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PHYTOCHEMICAL SCREENING OF ANTIFUNGAL BIOCOMPOUNDS FROM FRUITS AND LEAVES EXTRACT OF *Cerbera odollam* GAERTN

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ABSTRACT

Nowadays, heavy usage of fungicide in agricultural industries has resulted in environmental pollution in additional to expose a significant risk to human health. Hence, there is a need to develop alternatives to replace synthetic fungicides. In this research, phytochemical test was carried out on both fruits and leaves of *Cerbera odollam* Gaertn using standardized procedures to determine the antifungal compounds present in the ethanol extracts. Antifungal bioassay was performed through Kirby-Bauer disc diffusion method at various weight (0.5mg to 100mg) against fungi: *Aspergillus niger*, *Fusarium oxysporum* and *Penicilium citrum*. Fungi assay was assessed based on the minimum inhibitory amount of both ethanol extracts. The results of the research showed the presence of the active compounds such as alkaloid, cardiac glycoside, phenol, steroid, tannin and terpenoid in the extracts. Leaves extracts were found to have more phytoconstituents as compared to the fruits extracts. Besides, antifungal activity of leaves extract had moderate antifungal effects against *Aspergillus niger* (13.40mm inhibition zone) and *Penicilium citrum* (15.73mm inhibition zone). However, *Fusarium oxysporum* did not exhibit inhibition zone with this extract. Leaves extract showed best antifungal activity against *Penicilium citrum*, with the lowest dosage (<1mg) when compared to others. Meanwhile, fruits had showed weak antifungal activity (below 11.00mm inhibition zone) for all the tested fungi. The study on *Cerbera odollam*'s fruits and leaves extract indicated that they have an antifungal potential to be used as an alternative to synthetic fungicides. Investigation for its active biocompounds could be exploited further.

Key words: *Cerbera odollam*, fruits, leaves, phytochemical screening, antifungal

INTRODUCTION

Post-harvest diseases caused by *Fusarium*, *Alternaria* and *Penicilium* had led to significant damage and economic losses in fruits and vegetable industries (Coates & Johnson, 2013). The predisposing factors for the disease incidence are normally mechanical wounds or cuts produced during agricultural practices (Coates & Johnson, 2013).

Basically, agricultural diseases are largely controlled by chemical fungicide. Fungicides are chemicals agents or substances meant for inhibiting the growth or to kill fungi. They are extensively used in the agricultural industry to protect crops from diseases. Fungicide such as Imazalil, Benomyl, and Thiabenzole are extensively applied for post harvest disease control in fruits and vegetables (Harbant *et al*., 2011). However, prolonged usage of these chemicals are found to leave a residual effect in food for human consumption. Hence, the negative effects of synthetic fungicide has urged the market to look for the alternatives for it such as plant extracts (botanicals) or biological control (Harbant *et al*., 2011).

Cerbera odollam Gaertn belongs to Apocynaceae family and it is commonly known as "Pong-Pong tree" in English or "Buta-buta" in Malay. In peninsular Malaysia, *Cerbera odollam* are distributed mostly at the coastal mangrove swamps; sometimes found inland as a roadside tree at Perlis, Kedah, Perak, Selangor, Melaka, Pahang and Johor (Kiew *et al*., 2011). This species are able to flower and fruit throughout the year. In the past decade, *Cerbera odollam* has been widely studied for its effects as antiproliferative, anticancer, anti-estrogenic, antimicrobial, antinociceptive and sedative effect of different dosage no matter *in vitro*

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or *in vivo* (Ahmed *et al*., 2006; Ahmed *et al*., 2008; Shen *et al*., 2007). Useful phyto-constituents have been reported present and have potential of having anti-microbial effect (Shen *et al*., 2007; Cowan, 1999). Thus, the potential of *Cerbera odollam* as a replacement to conventional fungicide in agricultural management led the author to pursue in its anti-fungal effect in post harvest diseases. In this study, the objective were to explore the phytochemical constituents of the extracts and also to determine the antifungal activities of the extracts of fruits and leaves from *Cerbera odollam*.

MATERIALS AND METHODS

Plant materials collection and extraction

Fresh samples of the fruits and leave of *Cerbera odollam* were collected at Pengkalam Asam, Perlis, Malaysia, from January until March 2014. Samples were ground into small pieces before dried. 10g of dried materials from each sample was further extracted with ethanol for 6 hours (Hashim *et al*., 2009). The extract was then filtered and concentrated under reduced pressure. The crude extracts were kept in the refrigerator at 4°C until further used.

