (Baruah *et al*., 1996). To avoid these disadvantages, new strategies for fungicide use and disease management must be developed and identified. The alternatives of synthetic fungicides could be the development of effective phyto-chemicals from plant origin, which are expected to be more advantageous than synthetic fungicides (Davidson and Parish, 1989). The increased importance of the development and application of biological fungicides is recognized under the concept of Integrated Pest Management (IPM) (Del-Campo *et al*., 2002). Under this concept, all possible modes of plant disease control methods should be integrated to minimize the excessive use of synthetic fungicides (Bishop and Thornton, 1997).

It is well established that some plants contain active compounds which are able to inhibit the microbial growth (Naqui *et al*., 1994). These plant compounds have different structures and antimicrobial activities when compared with conventional fungicides (Nascimento *et al*., 2000). The potential antimicrobial properties of plant is related to their ability to synthesize several chemical compounds of relatively complex structure with antimicrobial activity, including alkaloids, flavanoids, isoflavanoids, tannins, cumanins, glycosides, terpenes and organic acids (Nychas, 1996). For examples, a solvent extract from clove flower buds that contains eugenol as main active compound was antifungal active against *Alternaria* sp., *Fusarium* sp., *Botrytis* sp. and

Rhizoctonia sp. (Soatthiamroong *et al*., 2003). Sage oil was active against *Botrytis* sp. (Carta *et al*., 1996) and thyme inhibited post harvest diseases of tomato (Plotto *et al*., 2003).

Thus, the aim of this study was to screen for the best *in vitro* antifungal activity of *Acorus calamus* L., *Stemona curtisii* HK.f., *Stemona tuberosa* L., *Memmea siamensis* Kost. and *Eugenia caryophyllus* (Spreng.) Bullock and S.G. Harrisoncrude extracts and eugenol essential oil against rice pathogenic fungi as a possible alternative for synthetic chemical antifungal compounds.

MATERIALS AND METHODS

The experiment was conducted at Department of Agricultural Technology, Faculty of Technology, Maha Sarakham University, Maha Sarakham, Thailand and Seed Science and Technology Laboratory, Section of Seed Science and Technology, Department of Agronomy, Faculty of Agriculture, Chiang Mai University, Thailand in 2008. Plant extracts and an essential oil were used as bio-fungicides. *Acorus calamus* Linn., *Stemona curtisii* Hk.f., *Stemona tuberosa* L., *Memmea siamensis* Kost. and *Eugenia caryophyllus* were stream extracted and eugenol essential oil was purchased from Fluka (Steinheim, Germany). The experiment was conducted in Factorial in CRD design with 4 replications. The mycelium growth and spore germination inhibition techniques were applied to record the efficiency of those essential oils at 0.10, 0.25, 0.50 and 1.00% (v/v) and un-used of essential oils were subjected as control. *Alternaria solani*, *Aspergillus flavus*, *Aspergillus niger*, *Bipolaris oryzae*, *Colletotrichum* sp., *Fusarium moniliforme*, *Nigrospora* sp. and *Rhizoctonia solani* were used as target fungi which was provided from the collection of the Department of Plant Pathology, Faculty of Agriculture, Chiang Mai University, Thailand.

Mycelium growth inhibition analysis: The experiments were conducted by agar overlay technique was described by Morris *et al*. (1979). The plant extract and essential oil at different concentrations (0.10, 0.25, 0.50 and 1.00% v/v) on Potato Dextrose Agar (PDA) medium was *in vitro* tested against the fungi mycelium growth. Medium (20 mL) was dispensed into Petri dish and 5 mm diameter of the test fungi cutted from the middle of 7-days-old cultures were incubated upside down separately to each assay plate and incubated for 96 h at 25±2°C. The colony diameter was measured and the mycelium inhibition percentage was calculated by following Deans and Svoboda (1990). Four replicates of each treatment were tested and the average was calculated. Control sets were simultaneously run without using the plant extract and essential oil and mycelium growth inhibition was calculated as Eq. 1.

