Effects of Garden Balsam (*Impatiens balsamina* L.) Extracts on Fungal Infection of Rice

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ABSTRACT

Garden balsam (*Impatiens balsamina* L.) is widely used as an ornamental plant in landscaping. Different parts of this plant are used traditionally to treat skin diseases. The objective of this study is to determine the effects of ethanol crude extract of garden balsam on rice disease and its total phenolic and flavonoid content. There are many rice diseases in Thailand caused by fungi; i.e dirty panicle disease (*Curvularia lunata*), brown spot disease (*Bipolaris oryzae*) and rice blast disease (*Pyricularia oryzae*). Screening of the antifungal activity of brown spot disease (*Bipolaris oryzae*) and dirty panicle disease (*Curvularia lunata*) with extracts from six parts of this plant was conducted by a disk diffusion test. The most active extract was from the pericarps of garden balsam, which inhibited *Curvularia lunata*. The inhibition diameter was 4 6 mm. The pericarp extract concentration was 1 1 . 3 µg/µl which showed the total phenolic compound 13.9 ± 0.5 (mg GAE/g cw), while the flavonoid content of pericarp was 33.4 ± 0.9 (µg QE/mg cw), the extract concentration was 11.3 µg/µl.

Keywords: Garden balsam, plant extracts, antifungal activity, phenolic compounds, and flavonoid compounds.

INTRODUCTION

Rice is the primary staple food for Thai people and the most important economic crop in Thailand. However, rice disease is the main problem in rice production impacting both yield and quality of rice. Farmers lose an average 37% of their crops to pests and disease every year (Rice Knowledge Bank website). Dirty particle disease is one of the main problems causing a reduced price for rice grains. Farmers have to constantly use large amounts of pesticide to control this rice disease. Intensive and indiscriminate use of pesticides in rice production has caused many problems for the environment such as soil, animal and food contamination and the poisoning of famers and consumers alike. Minimizing the negative effects of pesticides has been the goal of the development of alternative controls of rice disease, which includes the use of plant extracts as biological controls. Garden balsam (*Impatiens balsamina* L.), a member of the family Balsaminaceae, has been used as traditional medicine for a long time, i.e. treating skin diseases, ingrown nails and burns. Garden balsam extract has antifungal activity (Rajendran *et al*., 2014).

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Moreover, there are many studies using garden balsam extract to reduce fungal diseases (Rajendran *et al*., 2014; Yang *et al*., 2001). Garden balsam has the potential to be developed as a biological agent to control fungi. However, there are no studies using garden balsam extract against fungi disease in rice. To gain this information about the antifungal activity of the garden balsam extract, we examined the ability of the garden balsam extract against rice disease.

Garden balsam extract chemical compounds have been studied, and their extracts have been found to contain many functional compounds such as flavonoids, alkaloids and steroids (Manikandan e*t al*., 2016; Sakunphueak *et al*., 2013) . The presence of phenolic compounds in plants is responsible for antioxidant and antifungal activity (Herna´ndez *et al*., 2000) . This leads to the question whether phenolic compounds in garden balsam are related to antifungal activity. To gain further insight on this topic, we determined the phenolic compounds in different parts of garden balsam to study their relationships with antifungal activity. Flavonoid compounds are believed to promote physiological survival of the plant, protecting it from, for example, fungal pathogens and UV-B radiation (Harborne and Baxter, 1999; Harborne and Williams, 2000). This leads to the investigation of flavonoid contents in garden balsam related to the antifungal activity of this plant's extracts.

MATERIALS & METHODS

Plant samples and extraction

Garden balsam (*Impatiens balsamina* L.) samples are grown in greenhouses at the Department of Botany, Faculty of Science, Kasetsart University. Leaves, stems, roots, flowers, pericarps and seeds of garden balsam were collected after the plants were full grown for the plant extraction experiment.

