Antioxidant Activity, Total Phenolic Contents and Cytotoxicity Of Coix Lacryma-Jobi Extracts From Different Extraction Methods

Julinta Don-in¹, Tipparat Saejung² and Thitiphon Chimsook³ *

¹² Applied Chemistry Program, Faculty of Science, Maejo University, Chiangmai 50290, Thailand
³ Chemistry and Applied Chemistry Program, Faculty of Science, Maejo University, Chiangmai 50290, Thailand
*E-mail: thitiphon.cs@gmail.com

Abstract: The aim of this study was to determine the percentage yield of Coix lacryma-jobi extracts from different extraction methods and evaluate the antioxidant activity, total phenolic contents and anticancer activity of C. lacryma-jobi extracts. Two conventional extraction methods including maceration and reflux were used to perform the crude extracts from C. lacryma-jobi using 95% ethanol and distilled water to obtain ethanolic and aqueous extracts. The antioxidant activity was investigated by DPPH (1,1-diphenyl 2-picrylhydrazyl) assay. The results showed that the highest DPPH scavenging activity of ethanolic extracts were observed from reflux extraction for 2 hours (7.11±0.23%). The total phenolic contents of C. lacryma-jobi extracts were determined with Folin–Ciocalteu reagent. The results revealed that the reflux extraction for 2 hours exhibited the highest total phenolic contents (7.85±1.00 mg GAE/g extract) from ethanolic extract. Moreover, all extracts were tested the cytotoxicity against the human breast cancer (MCF-7) and human small lung (NCI-H187). The results demonstrated that ethanolic extract from reflux extraction for 2 hours displayed the strongest cytotoxicity, being more active against MCF-7 (IC₅₀ 48.25±0.11 μM) and NCI-H187 (IC₅₀ 30.05±1.11 μM).

Keywords: Coix lacryma-jobi, Conventional extraction methods, Antioxidant activity, Total phenolic contents, MTT assay

1. Introduction

Coix lacryma-jobi (called adlay or Job’s tears), a traditional Chinese medicinal plant, is regarded as a nutritive food source. It has been reported various pharmacological activities for example anti-inflammatory [1] and anti-allergic effects [2]. Several
physiological functions of adlay and its biological active components in different part of adlay, including hull, bran, testa and endosperm, have been investigated [3-6]. Moreover, recent studies demonstrated that adlay seed extracts have showed the antitumor and anticancer activities, inhibited the allergic diseases [7] and some physiological effects [8-9].

The conventional extraction methods such as maceration, reflux and soxhlet extractions have been used for several years to extract the active compounds from plants and other natural products. Reflux extraction is one of the hot continuous extractions which could extract the bioactive substances. This method requires a smaller quantity of solvent compared to maceration. However, numerous factors of extraction have been studied such as extraction time, temperature, solvent-sample ratio etc.

Accordingly, in this present study, we investigated the effects of extraction methods and solvent to percentage yield, antioxidant activity, total phenolic contents and cytotoxicity of C. lachryma-jobi extracts. Each extract of C. lachryma-jobi was carried out from two extraction methods including maceration and reflux extractions in two different solvents of ethanol and distilled water.

2. Materials and methods

2.1. Sample and chemicals

Fresh of C. lachryma-jobi was purchased from the market and tested the pesticide-free. C. lachryma-jobi was used as the raw material (Fig. 1) for preparing the crude extracts and studied their bioactivities. All solvents used for extraction were of analytical grade and purchased from Merck. The standards were purchased from Sigma-Aldrich.

![Sample of fresh C. lachryma-jobi.](image)

Fig. 1 Sample of fresh C. lachryma-jobi.

2.2. Sample extraction

C. lachryma-jobi extracts were carried out from two conventional extraction methods including maceration and reflux. Two solvents; 95% ethanol and distilled water; were used to extract fresh of C. lachryma-jobi. Briefly, 20 g of C. lachryma-jobi was rinsed with distilled water, dried and crushed using a blender before extracted using 500 ml of each solvent. For maceration, C. lachryma-jobi was mixed with each solvent and then kept in orbital shakers overnight before allowed to stand in dark bottles at room temperature for 3, 5, 7 days with occasional agitation. All extracts were filtered using filter paper. Filtrate was evaporated and dried using freeze dryer to obtain two powder of C. lachryma-jobi extracts. All C. lachryma-jobi extracts were evaluated the percentage of extraction yield and further analyzed the
antioxidant activity, total phenolic contents and cytotoxicity. For reflux extraction, 20 g of C. lacryma-jobi was extracted with 500 ml of 95% ethanol or distilled water. Different extraction time at 1.5, 2, and 3 hours was carried out in each samples at 65 °C. The mixtures were filtered, concentrated and freeze dry to obtain the crude of C. lacryma-jobi extracts. All extracts were analyzed the percentage of extraction yield and analyzed their bioactivities.