Spores inhibition analysis: Spores of *Alternaria solani*, *Fusarium moniliforme* and *Rhizoctonia solani* were produced by using a modification method of Mitchell and Yang (1966). The fungus was grown for 3 days at 25±2°C in PDA aqueous medium. To induce spores formation, fungal mycelium was rinsed 3 times with distilled water, for approximately 30 sec each time and after the final rinsed, cultures were kept at 25±2°C for 2 days. Spore concentration was adjusted to approximately to 10^6 cfu mL^{-1} by the hemacytometer. Sterile microscope slides were dropped 100 μL of PDA aqueous medium to obtain a thin agar layer on the slide, then 100 μL of spores suspension sample was gently spread on each slide. An uncovered watch glass containing either 100 μL of sterile water as control, or 100 μL of plant extract and an essential oil at different concentration (0.10, 0.25, 0.50 and 1.00% v/v) were dropped into slide. After that, slides were place in glass Petri dishes lined with moistened filter paper, covered and sealed with parafilm, incubated for 24 h at 25±2°C, all of the encysted spores on each slide were counted with compound microscope at magnification of x100 and spores inhibition was calculated as Eq. 1.

$$\text{Inhibition (\%)} = [(C-T)/C] \times 100 \quad (1)$$

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 treatment plate (mm).

Concentration response curves were obtained whereby the percentage of fungal inhibition was plotted against concentration and concentration required to give 50% inhibition of fungal growth (IC_{50}) was calculated from the regression equation.

Statistical analysis: The data are presented as Mean±Standard deviation. The analysis of variance was performed for data analysis and differentiated with Last Significant Different (LSD) test at $p<0.05$ using the software SX release 8.0 (Analytical software, Tallahassee, USA).

RESULTS AND DISCUSSION

The most of studied fungi were inhibited at 1% v/v of *A. calamus* Linn.; *A. solani*, *Colletotrichum* sp. and *R. solani* were completely inhibited (100%). Nevertheless, *F. moniliforme* was inhibited about 95.91% (Fig. 1). *R. solani* was slightly sensitive to *S. curtisii* Hk.f. extract

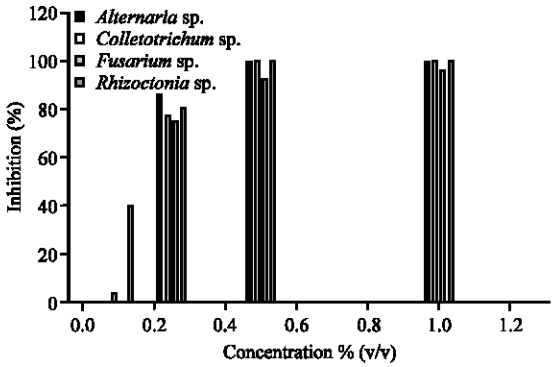

Fig. 1: The antifungal activity of *Acorus calamus* L. against economically rice pathogenic fungi

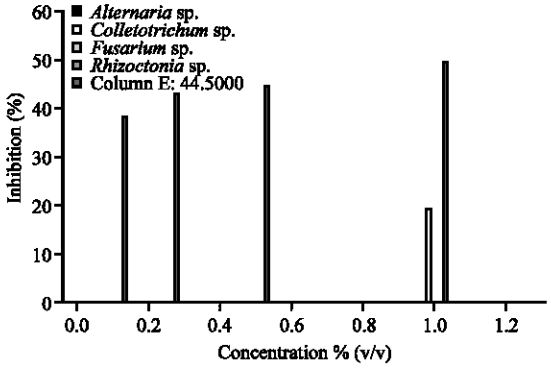

Fig. 2: The antifungal activity of *Stemona curtisii* Hk.f. against economically rice pathogenic fungi