Fresh samples (leaves, stems, roots, flowers, pericarps and seeds) were washed and padded to dry. Three grams of samples were ground and extracted with 500 ml of 95% ethanol. Samples were ground until homogenous suspension occurred. The mixtures of samples were macerated and shaken at room temperature for 7 days. The extracts were then filtered with Whatman No. 1 filter paper and evaporated by a rotary evaporator. After that, the samples were collected into vials and kept at 4 °C to preserve the extracted materials. (Figure 1)

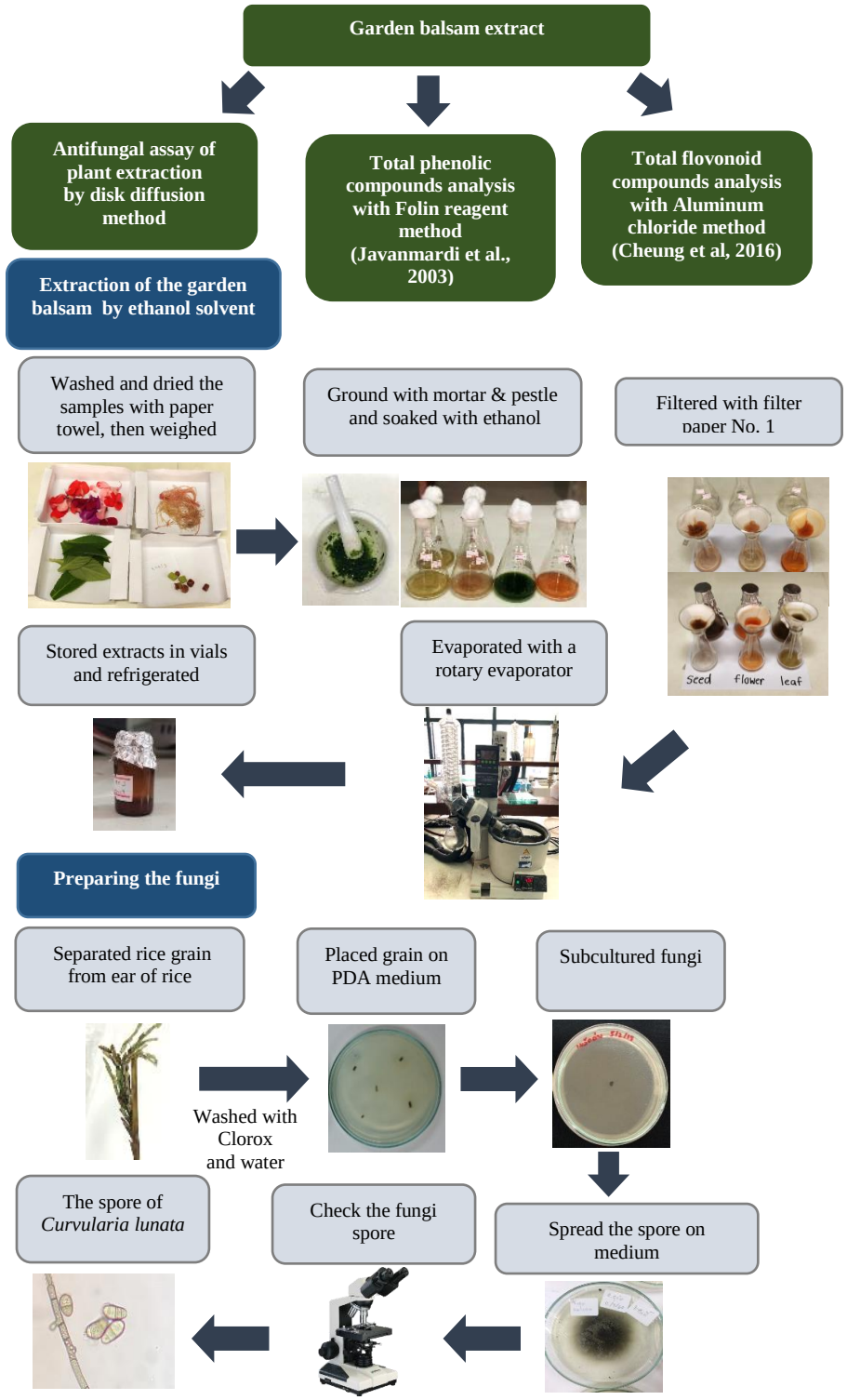

Figure 1 Flowchart of experimental method

Fungi of rice disease preparation

Rice disease samples were collected from rice leaves and grains. For the brown spot disease, leaves were cut with a wound of rice disease by half of healthy and half wound tissue. Then, the leaves were cut into small pieces 2x1 mm. In contrast, the rice grains were separated from the panicle and used for the following procedure in dirty panicle disease. The samples were washed with Clorox 1 time and sterile water 2 times, then the samples were placed on filter paper. After that, the leaf samples were cultured on PDA medium and incubated 3 days at room temperature. The fungi of interest were selected and cultured into PDA medium as a pure culture and incubated 3 days before stimulating spores by spreader. They were then incubated at 25 °C for 6 days before antifungal essay. Fungi spores were investigated under the microscope before antifungal testing (Figure 1).

Antifungal assay

Disk diffusion assays were used for antifungal testing activity. Plant extracts of different parts of garden balsam were filled into disks of filter paper No.1 and air dried. Fungi spore samples were suspended in 1 ml of sterile water and spread on PDA medium. Then, 3 replications of disks of plants extracts were placed on the medium and arranged as shown in Figure 2. The Petri dishes were incubated at room temperature for 3 days, and the inhibition zone was observed. The inhibition zone showed the ability of the antifungal plant extraction.

