Antioxidant Activity of Acidified Ethanolic Adlay (*Coix lacryma-jobi* L) Seed Extract *In Vitro* and Cell Culture Assays

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Abstract

Adlay (*Coix lacryma-jobi* L.), a Poaceae (Gramineae) plant, is cultivated in Thailand and other countries. It consists of numerous compounds, including phenolics, unsaturated fatty acids, triolein, coixenolide, and coixol, and possesses a variety of pharmacological activities. The adlay seed is the commonly used part of the plant in several foods and food supplements, but not in cosmetic products. The objectives of this study were to prepare the adlay seed extract and evaluate the total phenolic content. The antioxidant activity analyzed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and cell culture assays as well as the cytotoxicity assay were also investigated. The adlay seed extract was prepared by acidified ethanol using the maceration technique with the percent extraction yield of 7.72 ± 0.25% (w/w). The total phenolic content of the extract was 244.63 ± 1.19 mg gallic acid equivalent/g extract, and the concentration required to scavenge 50% of DPPH free radicals (IC_{50}) was 0.37 ± 0.00 mg/mL. The noncytotoxic concentrations of extract analyzed by sulforhodamine B assay were in the range of 0.0001-0.1 mg/mL. The antioxidant in cell culture assays showed the ability of the extract for the prevention of oxidative stress and treatment of oxidatively damaged cells in B16F10 melanoma cells. The study results suggested that adlay seed may potentially be used as an antioxidant ingredient in cosmetic preparations.

***Keywords**: adlay seed, total phenolic content, DPPH radical scavenging assay, cytotoxicity assay, cell culture assay*

1. Introduction

Adlay (*Coix lacryma-jobi* L.), also called job's tears, is a Poaceae (Gramineae) plant cultivated in the Himalayas, Europe, East Asia, China, Japan, and Thailand. Adlay has been used as a food and medicinal plant and is one of the nutrient-rich food that consists of proteins, polysaccharides, oils, fibers, minerals, and vitamins. Additionally, the essential components in adlay include phenolic compounds, unsaturated fatty acids, triolein, coixenolide, and coixol. The pharmacological effects of adlay are antioxidant, anticancer, anti-inflammatory, muscle relaxing, immunomodulating, and decreasing obesity activities (Xu et al., 2017; Ryu, 2008). As its health advantages have been reported, the products containing adlay, particularly in food and food supplement, are available in the market. The study showed that the adlay seed extract that consists of coixol and 2-O-β-glucopyranosyl-7-methoxy-2H-1,4-benzoxazin-3(4H)-one demonstrates a potent inhibitory activity on melanogenesis without significant cytotoxicity (Amen et al., 2017). The adlay extract also shows antioxidant activity in various antioxidant assays, including 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, ferric reducing antioxidant power (FRAP) assay, and *β*-carotene-linoleic acid bleaching assay (He et al., 2020). The major components in the extract that are responsible for the biological activity include the phenolic compounds such as *p*-coumaric acid, chlorogenic acid, syringic acid, syringaresinol, ferulic acid, coniferyl alcohol, mayuenolide, and ketopinoresinol (Kuo, Chen, & Chiang, 2012; Huang et al., 2014; Zhao et al., 2014).

In Thailand, the cultivation of adlay has been found in many provinces, particularly Loei, Chaiyaphum, Saraburi, Lopburi, Nakhon Ratchasima, and Phayao. Seed, the commonly used part of the plant, is used as a part of several food recipes and food supplements, but not in the cosmetic product. The objectives of this study were to evaluate the antioxidant activity of the adlay seed cultivated in Thailand for application as an ingredient in cosmetic products.

Proceedings of RSU International Research Conference (2021)

2. Objectives

The objectives of this study were to prepare the adlay seed extract and evaluate the total phenolic content. The antioxidant activity of the adlay seed extract was analyzed by *in vitro* 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and cell culture assays. The cytotoxicity assay of the extract was also evaluated.

3. Materials and Methods

3.1 Materials

Adlay seed was purchased from the local market in Chiang Rai province, Thailand. Vitamin C, sulforhodamine B (SRB), gallic acid, Folin-Ciocalteau reagent, sodium carbonate, and DPPH were obtained from Sigma Chemical Co. Ltd., USA. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin, and streptomycin solution were from Gibco, USA. All other chemicals and reagents were of analytical grade.

3.2 Preparation of adlay seed extract

The adlay seeds were dried in a hot air oven at 45 ± 2°C for 24 hours, then ground into a fine powder using a blender. The extraction of the adlay seed was performed as previously described with some modification (Zhao et al., 2014). Adlay fine powder (30 g) was macerated in the acidified ethanol (95% ethanol:1 M HCl = 85:15, v/v) for 2 hours. The mixture was filtered to collect the supernatant, and the residue was re-extracted twice. The supernatant was pooled and concentrated using a rotary evaporator. The extraction was performed in triplicate and calculated the percent extraction yield. The dry adlay seed extract was kept at -20°C until use. The solvent used for the extract solubilization in all assays was dimethyl sulfoxide (DMSO).

