Available online at https://www.rsu.ac.th/rjas

Rangsit Journal of Arts and Sciences, January-June 2016 RJAS Vol. 6 No. 1, pp. 17-21

ISSN 2229-063X (Print)/ISSN 2392-554X (Online)
DOI : 10.14456/rjas.2016.2

Allelopathic potential of *Citrus reticulata* peel extract for weed control

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Submitted 27 May 2015; accepted in final form 29 July 2015
Available online 21 June 2016

Abstract

The potential use of tangerine orange (*Citrus reticulata*) peel extract for controlling barnyard grass (*Echinochloa crus-galli*) was investigated. Crude extracts of dried powdered peel with 70% methanol before and after chlorophyll removal were compared. The crude extract without chlorophyll showed greater inhibition efficiency of seed germination than the extract containing chlorophyll. Moreover, the extract of chlorophyll-lacking fraction was shown to contain ABA, a known growth inhibitor in various plant species. This fraction also showed higher concentration of ABA than before chlorophyll removal which greatly correlated to the weeds germination inhibition test. This work suggested the ability of the crude extract of *Citrus reticulata*'s peel to control weed growth could be enhanced by chlorophyll removal.

Keywords**:** *Citrus reticulata, Echinochloa crus-galli, peel extract, weed control, allelopathy*

1. Introduction

Allelopathy is a biological phenomenon that exhibits any direct or indirect harmful effects by one plant and microorganism on another through the production of chemical compounds that escape to the environment (Rice, 1984). Recently, studies on the utilization of allelopathic substances, as natural products for weed control, have been documented in various plant species (Narwal, 1999; Duke, Dayan, Romagni, & Rimando, 2000; Kato-Noguchi & Seki, 2010).

According to Kato-Noguchi (2003), *Citrus junos* peel, extracted with 80% aqueous cold methanol, contained abscisic acid-β-D-glucopyranosyl ester (ABA-GE) that inhibited lettuce germination and growth. Kato-Noguchi and Tanaka (2004) further reported that the same extract of *Citrus junos* peel waste from the food processing industry, with the inside and seeds separated from the fruit waste, inhibited the growth of roots and alfalfa shoots (*Medicago sativa* L.), garden cress (*Lepidium sativum* L.), crabgrass (*Digitaria sanguinalis* L.), lettuce (*Lactuca sativa* L.), timothy *(Pheleum pretense* L.), and ryegrass (*Lolium multiflorum* Lam.). Moreover, in the Arabidopsis plant model, ABA-GE showed growth inhibition (Kato-Noguchi & Tanaka, 2008). These results suggested the potential of Citrus peel for use as a natural source of weed control.

The purpose of this study was to determine which substances extracted from peels contained active compounds efficient in weed control and provide a cheaper alternative to chemical herbicides, and thus reduce the cost of agricultural production in Thailand.

2. Objectives

There were two main objectives to this study: first, compare the inhibitory activity of the peel extract of tangerines (*Citrus reticulata*) before and after chlorophyll removal to suppress growth of lettuce (*Lactuca sativa*) and barnyard grass (*Echinochloa crus-galli*) which is a major weed in paddy rice, and second, to determine the ABA concentration of *Citrus reticulata* peel extract with and without chlorophyll.

3. Materials and methods

3.1 Extraction and materials

The tangerines peel (*Citrus reticulata*) were collected from local markets, cut in small pieces and freeze dried. The samples were ground

as dried powders and stored at -20ºC until testing or extraction. Next, the dried powders were extracted with 70% methanol as previously described (Kato-Noguchi, Tanaka, Murakami, Yamamura, & Fujihara, 2002). Solvent fractions were collected and evaporated at 40°C. A method of crude methanol extraction removed chlorophyll following a previous report (Kijjoa et al., 2002). Briefly, 30 g of crude extract was added with 600 ml warm methanol and added with 700 ml of mixed solution (30 g lead acetate and 8 ml glacial acetic acid in distilled water). The solution was kept under dark conditions for 48 hours and filtered. The filtered solution was evaporated under vacuum at 40°C. The powder was extracted with chloroform three times and water removed with anhydrous sodium sulfate. Finally, the extract was evaporated under vacuum at 40°C again before using for biological assay.