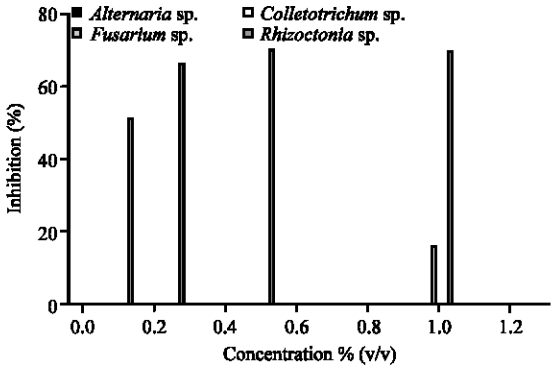

Fig. 3: The antifungal activity of *Stemona tuberosa* L. against economically rice pathogenic fungi

and was inhibited to 49.54% at 1.00% v/v concentration. The growth of *A. solani*, *Colletotrichum* sp. and *F. moniliforme* were uninhibited by *S. curtisii* Hk.f. extract (Fig. 2). Figure 3 shows the antifungal activity of *S. tuberosa* L. which had no inhibition effect on *A. solani*, *Colletotrichum* sp. and *F. moniliforme*. However, *S. tuberosa* L. could inhibited *R. solani* for 70%

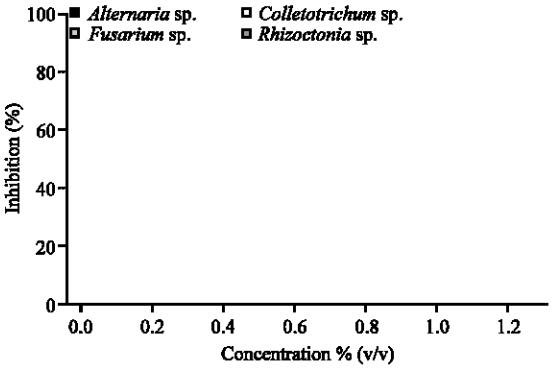

Fig. 4: The antifungal activity of *Memmea siamensis* Kost. against economically rice pathogenic fungi

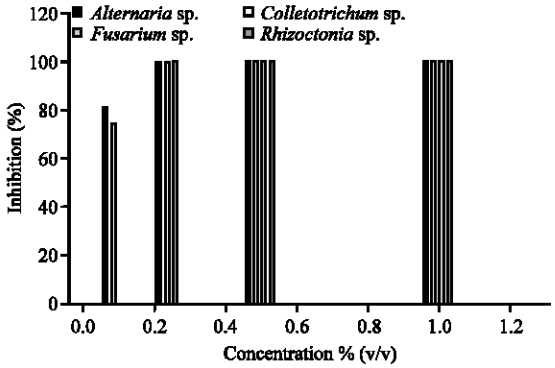

Fig. 5: The antifungal activity of *Eugenia caryophyllus* against economically rice pathogenic fungi

at 1.00% v/v concentration. As shown in Fig. 4, the *M. siamensis* Kost extract was unable to control all fungi species. Figure 5 showed that *E. caryophyllus* extract at 0.25% v/v completely inhibited *A. solani*, *Colletotrichum* sp. and *F. moniliforme*. Moreover, when the concentration increased to 0.50% v/v, this extract inhibited *Rhizoctonia solani* completely. The IC_{50} values indicated that *E. caryophyllus* crude extract showed strongly antifungal activity against pathogenic fungal as well as *A. calamus* Lin. However, *S. curtisii* Hk.f, *S. tuberosa* L. and *M. siamensis* Kost. were unable to inhibit all fungi species. Although, *R. solani* seemed to be less susceptible to the plant extracts than the other fungi (Table 1). Table 2 shows that, Eugenol showed the strongest antifungal activity at 1.00% v/v. Additionally, eugenol had the strongest antifungal activity (showed 100% inhibition zone) against the each fungal species as well as *E. caryophyllus* did (Table 3). Eugenol was similar effective in inhibiting the mycelium growth of each fungus as captan did, especially on *A. solani*, *A. flavus*, *A. niger*, *B. oryzae* and *F. moniliforme* (Table 4, Fig. 6). Nevertheless, captan was effective on inhibition of spore