3.3 Total phenolic content (TPC) assay

TPC of the extract was determined by Folin-Ciocalteau assay (Cheok et al., 2012). The reagent was mixed with sodium carbonate, and the absorbance was measured by a UV-Vis spectrophotometer at 765 nm. The TPC was compared with gallic acid and expressed as mg of gallic acid equivalent (GAE)/g extract. The determination was performed in triplicate.

3.4 *In vitro* DPPH radical scavenging assay

DPPH radical scavenging assay of the extract was determined, and vitamin C was used as a standard (Zhao et al., 2014). The mixture of the DPPH solution and samples, including the adlay seed extract and vitamin C, was mixed and incubated at room temperature for 30 minutes. The absorbance was then measured at 517 nm by the UV-Vis spectrophotometer. The determination was done in triplicate. The antioxidant of each sample was calculated and presented as the concentration required for 50% free radical scavenging (IC_{50}).

3.5 Cytotoxicity assay

The cytotoxicity of the extract was performed in B16F10 melanoma cells (ATCC® CRL-6475™) by sulforhodamine B (SRB) assay and compared with vitamin C (Chaikul, Lourith, & Kanlayavattanakul, 2017). The cells at 1×10^4 cells/well were seeded in the 96-well plate and incubated in a carbon dioxide incubator for 24 hours. The samples, including the extract and vitamin C, at 0.0001-1 mg/mL were added and incubated for 72 hours. Then, the cells were fixed with trichloroacetic acid and stained with SRB dye. The absorbance was measured at 540 nm. The cell viability was calculated compared with the control (solvent).

3.6 Antioxidant assays in cell culture

3.6.1 Prevention of oxidative stress

B16F10 melanoma cells were seeded in the 96-well plate and incubated overnight. The samples at noncytotoxic concentrations and the control (solvent) were added and incubated for 72 hours. Thereafter, all treated samples were replaced with a fresh medium containing 200 µM hydrogen peroxide and incubated for 4 hours. The cells were then fixed and evaluated the cell viability by SRB assay. The cell viability was calculated compared with the hydrogen peroxide untreated control (Chaikul et al., 2017).

3.6.2 Treatment of oxidatively damaged cells

The method was performed as previously described with some modifications (Chaikul et al., 2017). B16F10 melanoma cells were plated in the 96-well plate and incubated overnight. The cells were then exposed to 200 µM hydrogen peroxide. After the 4-hour incubation, the cells were treated with the noncytotoxic concentrations of the samples and the control (solvent) for 72 h. The cells were fixed and evaluated the cell viability by SRB assay. The cell viability was calculated compared with the hydrogen peroxide untreated control.

3.7 Statistical analysis

The data were shown as mean ± SEM of three independent experiments and compared the difference using one-way analysis of variance (ANOVA) and the least significant difference test (LSD). A p-value < 0.05 was considered statistically significant.

4. Results and Discussion

4.1 Preparation of adlay seed extract

The adlay seed extract was semi-solid with dark brown to black color as shown in Figure 1. The percent extraction yield was 7.72 ± 0.25%. The extraction method in this study was selected due to the high content of phenolics in the obtained extract (Zhao et al., 2014). The phenolics have been reported to influence several biological activities. However, the acidified ethanol was employed as an extraction solvent, owing to the safety consideration. The obtained extract resulted in the different characteristics and percent extraction yield, which may be due to different extraction conditions including the polarity of extraction solvent, the extraction time, as well as extraction temperature, which are the key parameters that govern the extraction efficiency (He et al., 2020; Hu et al., 2007).

Figure 1 Adlay seed extract

4.2 Total phenolic content assay

The plant phenolics, the secondary plant metabolites, play a major role in biological activity. Thus, it is reasonable to determine the total content in the selected plant (Miliauskas et al., 2004). The total

phenolic content of the adlay seed extract was 244.63 ± 1.19 mg GAE/g extract. A previous study showed that the total phenolic contents of the polished adlay seed and adlay oil extract samples were in the range of 0.604-0.866 mg GAE/g sample (He et al., 2020; Zhao et al., 2014). The different total phenolic content in the adlay extract compared with the previous study may be influenced by a variety of adlay seed plantation and the extraction methodology (He et al., 2020)

4.3 *In vitro* DPPH radical scavenging assay

The IC_{50} values of the adlay seed extract and vitamin C were 0.37 ± 0.00 and 0.002 ± 0.00 mg/mL, respectively. The less antioxidant activity of the adlay seed extract than vitamin C, a standard, was observed (p<0.001). The previous study reported the IC_{50} value of the polished adlay seed extract analyzed by DPPH radical scavenging assay at 5.49 ± 0.12 mg/mL (Tseng et al., 2006). The different potency of the extract for radical scavenging may be due to the different extraction conditions and the variation of plant agronomic conditions (Sonkar et al., 2020). For the application of the adlay seed extract in cosmetic products, the cytotoxicity assay and the antioxidant activity in cell culture were analyzed.