2.3 Determination of antioxidant activities

In this work, the antioxidant activities of extracts were performed using DPPH (1,1-diphenyl 2-picrylhydrazyl) assay. DPPH radical scavenging ability of sample was adjusted from the methods of Choi et al. [10] and Brand-Williams et al [11]. Briefly, a total of 100 mg of dry extract was leached using 200 ml of 70% ethanol and 0.2% HCl. The filtrate was filtered using filter paper. Aliquots of 0.8 mM DPPH in ethanol were mixed with 1.0 mL of the extracts. The mixtures were vigorously shaken and left to stand for 10 min in the dark. The absorbance at 517 nm was measured against water and ethanol as the blank and control respectively [12]. The scavenging ability was calculated as follows equation (1):

\[
\text{Scavenging ability (\%) = } \left( \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \right) \times 100 \quad \ldots \ldots \quad (1)
\]

The assay was performed in triplicate. Results are presented as mean± standard deviations in each case.

2.4 Determination of total phenolic contents

The total phenolic contents were determined using the Folin-Ciocalteu assay according to the method of Luo et al and Singleton et al. [13-14] and adjust from Marinova et al [15]. A total of 100 mg of dry extract was leached using 200 ml of 70% ethanol and 0.2% HCl. The filtrate was filtered using filter paper. A 0.5 mL of the extract or standard solution of gallic acid (GE) (20-200 mg/l) was added to test tubes containing 1.5 mL of distilled deionized (DI) water. A reagent blank of DI water was prepared. Folin- Ciocalteu’s phenol reagent (0.5 mL of 0.1 M) was added to the mixture and shaken. After 5 min, 2 mL of 15% sodium carbonate solution was added to the mixture. The solution was diluted with DI water and mixed. After incubation for 60 min at 37 °C, the absorbance at 750 nm was determined. The total phenolic contents of extracts were expressed as mg gallic acid equivalents (GAE)/g extracted sample.

2.5 Cytotoxicity studies by MTT assay.

All extracts were evaluated their cytotoxicity against human cancer cells. All extracts were tested for cytotoxic activity against two cell lines plus additionally the breast cancer (MCF-7) and human small lung (NCI-H187) for the evaluation of growth inhibition. Cell line growth was monitored using the MTT assay as reported and compared with doxorubicin as a positive control. Determination of the cytotoxic activity of the compounds was based on the reduction of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye by viable cells to generate a purple formazan product, whose
absorbance was detected at 540 nm. Cancer cells were seeded at $5 \times 10^3$ cells/well (200 μL; 96-well plates) and incubated at 37 °C for 24 h, after which 20 μL/well of the test extract dissolved in DMSO and water at various concentrations (0.001-1000 μM) was added to triplicate wells per concentration per test extract and incubated for 72 h. Next, 10 μL of MTT solution (5 mg/mL) was added into each well and incubated for 4 hours prior to removal of the medium and the addition of 150 μL of DMSO to each well with aspiration to lyse the cells and dissolve the formazan. The absorbance at 540 nm was then read using a 96-well plate reader, and the obtained value was assumed to represent the number of viable cells. The absorbance at 540 nm of the control cells (only DMSO added) was taken as 100% and all other values were expressed as a percentage of the control as the % cell viability [16,17]. The IC$_{50}$ values, the concentration of an inhibitor that reduces the response by half, were calculated using the GraphPad Prism 5 software. Cytotoxicity was derived after 12 h exposure to the compound by MTT assay. Data are shown as mean ± SD, derived from three independent trials. The percent cell viability was calculate for each compound using the following equation (2):

$$\% \text{ cell viability} = (A/B) \times 100 \quad \ldots \ldots \ldots \ldots \ldots \ldots (2)$$

Where, A and B were the absorbance of the experimental and control cells, respectively.

3. Results and discussion

In this work, C. lacryma-jobi extracts were carried out from maceration and reflux extractions using varied conditions and solvents. 95% ethanol and distilled water were used to extract C. lacryma-jobi in each extraction method. All extracts were evaluated the biological activities including antioxidant activity, total phenolic contents and cytotoxicity, respectively.