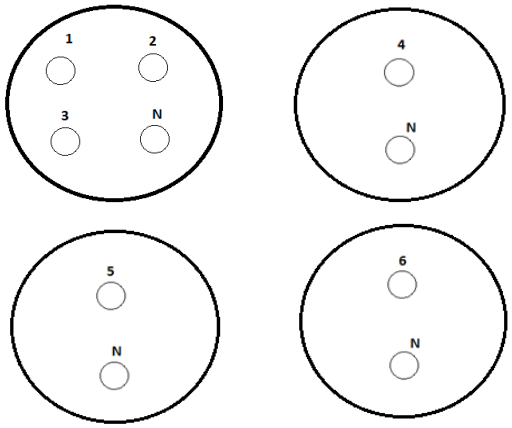

Figure 2 The disk diffusion of plants extracts were placed on the PDA medium. Numbers 1-6 were the extracts of different plant parts.
1) roots 2) stems 3) leaves 4) flowers 5) seeds 6) pericarps and N) Negative control (95% of ethanol)

Determination of total phenolic content

The amount of total phenolics in the ethanol extract of garden balsam (*I. balsamina*) leaves, stems, roots, flowers, pericarps and seeds were determined with Folin-Ciocalteu reagent using the method of Javanmardi et al. (2003). Gallic acid was used as a standard, and the total phenolics were expressed as mg/g gallic acid equivalents (GAE). For this purpose, the calibration curve of gallic acid was drawn. One milliliter of standard solution of concentration 50, 100, and 200 mg/ml of gallic acid were prepared in ethanol. Fifty microliters of extract solution or standard was introduced into test tubes and mixed with 2.5 ml of a 10-fold dilute Folin-Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The tubes were covered with parafilm and allowed to stand for 30 minutes at room temperature before the absorbance was at read at 765 nm spectrometrically. Results were expressed as milligrams of gallic acid equivalent per gram of crude weight (mg GAE/g cw).

Determination of total flavonoid content

Aluminum chloride method was used for flavonoid determination (Cheung et al, 2016). In this method Quercetin was used as the standard, and flavonoid contents were measured as quercetin equivalents. For this purpose, the calibration curve of quercetin was drawn. Four hundred microliters of standard (20, 40, 60, 80,100 mg/l) or extract solution was used. Three hundred microliters of 5% NaNO2 was added to the test tubes. After 5 min, 300 µl 10% AlCl3 w added to the mixture. At the 6^{th} min, 2 ml of 1 M NaOH were added. Then, the solution was incubated at room temperature for 15 min, and the absorbance was noted at 510 nm using UV-Visible spectrophotometer. Results were expressed as micrograms of quercetin equivalent per gram of crude weight (µg QE/mg cw).