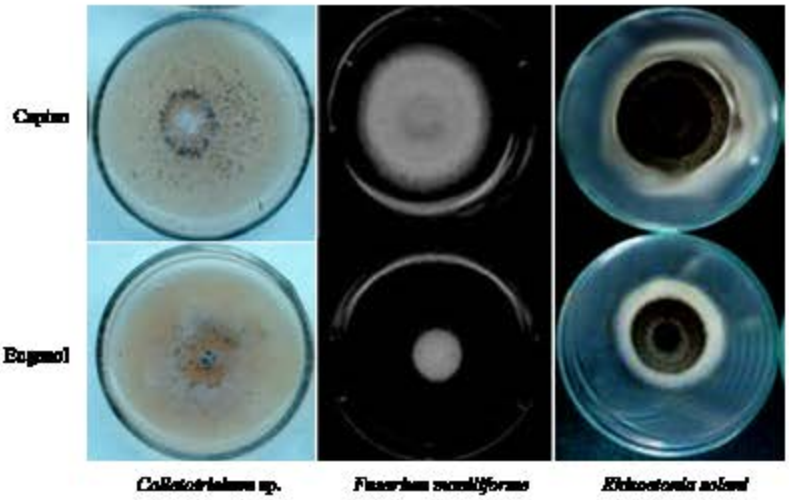

Fig. 6: Comparative the antifungal activity of synthetic fungicide; Captan and eugenol botanical fungicides; Eugenol treated at 1.00% v/v against fungal mycelium growth inhibition

Table 1: The 50% inhibitory concentration (IC_{50}) of botanical fungicides against some species of rice pathogenic fungi

	IC_{50} (% v/v)			
Botanical fungicides	*Alter**	*Collet***	*Fus****	*Rhi*****
Eugenia caryophyllus	0.0087	0.0219	0.1968	0.1894
Acorus calamus Linn.	0.2076	0.2097	0.2220	0.1445
Stemona tuberosa L.	-	-	-	0.0846
Stemona curtisii Hk f.	-	-	-	-
Mammea siamensis Kost.	-	-	-	-

Alternaria solani, **Colletotrichum sp., ***Fusarium moniliforme*, *****Rhizoctonia solani*. IC_{50}: The concentration required to give 50% inhibition of fungal growth, - : Not inhibited

Table 2: The concentration of botanical fungicide; eugenol against rice pathogenic fungi

	Inhibition (%)	
Fungi	0.25% (v/v) Eugenol	1% (v/v) Eugenol
Alternaria solani	100.00	100.00
Aspergillus flavus	44.22	100.00
Aspergillus niger	70.58	100.00
Bipolaris oryzae	86.98	100.00
Curvilaria sp.	50.04	97.04
Fusarium moniliforme	100.00	100.00
Nigrospora sp.	50.04	98.23
Rhizoctonia solani	100.00	100.00

Table 3: Comparative the antifungal activity of *Eugenia caryophyllus* crude extract and eugenol (purified) active compound against rice pathogenic fungi

	Pathogenic fungi inhibition (%)			
Treatment (at 1% v/v)	*Alter**	*Collet***	*Fus****	*Rhi*****
Eugenia caryophyllus crude extract	85.66	77.24	74.42	80.44
Eugenol	100.00	100.00	100.00	100.00

Alternaria solani, **Colletotrichum sp., ***Fusarium moniliforme*, *****Rhizoctonia solani*

germination completely at 0.10% v/v but eugenol significantly inhibited fungal spore germination only at 0.50 or 1.00% v/v (Table 5, Fig. 7).