4.4 Cytotoxicity assay

Figure 2 shows the cytotoxicity assay of the adlay seed extract and vitamin C. The cell viabilities after treatment with 0.0001-0.1 mg/mL adlay seed extract and vitamin C were greater than 80%, indicating the noncytotoxic concentrations. The increased concentration of the adlay seed extract and vitamin C led to the decreased cell viability, which showed the cytotoxic concentration. The cell viabilities of 1 mg/mL adlay seed extract and vitamin C were 12.64 ± 0.24 and 73.43 ± 1.82%, respectively.

The cytotoxic effect of the adlay seed extract may be attributed to the phenolic acids in the extract. The major free phenolics in the polished adlay extract, including chlorogenic acid (0.20 ± 0.02 mg/g dry weight) and *p*-coumaric acid (0.04 ± 0.00 mg/g dry weight), have been associated with the antiproliferative effect in the cancer cells (Hernandes et al., 2020; Kanlayavattanakul, Lourith, & Chaikul, 2016; Zhao et al., 2014). Besides, the adlay seed extract demonstrates an anticancer effect *in vitro* and animal assays by the inhibition of cell proliferation and induction of cell apoptosis (Chang, Huang, & Hung, 2003). The cytotoxicity of vitamin C was in agreement with the previous study (Chaikul et al., 2020). The noncytotoxic concentrations of the adlay seed extract and vitamin C, 0.0001-0.1 mg/mL, were further analyzed regarding the antioxidant assays in the cell culture.

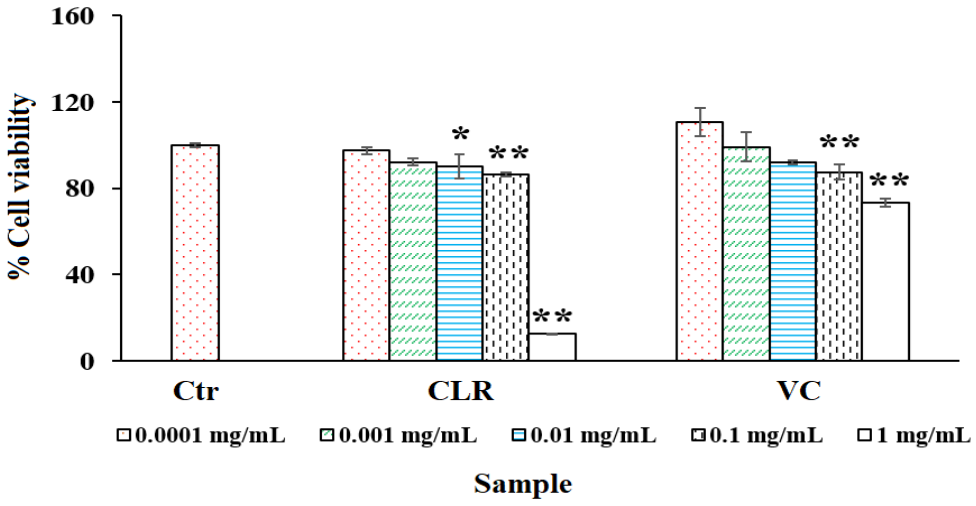
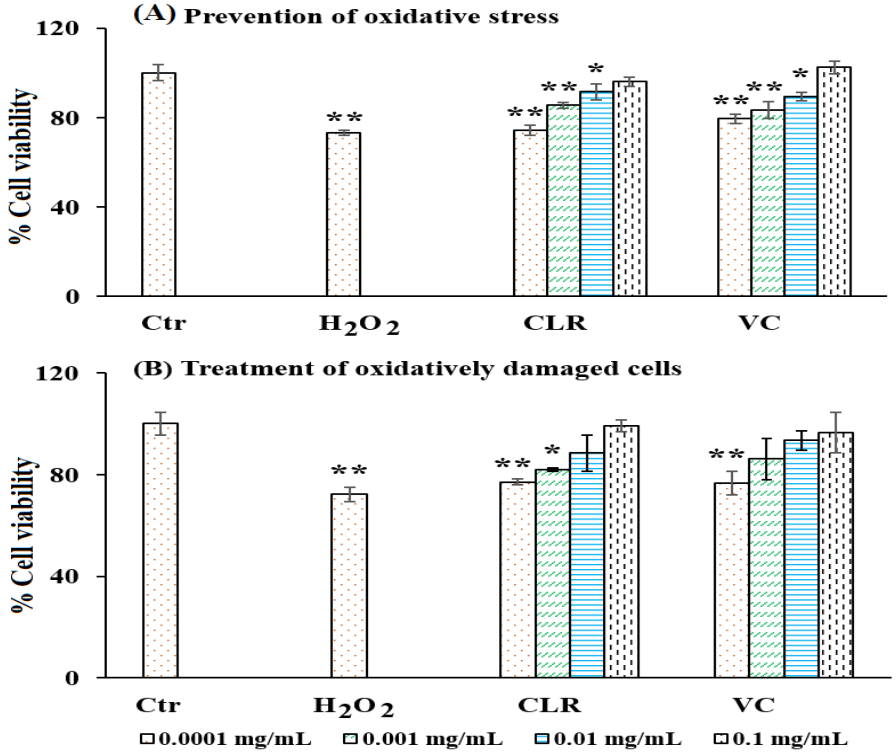