3.1 Sample extraction

For maceration and reflux extraction, effects of extraction conditions such as solvent and extraction time were studied and the optimum experiment conditions were obtained. C. lacryma-jobi was extracted from maceration and reflux extraction using two different solvents (95% ethanol and distilled water) and extraction time (3, 5, 7 days for maceration and 1.5, 2, 3 hours for reflux). The obtained extracts called ethanolic extract and aqueous extract. For maceration, three varied conditions composed of the extraction time for 3, 5 and 7 days in different solvents. As showed in Table 1, the results indicated that the highest percentage yield of extraction performed for 7 days using 95% ethanol (9.85 ± 1.14%). For reflux extraction, the highest percentage yield was 12.08 ± 0.04% which obtained from sample extracted for 2 hours using 95% ethanol. The results as showed in Table 1 indicate that for reflux extraction time of 2.0 hours was enough to obtain the maximum percentage yield of extract, while 7 days was needed for maceration to reach the maximum percentage yield. Also the percentage yield obtained by reflux extraction are higher than that obtained.
by maceration for most time settings. Previous studies of *C. lacryma-jobi* extract indicated that the optimization of the extracting technology of polyphenols from *C. lacryma-jobi*. The results showed that, the impact of the influence factors was ethanol concentration, extraction time, extraction temperature. Different conditions of *C. lacryma-jobi* extractions have been reported and used those extracts for evaluated various biological activities. The extraction methods of *C. lacryma-jobi* performed both conventional (maceration, reflux) and non-conventional extraction methods (supercritical fluid extraction, ultrasound assisted supercritical fluid extraction) and using various solvents (water, methanol, ethanol) [7]. The optimum extraction conditions were 60% ethanol, extraction time for 1.5 hours, extraction temperature at 40 °C and ratio of liquid to material 15:1 [18]. Moreover, *C. lacryma-jobi* extract was performed with hot ethanol and using an ultrasonic apparatus for 3 hours and then concentrated the crude ethanol before successively partitioned with hexane, ethyl acetate, and *n* - butanol. The obtained each extract was used to study increasing of glucose uptake in 3T3-L1 Cells [19]. In addition, various cultivar of *C. lacryma-jobi* were processed before solvent extraction by non-cooking, roasting, boiling and steaming. Each part of the *C. lacryma-jobi* was extracted by the cold and hot process by refluxing with methanol and hexane. The obtained extracts included methanol extracts and hexane extracts were investigated for anti-proliferative activity on human colon adenocarcinoma cell line (HT-29) by the sulforhodamine B (SRB) assay and indicated free radical scavenging activity at the SC50 [20].

**Table 1** Percentage extract yield of *C. lacryma-jobi* extracts obtained by each extraction methods.

<table>
<thead>
<tr>
<th>Extraction methods</th>
<th>Solvent</th>
<th>Conditions</th>
<th>% Extraction yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maceration</td>
<td>Water</td>
<td>3 days</td>
<td>4.05 ± 0.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 days</td>
<td>4.68 ± 0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 days</td>
<td>5.06 ± 0.55</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>3 days</td>
<td>5.11 ± 1.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 days</td>
<td>7.01 ± 1.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7 days</td>
<td>9.85 ± 1.14</td>
<td></td>
</tr>
<tr>
<td>Reflux</td>
<td>Water</td>
<td>1.5 hours</td>
<td>4.59 ± 0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 hours</td>
<td>6.53 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 hours</td>
<td>6.21 ± 0.41</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>1.5 hours</td>
<td>8.99 ± 0.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 hours</td>
<td>12.08 ± 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>11.52 ± 0.18</td>
<td></td>
</tr>
</tbody>
</table>