Table 4: Comparative the antifungal activity of synthetic fungicide; captan and botanical fungicide; eugenol treatment on fungal mycelia growth inhibition

Pathogenic fungi	Treatment	Mycelia growth (cm)	Inhibition (%)
A. solani	Captan	7.25	4.60
	1% Eugenol	7.40	2.63
A. flavus	Captan	7.90	8.13
	1% Eugenol	7.05	18.02
A. niger	Captan	8.25	4.06
	1% Eugenol	7.40	13.95
Bipolaris oryzae	Captan	8.90	1.11
	1% Eugenol	7.95	21.10
Curvilaria sp.	Captan	6.50	8.45
	1% Eugenol	6.45	9.15
F. moniliforme	Captan	7.50	12.28
	1% Eugenol	6.55	23.39
Nigrospora sp.	Captan	6.95	2.82
	1% Eugenol	6.80	6.97
Rhizoctonia solani	Captan	2.60	13.78
	1% Eugenol	2.50	17.06

The antifungal activity of plant crude extracts, or essential oil against pathogenic fungi can be lined up in order from strongest to lowest effectiveness as follows: eugenol>*Eugenia caryophyllus*>*Acorus calamus* Linn.>*Stemona tuberosa* L.> *Mammea siamensis* Kost.= *Stemona curtisii* Hk.f.

At low concentration, *S. tuberosa* L. and *S. curtisii* Hk.f. crude extracts were unable to control all species of fungi. Although, at the highest assayed concentration (1.0% v/v), *S. tuberose* L. extract inhibited only *Rhizoctonia* sp. The previous studies reported that *S. curtisii* Hk.f. and *S. tuberose* L. crude extracts had much stronger insecticidal activity than fungicidal activity (Chantawannakul *et al*., 2005). Furthermore, Issakul *et al*. (2004) found that *A. calamus* Linn. extract showed strong antifungal activity but the *M. siamensis* Kost. crude

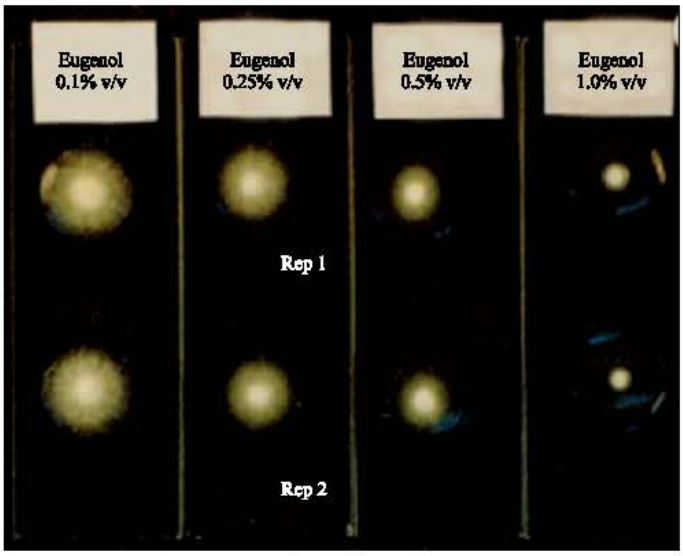

Fig. 7: The antifungal activity at various concentrations of botanical fungicide; eugenol treatment against *Fusarium moniliforme* spores germination, studies showed that eugenol at concentration higher than 0.5% (v/v) can completely inhibit the spore germination

Table 5: Comparative the effect of Captan, *Eugenia caryophyllus* and eugenol treatment on the inhibition of spore germination of some species of rice pathogenic fungi

		Spore inhibition (%)					
Treatment	Conc. (%v/v)	*A. flavus*	*A. niger*	*A. solani*	*F. moniliforme*	*Curvularia* sp.	*R. solani*
Captan	0.10	100.00	100.00	100.00	100.00	100.00	100.00
	0.25	100.00	100.00	100.00	100.00	100.00	100.00
	0.50	100.00	100.00	100.00	100.00	100.00	100.00
	1.00	100.00	100.00	100.00	100.00	100.00	100.00
Eugenol	0.10	54.00	37.59	14.41	21.88	44.94	93.53
	0.25	65.60	65.60	24.16	74.11	85.39	100.00
	0.50	100.00	100.00	65.68	100.00	100.00	100.00
	1.00	100.00	100.00	100.00	100.00	100.00	100.00

extract has no antifungal activity. However, *M. siamensis* Kost was previous found to have a strong insecticidal activity.