Phytochemical test

Phytochemical analysis for each sample's crude extract was carried out according to the standard procedure methods as previously described (Alabri *et al*., 2014; Mandal *et al*., 2013):

Test for alkaloids

1mL of crude extract was mixed with 5mL of diluted hydrochloride acid (HCL) and was placed in a water bath at 60°C for 15 minutes. 1mL of Wagner's reagent was added into 1mL of filtered suspension. Appearance of reddish-brown precipitate showed the presence of alkaloids.

Test for anthraquinone

1mL of diluted Ammonia was added into crude extract. Pink-red color in the lower layer confirms the presence of anthraquinone.

Test for cardiac glycoside

Crude extract was treated with 1mL of glacial acetic acid diluted (3%) ferric chloride ($FeCl_2$) follow by adding concentrated sulphuric acid (H_2SO_4) at the side of the test tube. The formation of brown ring indicated the presence of cardiac glycoside.

Test for flavonoid

Intense yellow color formed when 1mL of sodium hydroxide (NaOH) solution was added into 1mL of crude extract. The yellow color turned colorless upon addition of diluted HCL, which indicated the presence of flavonoid flavoniod.

Test for phenol

2mL of crude extract was boiled in water bath at 45 to 50°C for 5 minutes. Then, 2mL of 3% $FeCl_2$ was added into the suspension. Formation of blue or green grey colour indicated the presence of phenol.

Test for saponin

0.5mL of crude extract was diluted with 5mL of distill water. The suspension was shaken vigorously for a few minutes. Development of foam which is able to persist for 10 minutes shows the presence of saponin.

Test for steroid

Crude extract was treated with 2mL of acetic anhydride and a few drops of concentrated H_2SO_4. Formation of dark green or blue confirms the presence of steroid.

Test for tannin

A drop of 10% $FeCl_2$ was added to 2mL of crude extract. Development of dark blue or greenish grey showed the presence of tannin.

Test for terpenoid

0.5mL of crude extract was treated with 1mL of concentrated H_2SO_4. Formations of reddish brown confirm the presence of terpenoid.

Antifungal assay

The antifungal activities of each crude extracts was evaluated against fungus using Kirby-Bauer disc diffusion assay method (De Beer & Sherwood, 1945). The plant extracts (fruit and leaves) were treated with ethanol and pipetted out onto autoclaved 6mm Whatman Paper discs for the bio-assay. The discs were left in the laminar flow for 1 hour in order to let the solvent evaporate, leaving behind the extracts on the paper discs. The dried fruits and leaves extracts (in mg) on the discs were used as the weight of the extracts (Hashim *et al*., 2009).

The weights of the dried extracts were 100mg, 50mg, 10mg, 5mg, 1mg, and 0.5mg. *Aspergillus niger* (AY 939783.1), *Fusarium oxysporum* (JF 740775), *Penicilium citrum* (HQ 696034) were cultivated at 25±3°C for 5 to 7 days on Potato Dextrose Agar (PDA) medium. Spores were counted with haemocytometer and adjusted to $1x10^7$ spore/mL before performing the experiment (Dhillion *et al*., 2014). The preparation of the test plate techniques was modified from Edwin and De Beer method (De Beer & Sherwood, 1945; Raahave,

1974). Each plate of growing fungus was scraped with spatula and mixed with a fresh autoclaved PDA solution.

The mixed agars were poured into the petri dish and left to solidify in laminar flow for 30 minutes before ready to be used for the antifungal test. Paper disc treated with crude extracts were placed on the mixed agar and incubated at 25±3°C for7 days. Benomyl (commercial fungicide) and ethanol were used as a positive and negative control for the test. Antifungal activities of each extract were evaluated after 24 hours for 7 days. All the experiment were done in triplicates. Zone of inhibition was measured in diameter and analyzed using Sigma Scan Pro software. Data collected were analyzed using ANOVA.

RESULTS AND DISCUSSION

Phytochemical analysis

Phyto-constituents in natural plant were reported to have active compounds which are responsible for biological activities such as antimicrobial, anticancer or antioxidant (Cowan, 1999; Hilal *et al*., 2014). Presence of alkaloid, phenol and tannin are an indication of the ability of plants in antimicrobial activity (Cowan, 1999). Table 1 shows the phytochemical analysis for both fruits and leaves crude extracts of *Cerbera odollam*. Phytochemical screening results showed that steroid, tannin and terpenoids were reported to be present in the dried fruits and leaves.

On the other hand, alkaloid and phenol only existed in leaves extracts while cardiac glycoside was found in fruit extracts. Result of current investigation agreed with previous study which revealed the existence of alkaloid and tannins, but inexistence of flavonoid in seed extract of *Cerbera odollam* (Ahmed *et al*., 2008).