Figure 2 Cytotoxicity assay in B16F10 melanoma cells treated with adlay seed extract (CLR) and vitamin C (VC) at 0.0001 – 1 mg/mL. Ctr represents the solvent-treated cells. * indicates the significant difference from the control (* $p<0.05$ and ** $p<0.001$).

4.5 Antioxidant assays in cell culture

The antioxidant analysis was performed in the prevention of oxidative stress and the treatment of oxidatively damaged cells. Figure 3 presents the antioxidant activity of the adlay seed extract and vitamin C in the prevention of oxidative stress and the treatment of oxidatively damaged cells. In the prevention of the oxidative stress assay, the cell viability after the addition of hydrogen peroxide decreased to 73.18 ± 1.08%. The greater cell viability than the hydrogen peroxide treated group was observed when treated with the adlay seed extract and vitamin C at 0.001 mg/mL. The increased cell viability was comparable to the hydrogen-peroxide untreated control when treated at 0.1 mg/mL adlay seed extract and vitamin C. In the treatment of the oxidatively damaged cells, hydrogen peroxide was added to cause cell damage. The cell viability of the hydrogen peroxide treated group was 72.29 ± 2.77%. The increase in the cell viability after treatment with the adlay seed extract and vitamin C was shown to be in a concentration-dependent manner. 0.01 mg/mL adlay seed extract and 0.001 mg/mL vitamin C appeared to treat the oxidatively damaged cells and increase the cell viability, which was comparable to the hydrogen peroxide untreated group. The cell response to the adlay seed extract and vitamin C was observed in the treatment of the oxidatively damaged cells assay greater than in the prevention of oxidative stress assay, which may be due to the increased permeation of the samples through the damaged cell membranes, resulting in a greater effect at low treated concentrations.

At present, free radicals have been associated with several diseases, including skin aging and skin inflammation. To combat the formation of free radicals, one of the well-known strategies is to utilize the antioxidant. The antioxidant can prevent, scavenge, or slow down the formation of free radicals, leading to decelerating oxidative damage (Chaikul et al., 2020). Due to the safety consideration and environmental awareness, several studies have investigated antioxidants from natural sources. Hydrogen peroxide, an oxidant, was reported to induce oxidative stress by leakage of the cell membrane and DNA damage (Whittemore et al., 1995). The adlay seed extract in this study demonstrated to protect the cell damage and treat the damaged cells in a concentration-dependent manner. The antioxidant activity of the extract may be attributed to the phenolic compounds. The phenolic profiles in adlay, including chlorogenic and *p*-coumaric acids, have been reported and are associated with the antioxidant activity (He et al., 2020; Zhao et al., 2014). Vitamin C has mediated the antioxidant activity via reacting with aqueous peroxyl radicals and restoring the antioxidant properties of lipid-soluble vitamin E (Bendich et al., 1986).



Figu : (CLR) and vitamin C (VC) at 0.0001-0.1 mg/mL: (A) A prevention of oxidative stress and (B) treatment of oxidatively damaged cells. Ctr and H_2O_2 represent the hydrogen peroxide untreated and hydrogen peroxide treated groups, respectively. * indicates the significant difference from the control (* p<0.05 and ** p<0.001).

5. Conclusion

The adlay seed cultivated in Thailand was extracted by acidified ethanol using maceration and showed the noncytotoxic effect at 0.0001-0.1 mg/mL. The extract demonstrated the antioxidant activity *in vitro* DPPH radical scavenging and cell culture assays. The study results suggested that the adlay seed can be used as an antioxidant ingredient in cosmetic preparations. The suggestion for further study may include the evaluation of the phenolic constituents in the adlay seed extract and other biological activities related to the cosmetic application, including melanogenesis assay and anti-aging effect.

6. Acknowledgements

The authors would like to thank Mae Fah Luang University for the financial support and facilities used in the study.

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