Weed seeds were collected from paddy fields in Kanchanaburi province, Thailand. The tested seeds were sterilized in 2% (w/v) solution of sodium hypochlorite for 15 min rinsed in distilled water 4 times according to the method of Kato-Nagochi (2003).

3.2 Dried peel powders assay

Dried powders of *C. reticulata* were initially investigated for inhibition efficiency of *Lactuca sativa, Echinochoa crus-galli* and *Oryza sativa*. Each peel powder was mixed with sterilized quartz sand (24 g.) in 9 cm petri dish and moistened with 8 ml of distilled water. The concentration of dried peel powder was 0, 250, 500, 1000, 2000 and 3000 mg per petri dish. Four replications of 20 seeds of tested species were placed on the petri dish and allowed to germinate at 25ºC for 7 days. The germinated seeds were counted and the percentage of germination was calculated by comparing control seeds which had not been treated with the dried powder.

3.3 Peel extract assay

Fractions from every step of extraction were drained on Whatman filter paper No. 2 in a petri dish and dried at normal room conditions. Sterilized distilled water with 0.005% Tween20 was added to the dried filter paper. Weed seeds species were added into each petri dish, 20 seeds/dish. The petri dishes were incubated for 48 hours in the dark at 25°C. The percentage of seed germination was recorded for 4 replications.

3.4 Purification and determination of ABA

Approximately 100 mg of orange peel crude extract with or without chlorophyll were dissolved in 2.5ml of 1% acetic acid and filtered through a 0.2 µm filter before HPLC analysis. Hydrolysis of bound ABA (ABA-GE) to free ABA was performed according to the method of Dietz (Dietz, Sauter, Wichert, Messdaghi, & Hartung, 2000). Briefly the crude extract was dissolved in distilled water, adjusted to pH 12 with 1M NaOH and incubated at ambient temperatures for 1 hour. The hydrolyzed sample was adjusted to pH 3 with 2N HCl, filtered through a 0.2µm nylon filter before HPLC analysis. Determination of ABA was performed according to Nakurte, Keisa, and Rostoks (2012) using HPLC system, Agilent 1100 Series (Agilent Technologies, USA) equipped with quaternary pump, auto-sampler and Diode array detector. ABA separation were done on Apollo C18 column (Alltech Associates Inc., USA) 4.6 x 150 mm., 5 µm. The mobile phase was composed of methanol and 1% acetic acid (60:40, v/v) in isocratic mode at a flow rate of 1 ml/min. ABA was detected at 270 nm. Chromatographic results were evaluated by Chemstation Plus Software (Agilent Technologies, USA) using (±)ABA (Sigma-Aldrich, USA) at the concentration ranges of 10, 100, 1,000 to 10,000 ng ml^{-1} as a calibration standard.

3.5 Statistical analysis

Analysis of variance was performed for all data. Comparisons between treatments were made at 0.05 probability level using the Duncan's multiple-range test. Inhibition percentage was calculated as (control data – treatment data/control data x 100).

4. Results

4.1 Ability of *Citrus reticulata* peel powders on weed control

The peel powder of *Citrus reticulata* inhibited 50% seed germination of lettuce (*Lactuca sativa*) and barnyard grass (*Echinochloa crus-galli*) at the concentrations of less than 400 mg and 1600 mg dried peel powder respectively. Meanwhile, an inhibition of seed germination on rice (*Oryza sativa*) required a concentration higher than 2400 mg. (Table 1). These results revealed that the dried powder of *C. reticulata* exhibited a high potential of weed control in paddy rice.