RESULTS AND DISCUSSION

Antifungal assay of plant extraction

The disk diffusion method for antifungal testing was performed to determine the antifungal activity of the garden balsam extracts against the brown spot disease (*Bipolaris oryzae*) and the dirty panicle disease (*Curvularia lunata*). There were no inhibition zones for *Bipolaris oryzae* from all extracts of garden balsam (Table 1). The extracts from roots, stems, leaves, flowers and seeds had no inhibition zone on *Curvularia lunata* (Table 1 and Figure 3 A-C). Therefore, it was concluded there was no antifungal activity of the extracts from roots, stems, leaves, flowers and seeds. However, the extracts from pericarps showed an inhibition zone. The inhibition diameter was equal to 46 mm. The inhibitory concentration was 0.348 mg/ µl (Figure 3 D).

Recently, no reports found that garden balsam extract can inhibit *Curvularia lunata*. However, extracts of cinnamon (*Cinnamomum zeylanicum*), thorn apple (*Datura stramonium*) and rooster tree (*Calotropis procera*) have been found to inhibit *Curvularia lunata* (Gurjar *et al*., 2012; Mishra *et al*., 2009). The groups of compounds found in these plants contained phenolic compounds such as phenols, polyphenols and flavonoids (Mishra *et al*., 2009). Phenolic compounds are a

secondary metabolite that plants synthesize to respond to biotic and abiotic stress (Balasundram *et al*., 2005; Cheng *et al*., 2007). Moreover, the position and number of hydroxyl groups of the aromatic ring make phenolic compounds which can inhibit the growth of microorganisms such as fungi (Gurjar *et al*., 2012). To gain further information whether phenolic compounds in garden balsam is related to the antifungal activity, we determined the phenolic compounds in different parts of the garden balsam to study their relationship with antifungal activity.

Flavonoid compounds are believed to promote physiological survival of the plant, protecting it from, for example, fungal pathogens and UV-B radiation (Harborne and Baxter, 1999; Harborne and Williams, 2000). This leads to the investigation of flavonoid contents in garden balsam related to the antifungal activity of this plant's extracts.

Table 1 Antifungal assay of garden balsam extracts against brown spot disease (*Bipolaris oryzae*) and dirty panicle disease (*Curvularia lunata*).

Fungi	Garden balsam extracts					
	roots	stems	Leaves	flowers	pericarps	seeds
Bipolaris oryzae	**X**	**X**	**X**	**X**	**X**	**X**
Curvularia lunata	**X**	**X**	**X**	**X**	✓	**X**

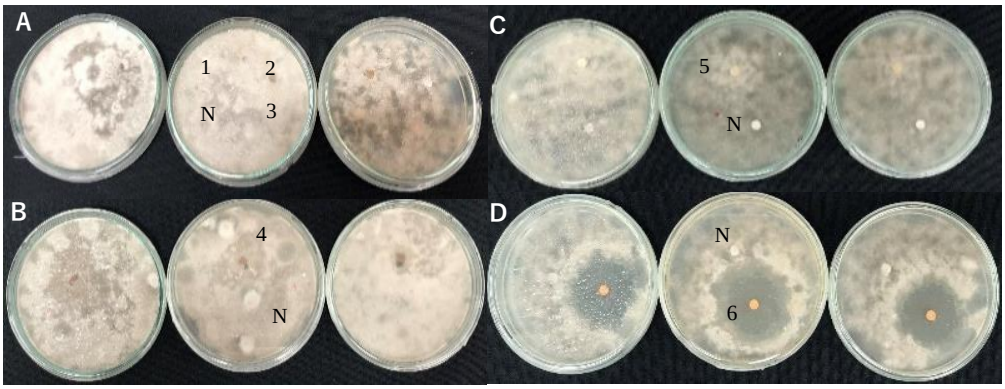

Figure 3 Antifungal assay of the extracts from 6 parts of garden balsam
A: the extract from roots (1), stems (2) and leaves (3), B: flowers (4), C: seeds (5) and D: pericarps (6) N: ethanol 95%)