3.2 Determination of antioxidant activities

In this work, the antioxidant activities of aqueous and ethanolic extracts of *C. lacryma-jobi* from both maceration and reflux extraction were carried out using DPPH assay. Two extraction methods were performed using different solvents and extraction time. The results were shown in Fig. 2 and 3. For ethanolic extract, DPPH radical scavenging ability
of extracts were shown in Fig. 2. As shown in Fig. 2, it could be considered that the DPPH radical scavenging ability of ethanolic extracts from maceration at 3 and 5 days was not significantly different (p > 0.05). The highest DPPH scavenging activity was observed from maceration at 7 days (6.55±0.28%). Moreover, the highest DPPH scavenging activity of ethanolic extracts were observed from reflux extraction for 2 hours (7.11±0.23%). The DPPH radical scavenging ability of ethanolic extracts from reflux extraction for 3 hours was less than that of 2 hours and it was not significantly different (p > 0.05) of radical scavenging ability when compared to extraction time for 2 hours. For aqueous extract, DPPH radical scavenging ability of extracts was shown in Fig. 3. As compared the results with ethanolic extracts, the DPPH radical scavenging ability of aqueous extracts from both maceration and reflux extraction was lower than that of ethanolic extracts in all conditions. As shown in Fig. 3, the highest DPPH scavenging activity of aqueous extract from maceration was 4.25±0.11% which performed for 7 days and that of aqueous extract from reflux extraction was 6.29±1.00% which done only 3 hours, respectively. It can be observed that the highest DPPH scavenging activity of aqueous extract from maceration for 7 days were lower than that of aqueous extract from reflux extraction for 3 hours. Interestingly, the DPPH radical scavenging ability of aqueous extracts from reflux extraction for 3 hours had slightly higher than that of 2 hours. However, the DPPH scavenging activity of aqueous extract from reflux extraction for 2 and 3 hours was not significantly different (p > 0.05).

![Graph showing DPPH radical scavenging activity for different extraction methods (Maceration vs Reflux) and extraction times.](image)

**Fig. 2** DPPH radical scavenging ability of all extracts from maceration and reflux using 95% ethanol as solvent.
The seed flour of *C. lacryma-jobi* was extracted with ethanol whereby the extract was subjected to different phytochemical tests. The phytochemical tests included phenols estimation, flavanol content, and antioxidant activity and tannin content. The results revealed that the seed of *C. lacryma-jobi* was found to be a very rich source of flavonoids. Moreover, antioxidant activity of *C. lacryma-jobi* seed using DPPH scavenging assay exhibited high value of 91.35% inhibition [21].

3.3 Determination of total phenolic contents

The results of total phenolic contents of all extracts were shown in Fig. 4 and Fig. 5. As shown in Fig. 4, all extracts were performed using 95% ethanol (called 95% ethanolic extract as solvent whereas Fig. 5 was carried out using distilled water (called aqueous extract). The results showed that the total phenolic contents of all ethanolic extracts from maceration were less than that of all ethanolic extracts from reflux extraction. It can be observed that the reflux extraction for 2 hours exhibited the highest total phenolic contents (7.85±1.00 mg GAE/g extract) from ethanolic extract. As shown in Fig. 5, the results showed that the total phenolic contents of all aqueous extracts were lower than that of all 95% ethanolic extract. However, higher value of total phenolic contents of aqueous extract was investigated from reflux extraction. It could be possible that the total phenolic contents of each sample depended on the solvent, extraction time, the ratio of sample and solvent and also cultivar [22]. In addition, Chhabra, D et. al. reported the total phenolic contents of *C. lacryma-jobi* seed extracted from soaking seed for 2 days in an incubator shaker (at 50 °C) using ethanol as solvent had high value of 20.5 mg GAE/g sample [21].
3.4 Cytotoxicity studies by MTT assay

In this work, all extracts were tested for cytotoxic activity against the human breast cancer (MCF-7) and human small lung (NCI-H187) for the evaluation of growth inhibition. Cell line growth was monitored using the MTT assay and compared with doxorubicin as a positive control. The IC$_{50}$ values of all extracts were summarized in Table 2, along with that of the doxorubicin for comparison. For doxorubicin, the results indicate significant cytotoxic activities with the IC$_{50}$ value of 13.50 and 0.03 μM against MCF-7 and NCI-H187, respectively. The results showed that all aqueous extracts from maceration and ethnaolic extracts from maceration for 3 days showed inactive activity for all tested cell lines. In
addition, ethanolic extracts from maceration for 5 and 7 days displayed weak cytotoxicity for MCF-7 but showed inactive for NCI-H187. For reflux extraction, the aqueous extract obtained from extracting for 1.5 hours displayed inactive activity for all tested cell lines. In contrast, only aqueous extract obtained from reflux extraction for 2 hours showed the cytotoxicity against MCF-7. The weak cytotoxicity of aqueous extracts were obtained from reflux extraction for 3 hours against MCF-7 (IC$_{50}$ 71.05±0.15 μM) and NCI-H187 (IC$_{50}$ 75.51±1.02 μM), respectively. Interestingly, all ethanolic extracts from reflux extraction exhibited the cytotoxicity against both MCF-7 and NCI-H187. The results showed that ethanolic extract from reflux extraction for 2 hours displayed the strongest cytotoxicity, being more active against MCF-7 (IC$_{50}$ 48.25±0.11 μM) and NCI-H187 (IC$_{50}$ 30.05±1.11 μM) and at a higher level (lower IC$_{50}$) than other conditions of reflux extraction. Interestingly, the extraction time for 3 hours displayed lower cytotoxicity than the extraction time for 1.5 and 2 hours. These results revealed that ethanolic extracts from reflux extraction showed promising cytotoxic activities against MCF-7 and NCI-H187, with IC$_{50}$ values ranging from 30.05 to 65.01 μM. In addition, the results showed that not only solvents that effected to the obtained bioactive compounds, but also the extraction time that can help to improve the cytotoxicity. However, this preliminary correlation certainly requires further work for conformation and topoisomerase II inhibitory activities. For previous cytotoxicity studies, Manosroi et al. have reported the biological activities of C. lacryma-jobi extracts obtained from each parts of C. lacryma-jobi such as whole seed, endosperm and hull. The results showed that C. lacryma-jobi extracts performed by hot methanol after pretreatment showed the highest anti-proliferative activity on HT-29 with the IC$_{50}$ values of 11.61 ± 0.95 μg/ml, while the extract from cold methanol extraction after pretreatment gave the highest apoptosis (8.17 ± 1.18%) with no necrosis [20].