Table 1 Effect of *Citrus reticulata* peel powders on seed germination of tested plants

Concentration (mg/100 ml)	*Lactuca sativa*		*Echinochloa crus-galli*		*Oryza sativa*	
	% Seed Germination	% Seed Inhibition	% Seed Germination	% Seed Inhibition	% Seed Germination	% Seed Inhibition
0	88.00±1.53^{a}	0.00±0.00^{a}	81.67±2.51^{a}	0.00±2.51^{a}	93.75±1.00^{a}	0.00±0.00^{a}
400	1.25±0.57^{b}	98.59±0.57^{b}	68.33±1.52^{a}	16.33±1.52^{a}	88.78±1.52^{a}	11.22±1.52^{a}
800	0.00±0.00^{b}	100.00±0.00^{b}	48.33±2.88^{b}	40.82±2.88^{b}	75.34±2.00^{b}	24.66±2.00^{b}
1600	0.00±0.00^{b}	100.00±0.00^{b}	28.33±1.52^{b}	65.31±1.52^{c}	48.46±1.15^{c}	51.54±1.15^{c}
2400	0.00±0.00^{b}	100.00±0.00^{b}	6.67±1.52^{d}	91.84±1.52^{d}	21.58±0.57^{d}	78.42±0.57^{d}
3200	0.00±0.00^{b}	100.00±0.00^{b}	0.00±0.00^{d}	100.00±0.00^{d}	0.00±0.00^{d}	100.00±0.00^{d}

Values represent mean ± standard deviation of means.
Means values within the same column sharing the same superscript were not significantly different at $p \leq 0.05$ by Duncan.

4.2 Increasing potential of extracted *Citrus reticulata* peel on seed inhibition of lettuce (*Lactuca sativa*) and barnyard grass (*Echinochloa crus-galli*)

Various concentrations of *Citrus reticulata* peel extractwith and without chlorophyll were tested in *Lactuca sativa* and *Echinochloa crus-galli*. In *Lactuca sativa* germination tests, extracts containing chlorophyll inhibited 100% germination at 1000 mg/100ml (Table 2) while those without chlorophyll inhibited 100% germination at 500 mg/100ml (Table 3). In *Echinochloa crus-galli* germination tests, 2500 mg/100ml of *Citrus reticulata* peel powder extracts with chlorophyll inhibited 79.07% of germination (Table 2) while extracts without chlorophyll reached 100% inhibition (Table 3). These results indicated that the potential of extracts of *Citrus reticulata* peel on weed control could be enhanced by chlorophyll removal after extraction.

4.3 Determination of ABA in *Citrus reticulata* peel powder extracts

The ABA content of orange peel crude extracts, with and without chlorophyll removal, is showed in Table 4. Higher ABA content (46,196.7 ng ml^{-1}) was obtained from the extract after chlorophyll removal compared to the extract without chlorophyll removal (9,542.2 ng ml^{-1}). The results suggested approximately 5 fold concentration was achieved during the chlorophyll removal process. A significant increased (6.7 to 9.6 fold) in ABA content was observed from the extract with or without chlorophyll removal after hydrolysis step indicating that bound-ABA (ABA-GE) is the major form of ABA contained in the orange peels. Chlorophyll removal after hydrolysis tended to yield slightly lower amounts of free-ABA compared to extracts with chlorophyll. The results may suggest some of the bound-ABA might have been released to free-ABA during the Chlorophyll removal.

Table 2 Effect of *Citrus reticulata* peel powder extract without chlorophyll removal on seed germination of tested plants

Concentration (mg/100 ml)	*Lactuca sativa*		*Echinochloa crus-galli*	
	% Seed Germination	% Seed Inhibition	% Seed Germination	% Seed Inhibition
0	80.00±1.00^{a}	0.00±0.00^{a}	71.67±1.15^{a}	0.00±0.00^{a}
250	20.83±1.52^{b}	63.33±1.52^{b}	63.33±1.15^{b}	11.63±1.15^{b}
500	10.42±1.00^{b}	78.33±1.00^{b}	48.33±1.52^{c}	32.56±1.52^{c}
1000	0.00±0.00^{c}	100.00±0.00^{c}	48.33±2.08^{c}	32.56±2.08^{c}
2000	0.00±0.00^{c}	100.00±0.00^{c}	35.00±1.00^{c}	37.21±1.00^{c}
2500	0.00±0.00^{c}	100.00±0.00^{c}	15.00±1.00^{c}	79.07±1.00^{c}

Values represent mean ± standard deviation of means.
Means values within the same column sharing the same superscript were not significantly different at $p \leq 0.05$ by Duncan.