The present study found that *A. calamus* Linn. (Sweet flag) has an effective antifungal activity, may be β-asarone and a-asarone are the major constituents in the rhizomes and leaf oils of *A. calamus* Linn (Raina *et al*., 2003). These compounds are provided antifungal active against *Curvularia* sp. and *Alternaria* sp. (Janssen *et al*., 1988). Ghosh (2006) reported the *A. calamus* Linn. crude extract inhibited hyphal growth of *F. moniliforme*, whereas, Lee (2006) found that methanolic extract of *A. calamus* L. had strong fungicidal activity against *R. solani*. Nevertheless, the results suggested that *E. caryophyllus* crude extract had the strongest antifungal activity at its lowest concentration.

The concentration and active compound of plant products are the most important factors in the antifungal activity. Obviously, the results indicated that at high concentration (1.00% v/v) of *E. caryophyllus* crude extract could inhibit the pathogenic fungi. However, in term of spore germination and mycelia growth inhibition, eugenol was found to have stronger inhibition ability than *E. caryophyllus* crude extract, which was in agreement with Viollon and Chaumont (1994). Eugenol is the major phenolic compound of *E. caryophyllus* and have a strong antibacterial and antifungal activity, e.g., against *Aspergillus* sp. and *Fusarium* sp. (Pauli and Knobloch, 1987), as well as against *Alternaria* sp., *Fusarium* sp., *Curvularia* sp. and *Rhizoctonia* sp. (Beg and Ahmed, 2002). The present study established the high effectiveness of eugenol at the Minimum Fungistatic Concentration (MFC) of 0.50% v/v. At concentrations higher than 0.50% v/v, lysis of spore and inhibition of mycelia growth were detected and confirm the results of Neni *et al*. (2006). However, the MFC and toxicity concentration varied from study to study. This is probably due to the different extraction methods of

essential oils and different sensitivity of the test strains used (Saikia *et al*., 2001). The plant extracts showed clear antimicrobial properties, although the mechanistic of action are poorly understood. However, it must be pointed out that the intrinsic activity of a compound is very important for its effectiveness. In this context, the essential oil containing phenolic compound was reported to exhibit a high inhibitory effect (Bennis *et al*., 2004).

The mode of action of antifungal agents depends also on the type of target microorganisms and is mainly related to their cell wall structure and the outer membrane arrangement (Dorman and Deans, 2000). These observations suggested that the physical and chemical properties (solubility and volatility) might have considerably effects on the *in vitro* antimicrobial activity of plant extracts (Inouye *et al*., 2000). Eugenol was found in this study to strong activity due to their relatively low capacity to dissolve in water, which supported by Hilli *et al*. (1997). The effectiveness of eugenol depends on the structure of the phenolics where in previous studies identified as active compounds, e.g., the hydroxyl group and its relative position within the molecule (Tullio *et al*., 2006). High hydrophobic compounds are generally reported to be very effective on the primary site at the cytoplasmic membrane (Sikkema *et al*., 1995). The effect of eugenol when separated from the fungi membranes suggests that its activity is based on the lipophilic properties. The interactions between antimicrobial compounds and cell membranes affect both the lipid ordering and the bilayer stability (Ben-Arfa *et al*., 2006). Their mode of action appeared to be at the phospholipid bilayer, caused by biochemical mechanisms, catalysed by the phospholipid bilayers of the cell and related to the cell membrane disruption. These processes include the inhibition of electron transport, protein translocation, phosphorylation steps and other enzyme-dependent reaction (Knobloch *et al*., 1988). Eugenol is able to inhibit the respiration and ion transport processes, increase membrane permeability and the releasing of cellular content (Uribe *et al*., 1985). Moreover, eugenol is able to inhibit the respiration of cell suspensions and to disrupt the permeability barrier of microbial membrane structure (Cox *et al*., 2000).