The total phenolic compound analysis

The amount of total phenolic compounds in extracts was determined with the Folin-Ciocalteu reagent (Javanmardi *et al*., 2003). Gallic acid was used as a standard compound, and the total phenolic compounds were expressed as ppm Gallic acid equivalent (GAE) using the standard curve equation: y = 0.0016x + 0.0021, R^2=0.9997. The pericarp extract concentrations were 11.3, 8.5 and 6.8 mg/µl, and the total phenolic compounds were 157.31, 126.48 and 97.72 ppm GAE respectively (data not shown). The maximum phenolic content was found in the flower extract (88.34 ± 1.43 g/mg GAE) (Table 2). This was followed by the extract from leaves, pericarps, seeds, stems and roots, respectively (Figure 4 and Table 2). A significant difference ($p<0.05$) was found for the total contents among flowers, leaves and pericarps. The pericarps had a significantly lesser amount of phenolic compounds than flowers and leaves.

Table 2 Total phenolic compound analysis

Treatment	Concentration of extracts (µg/µl)	Total phenolic content (mg GAE/g cw)
Flowers	1.9	88.3 ± 1.4a
Leaves	2.9	55.7 ± 2.8b
Pericarps	11.3	13.9 ± 0.5c
Seeds	6.9	11.5 ± 0.2cd
Stems	8.1	7.4 ± 0.3de
Roots	7.2	4.3 ± 0.0e

*Significant difference ($p < 0.05$) when compared within the same column. Mean values followed by the same letter within the column are not significantly different.

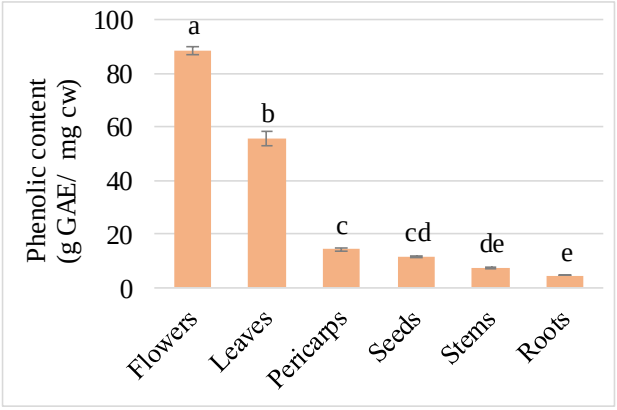

Figure 4 Total phenolic compound in garden balsam

The total flavonoid compound analysis

The total flavonoid contents in extracts was determined with aluminum chloride reagent (Cheung et al, 2016). Quercetin was used as a standard compound, and the total flavonoid contents were expressed as ppm Quercetin equivalent (QE) using the standard curve equation: $y = 0.0009x + 0.0065$, $R^2=0.9893$. The pericarp extract concentrations were 4.35, 3.48 and 2.9 µg/µl, and the total flavonoid compounds were 145.4, 122.5 and 97.8 ppm QE, respectively (data not shown). The maximum flavonoid compound was found in the leaf extract (43.5 ± 0.5 µg QE/mg cw) (Table 3). This was followed by the extract from pericarps, seeds, roots, flowers, and stems, respectively (Figure 5 and Table 3). A significant difference ($p<0.05$) was found for the total contents among leaves, pericarps and seeds. The pericarps had a significantly lesser amount of flavonoid compounds than the leaves.

Table 3 Total flavonoid compound analysis

Treatment	Concentration of extracts (µg/µl)	Total flavonoid content (µg QE/mg cw)
Leaves	2.9	43.5 ± 0.5 a
Pericarps	11.3.	33.4 ± 0.9 b
Seeds	6.9	17.4 ± 0.6 c
Roots	7.2	15.9 ± 1.1 cd
Flowers	1.9	14.6 ± 0.5 d
Stems	8.1	7.5 ± 0.4 e

*Significant difference ($p < 0.05$) when compared within the same column. Mean values followed by the same letter within the column are not significantly different.