Antifungal Activities

Table 2 shows the antifungal activity of fruits and leaves crude extract of *Cerbera odollam* against *Aspergillus niger, Fusarium oxysporum and Penicilium citrum* at 100mg since the other treatment (50mg, 10mg, 5mg, 1mg, and 0.5mg) did not exhibit clear measuring zone. Statistical analysis showed weak significant difference ($P \leq 0.05$) between the interaction of different parts of extracts and fungi. Antifungal effects of leaves extracts towards *Aspergillus niger* and *Penicilium citrum* were highest between the extracts. They were reported to have "moderate" inhibitory activity with inhibition zone of 13.40mm and 15.73mm.

However, there was no antifungal activity of leave extracts reported on the inhibition of *Fusarium*

Table 1. Phytochemical analysis of both fruits and leaves extracts of *Cerbera odollam*

Phyto-constituents	Observation	
	Fruits extracts	Leaves extracts
Alkaloid	–	+
Anthraquinone	–	–
Cardiac glycoside	+	–
Flavonoid	–	–
Phenol	–	+
Saponin	–	–
Steroid	+	+
Tannin	+	+
Terpenoid	+	+

Presence (+); Absence (–)

Table 2. Antifungal activities of 2 different crude extract (fruits and leaves) of *Cerbera odollam* against *Aspergillus niger, Fusarium oxysporum and Penicilium citrum* at 100mg

Fungal	Positive Control/ Benomyl (mm)	Negative control/ Ethanol (mm)	Inhibition zone (mm)	Inhibitory activity
Aspergillus niger				
Fruit	22.12	–	10.72 ± 1.19	Weak
Leave	22.12	–	13.40 ± 0.43	Moderate
Fusarium oxysporum				
Fruit	22.62	–	9.39 ± 9.39	Weak
Leave	22.62	–	–	Negative
Penicilium citrum				
Fruit	55.68	–	8.67 ± 0.76	Weak
Leave	55.68	–	15.73 ± 1.31	Moderate

*Zone of inhibition-strong= >17mm, moderate= 12 to 16mm, weak= 7 to 11mm, negative= 6 or 0mm (Segismundo, 2008)

Table 3. Minimum inhibitory amount of two different crude extract (fruits and leaves) of *Cerbera odollam* against *Aspergillus niger, Fusarium oxysporum and Penicilium citrum*

Parts	Minimum Inhibition Amount (mg)		
	Aspergillus niger	*Fusarium oxysporum*	*Penicilium citrum*
Fruits	5	50	5
Leaves	50	–	1

oxysporum. This fungus seems to be tolerant to the active bio-compounds present in leaves extracts as it is highly diverse and resistance to many antifungal agents (Barik *et al*., 2011). Next, fruits extract had weak antifungal activities for all the fungi with inhibition zones between 7mm to 11mm. Weaker activities may due to fewer active compounds present in the crude extracts. This result agrees with previous study regarding the failure in recording significant antimicrobial effect in fruits of *Cerbera odollam* (Shankar *et al*., 2009).

Minimum inhibitory amount is the lowest weight of extract (in mg) with positive antifungal activity (Hashim *et al*., 2009). The minimum inhibitory amount for crude extracts from fruits and leaves against *Aspergillus niger, Fusarium oxysporum and Penicilium citrum* were observed and tabulated in Table 3. Fruit extracts denoted a better performance in obtaining a minimum amount of 5mg for *Aspergillus niger* and 50mg for *Fusarium oxysporum* suppression as compared with leaves extracts. On the other hand, leaves extracts showed better antifungal activity (15.73mm) than fruits extracts (8.67mm) in attaining lowest minimum inhibitory amount value (1mg) for *Penicilium citrum*. Furthermore, the minimum inhibitory amount value of leaves extract in our study for *Penicilium citrum* was lower than previous studies (Atiyah, 2014) where the author obtained a higher minimum inhibitory concentration value (3.125 mg/L). The improved result is probably due to the difference in techniques or approaches applied during the experiment.

CONCLUSIONS

In the current research, phytochemical screening denoted that steroid, tannin and terpenoids were reported to be present in both fruits and leaves extracts. Crude extracts of leaves had more phyto-constituents and higher inhibitory effects compared to fruits' extract. Hence, the bio-compounds are related to their ability in antimicrobial activity. Besides, crude extracts of fruits and leaves do have positive effects on inhibiting towards fungi in antifungal bioassay, except for leaves extracts on *Fusarium oxysporum* combation. Thus, application of natural plants to control post harvest fungi in agricultural industry is possible and could also be an emerging technology to be used as an alternative to synthetic fungicides as eco-friendly and pollution free bio-fungicide.

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