Morris *et al*. (1979) reported that the fungi after treated with eugenol, decreased its size, appeared in irregular shape with cell wall modifications and the cell surface depressions. Such modifications may be related to the interference of the oil components with enzymatic reactions of cell wall synthesis, which affects fungal morphogenesis and inhibited growth.

According to Helal *et al*. (2006), the antifungal activity of eugenol may take place via two steps. The first step involves the passive entry of the oil into the plasma membrane in order to initiate cell membrane disruption. The second step, the accumulation of oil in the plasma membrane results in the inhibition of cell growth. This can be ascribed as combination of membrane effects such as increased bi-layer disorder and ion leakage. These effects disturb the osmotic balance of the cell through the loss of ions, making its membrane associated proteins inefficient due to increased membrane disorder eventually leading to inhibition of cell growth. The cytoplasmic, plasma and mitochodrial membrane of fungal provide a barrier to the passage of small ion such as H^+, K^+, Na^+ and Ca^{2+} and allow cells and organelles to control the entry and exit of different compounds (Suhr and Nieken, 2003). This permeability barrier role of cell membranes is integral to many cellular functions, including the maintenance of the energy status of the cell, other membrane-coupled energy-transducing process, solute transportation, regulation of metabolism and control of turgor pressure (Trumpower and Gennis, 1994). Cox *et al*. (2000) observed the leakage of the K^+, Ca^{2+} and Mg^{2+} from exposed fungal cells with eugenol. Some changes in the cell membrane may occur in spite of the damage to the plasma membrane. These changes are accompanied with the loss of chemiosmotic control disrupted the permeability barrier of cell membrane structure. Ultee *et al*. (2002) hypothesized that the hydroxyl group and the presence of a system of delocalized electrons are important for the antimicrobial activity of the eugenol. Such a particular structure would allow compounds to act as proton exchanger, thereby reducing the pH gradient across the cytoplasmic membrane. Eventually, the reduction of the proton motive force and the depletion of the ATP pool lead to the cell death. Hence, these findings supported our results that eugenol was identified as the best antifungal effect of all studied plant extracts.

CONCLUSION

The crude plant extracts from *Stemona curtisii* Hk.f., *Stemona tuberosa* L. and *Mammea siamensis* Kost. did not have an antifungal activity. However, high concentrations of *Acorus calamus* Linn. and the *Eugenia caryophyllus* crude extracts can inhibit the studied pathogenic fungi. Eugenol, an active compound of *Eugenia caryophyllus*, showed the strongest antifungal activity in inhibiting the growth of pathogenic fungi. The mode of action based on the deterioration of the fungal cellular structure leading to complete cell death, even at lower concentration of eugenol. Therefore, based on the antifungal activity against pathogenic fungi, the following order from strongest to lowest effectiveness was found:

eugenol > *Eugenia caryophyllus* > *Acorus calamus* Linn. > *Stemona tuberosa* L. > *Mammea siamensis* Kost. = *Stemona curtisii* Hk.f. This finding increases the possibility of exploiting eugenol as a promising candidate for safe natural antifungal agent.

ACKNOWLEDGMENTS

Authors acknowledge the Nation Research Council of Thailand (NRCT), Royal Thai Government, for research financial supported of the present studies, gratefully thanks to Dr. Suchada Vearasilp, Prof. Dr. Elke Pawelzik, Prof. Dr. Udo ter Meullen, Prof. Dr. Wolfgang Lüke for theirs are valuable suggestions through out the research project.

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