Table 3 Effect of *Citrus reticulata* peel powder extracts with chlorophyll removal on seed germination of tested plants

Concentration (mg/100 ml)	*Lactuca sativa*		*Echinochloa crus-galli*	
	% Seed Germination	% Seed Inhibition	% Seed Germination	% Seed Inhibition
0	80.00±2.00[a]	0.00±0.00[a]	71.67±1.15[a]	0.00±1.15[a]
250	6.67±0.57[b]	91.67±0.57[b]	60.00±1.00[b]	16.28±1.00[b]
500	0.00±0.00[b]	100.00±0.00[b]	48.33±2.51[c]	32.56±2.51[c]
1000	0.00±0.00[b]	100.00±0.00[b]	28.33±1.54[d]	60.47±1.54[d]
2000	0.00±0.00[b]	100.00±0.00[b]	3.33±1.54[e]	95.35±1.54[e]
2500	0.00±0.00[b]	100.00±0.00[b]	0.00±0.00[e]	100.00±0.00[e]

Values represent mean ± standard deviation of means.
Means values within the same column sharing the same superscript were not significantly different at $p \leq 0.05$ by Duncan.

Table 4 HPLC analysis of ABA content of orange peel extract with or without chlorophyll removal before and after hydrolysis

Extraction condition	ABA content (ng ml^{-1})	
	- hydrolysis	+ hydrolysis
Extract without chlorophyll removal	9,542.2	92,393.4
Extract with chlorophyll removal	46,196.7	309,828.1

5. Discussion

Many seeds do not germinate immediately after dispersal from mother plants because of seed dormancy. ABA and bioactive gibberellins (GA) levels in seeds play important roles to control seed germination, a process that is also seen in various weed species. Light and cold treatments have been shown to decrease levels of ABA and increase GA for promoting seed germination (Piskurewicz et al., 2008; Seo et al., 2006). GA levels in seeds have long been known to induce the synthesis of hydrolytic enzymes which degrade accumulated reserves of stored food in endosperm for providing carbohydrates and protein as energy sources for seedling growth. Conversely, increasing the ABA level promotes seed dormancy by inhibiting GA by two mechanism: direct inhibition by inducing expression of a transcriptional repressor of some GA-regulated genes (Hoecker, Vasil, & McCarty, 1995) and indirect inhibition by repressing a transcription factor which mediates the GA induction of α-amylase expression (Gómez-Cadenas, Zentalla, Walker-Simmons, & Ho, 2001). During citrus fruit ripening, expression of ABA induced genes were increased in the late stage of ripening suggesting that levels of ABA in late stage were higher than the early stage (Wu et al., 2014). ABA also is involved in chlorophyll degradation by inhibiting transcription of chloroplast genes (Yamburenko et al., 2013) supporting our hypothesis that removal of chlorophyll from extracted Citrus peel will significantly increasing potential of weed germination control. Further applications of using industry waste *Citrus reticulata* peel powder instead of using chemical herbicides in paddy rice in Thailand will be optimized.

6. Conclusion

After extraction of *Citrus reticulata* peel and removal of chlorophyll, the potential of weed germination control is higher than before chlorophyll removing. Additional experiments indicated that the ABA levels increased after chlorophyll removal. This research suggested an alternative way to increase the potential of weed control for allelopathic substances by removing chlorophyll.

7. Acknowledgements

Special thanks to Research Institute of Rangsit University for funding this research.

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