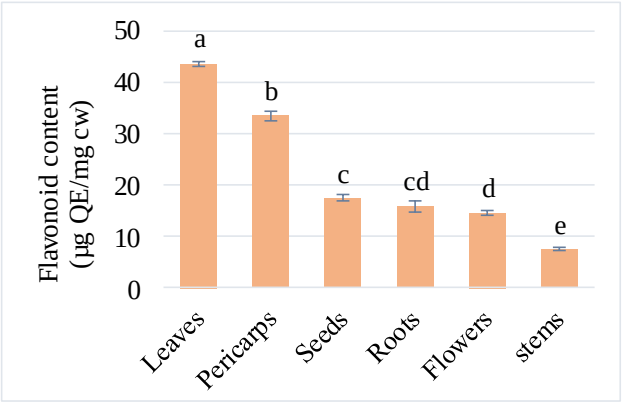

Figure 5 Total flavonoid content in garden balsam

Flavones, flavonoids and flavonols are derivative of phenolic compounds comprised of phenols and carbonyl group. There are 2 groups of flavonoids involved in plant defense mechanisms--preformed and induced compounds. Preformed flavonoids are innate compounds which are synthesized during the normal development of plant tissue. The induced compounds are synthesized by plants in response to physical injury, infection or stress. Flavonoids in plant play an important role by signaling transduction or the main function of the defense mechanism (Treutter, 2005). The role of flavonoids in resistance against fungi is related to the following mechanisms: cross-linking of microbial enzymes; inhibition of microbial cellulases, xylanases, and pectinases; chelation of metals necessary for enzyme activity; and/or formation of a hard, almost crystalline, structure as a physical barrier against pathogen attack (Treutter, 2006). Moreover, Padmavati *et al.* (1997) found that naringenin, kaempferol, quercetin and dihydroquercetin can inhibit the growth of *Pyricularia oryzae* which causes the rice blast disease.

Pericarp extracts of garden balsam were composed of balsaminone A, balsaminone B and 2-methoxy 1,4naphthoquinone. 2-methoxy 1,4-naphthoquinone. (Ishiguro *et al*., 1998; Tandon *et al*., 2004). The main contents of leaf extracts of garden balsam (*I. balsamina* L.) are napthoquinones, which are lawsone, methyl ether and methylene-3, 3'-bilawsone. These three naphthoquinones can inhibit dermatophyte fungi and *Candida albicans* (Sakunphueak and Panichayupakaranantab, 2012). Moreover, Skadhauge *et al*. (1997) showed that flavonoid is a strong inhibitor of *Fusarium* growth and macrospore formation.

The results showed that the pericarp extract can inhibit *Curvularia lunata*. However, the flavonoid contents in pericarps was lesser than in leaves. According to this result, the pericarp extract was still shown to have higher flavonoid contents than other parts of garden balsam. This flavonoid might affect the fungal growth of *Curvularia lunata*. According to Stevenson and Haware (1999) the maackiain (isoflavonoid) was increased in *Cicer bijugum.* This resistant strain of chickpea contains higher maackiain than is found in a susceptible strain--*Cicer arietinum*--which was susceptible to *Botrytis cinerea*. Maachkiain can inhibit the spore germination of fungi (*Botrytis cinerea*). So, this isoflavonoid was an important component of fungal resistance in wild chickpeas. However, further study should examine how pericarp extracts can inhibit the growth of *Curvularia lunata*. In order to verify which flavonoid compounds can inhibit the growth of fungi, further study of chemical compounds in the garden balsam is needed. High Performance Liquid Chromatography (HPLC) technique could be used to analyze the compound in garden balsam and study the relationship of their antifungal activity.

CONCLUSIONS

Pericarp extracts of garden balsam can inhibit *Curvularia lunata*. The inhibition diameter is equal to 46 mm, and the inhibitory concentration is 0.348 mg/ µl. Therefore, the pericarp extract is a potential agent for developing an inhibitor for the dirty panicle disease in rice. However, the total phenolic compounds in pericarps is less than in the extract from flowers. The pericarps also had a significantly lesser amount of flavonoid compounds than did the leaves. It is possible that the main

factor of inhibiting growth of *Curvularia lunata* could be involved with other chemical compounds in the pericarp. Further research is needed to determine these factors. For example, HPLC analysis of pericarp extract could provide the explicit compounds in pericarp.

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