Table 2 Cytotoxicity (IC$_{50}$) of all extracts from each extraction methods in different solvents against MCF-7 and NCI-H187 cells.

<table>
<thead>
<tr>
<th>Extraction methods</th>
<th>Conditions</th>
<th>Cytotoxicity (IC$_{50}$ μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MCF-7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCI - H187</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td></td>
<td>13.50</td>
</tr>
<tr>
<td>Maceration (aqueous extract)</td>
<td>3 days</td>
<td>inactive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>inactive</td>
</tr>
<tr>
<td>Maceration (95% ethanol extract)</td>
<td>3 days</td>
<td>inactive</td>
</tr>
<tr>
<td></td>
<td>5 days</td>
<td>inactive</td>
</tr>
<tr>
<td></td>
<td>7 days</td>
<td>inactive</td>
</tr>
<tr>
<td>Maceration (95% ethanol extract)</td>
<td>5 days</td>
<td>75.11±0.05</td>
</tr>
<tr>
<td></td>
<td>7 days</td>
<td>82.05±1.11</td>
</tr>
<tr>
<td>Reflux (aqueous extract)</td>
<td>1.5 hours</td>
<td>inactive</td>
</tr>
<tr>
<td></td>
<td>2 hours</td>
<td>inactive</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>inactive</td>
</tr>
<tr>
<td>Reflux (95% ethanol extract)</td>
<td>1.5 hours</td>
<td>59.11±2.11</td>
</tr>
<tr>
<td></td>
<td>2 hours</td>
<td>48.25±0.11</td>
</tr>
<tr>
<td></td>
<td>3 hours</td>
<td>65.01±0.41</td>
</tr>
</tbody>
</table>
4. Conclusions

In this work, *C. lacryma-jobi* extract was prepared using different extraction methods including maceration and reflux extraction. Two solvents (95% ethanol and water) were used in each extraction method to obtained ethanolic and aqueous extract. The optimum extraction conditions which gave the maximum yield of *C. lacryma-jobi* extract were ethanol concentration 95% and extraction time 2 hours at 65 °C. The yield of *C. lacryma-jobi* extract was up to 12.08 ± 0.04% under this condition. The antioxidant activity and total phenolic contents of *C. lacryma-jobi* extracts were investigated. The results showed that the highest DPPH scavenging activity and total phenolic contents were observed from ethanolic extracts obtained from reflux extraction for 2 hours. The cytotoxicity of each extract was investigated against two cancer cell lines including MCF-7 and NCI-H187. The results revealed that the ethanolic extract obtained from reflux extraction for 2 hours exhibited moderate cytotoxicity against MCF-7 (IC₅₀ 48.25±0.11 μM) and NCI-H187 (IC₅₀ 30.05±1.11 μM). This ethanolic extract gave higher cytotoxicity than the other extracts but lower than the anticancer drug of doxorubicin. The finding of the present study suggested that 95% ethanolic extract of *C. lacryma-jobi* from reflux extraction for 2 hours at 65 °C could be used as a potential source of antioxidants.

Acknowledgements
We are grateful for financial support from the National Research Council of Thailand, Applied Chemistry Program, Chemistry Program, Faculty of Science, Maejo University.

References