Induction of Apoptotic Cell Death in HL-60 Cells by Jacaranda Seed Oil Derived Fatty Acids

Masao Yamasaki, Chihiro Motonaga, Marino Yokoyama, Aya Ikezaki, Tomoka Kakihara, Rintaro Hayasegawa, Kaede Yamasaki, Masanobu Sakono, Yoichi Sakakibara, Masahito Suiko and Kazuo Nishiyama

Division of Food Science and Nutrition, Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan

Abstract: Various fatty acids are attracting considerable interest for their anticancer effects. Among them, fatty acids containing conjugated double bonds show one of the most potent cytotoxic effects on cancer cells. Here, we focused on the cancer cell killing activity of jacaranda seed oil. The seed oil of jacaranda harvested from Miyazaki in Japan contained 30.9% cis-8, trans-10, cis-12 octadecatrienoic acid, called jacaric acid (JA). Fatty acid prepared from this oil (JFA) and JA strongly induced cell death in human leukemia HL-60 cells. On the other hand, linoleic acid and trans-10, cis-12 conjugated linoleic acid (<10 μM) did not affect cell proliferation and viability. An increase in the sub-G1 population and internucleosomal fragmentation of DNA was observed in JA- and JFA-treated cells, indicating induction of apoptotic cell death. Finally, the cytotoxic effects of JA and JFA were completely abolished by α-tocopherol. Taken together, these data suggest that jacaranda seed oil has potent apoptotic activity in HL-60 cells through induction of oxidative stress.

Key words: jacaric acid, jacaranda seed oil, apoptosis, oxidative stress

1 INTRODUCTION

Accumulating data strongly suggest that various fatty acids exert anticancer effects, which can be attributed to their ability to induce apoptotic cell death, regulate cell cycle, and manipulate eicosanoid production. Among them, fatty acids with conjugated double bonds are attracting considerable attention. Conjugated linoleic acids (CLAs) are the most well-studied conjugated fatty acids, and data show the cytotoxic activity of trans-10, cis-12 (t10, c12)-CLA against various cancer cells. Interestingly, several studies showed that c9, t11-CLA did not exert anticancer effects, suggesting the importance of the molecular structure of the fatty acid. Moreover, naturally occurring conjugate linolenic acids (CLNs) have been reported to exert potent cytotoxic effects on cancer cells. Jacaranda is a subtropical plant originating from South America and is grown in the southern part of Kyushu in Japan. It is the sole source of natural c8, t10, c12 octadecatrienoic acid, called jacaric acid (JA), which is a type of CLN. A recent study revealed that JA has the most potent cytotoxic effects on human colon cancer cells. On the other hand, other fatty acids such as linoleic acid (LA), oleic acid, and palmitoleic acid, interfere with the cytotoxic effects of CLA on cancer cells. This interaction is at least partially illustrated by the inhibitory effect of CLA on stearoyl-CoA desaturase (SCD) that mediates delta-9 desaturation of saturated fatty acids. Naturally, because jacaranda seed oil contains several fatty acids aside from JA, there may be differences in the anticancer effects of jacaranda seed oil and purified JA. Here, we harvested jacaranda seeds to prepare seed oil and evaluated its cytotoxic effect on human leukemia HL-60 cells.

2 EXPERIMENTAL PROCEDURES

2.1 Materials

All fatty acids used in this study (LA, t10, c12-CLA, JA) were purchased from Cayman Chemical Company (Ann Arbor, MI). α-Tocopherol (Toc) was purchased from Wako Pure Chemicals (Osaka, Japan).
2.2 Preparation of jacaranda seed oil and preparation of fatty acids

Jacaranda seeds were kindly provided by the Miyazaki prefectural government agricultural research institute, subtropical plants branch facility (Nichinan, Japan). Twenty grams of jacaranda seeds was crushed with a food processor, and 100 ml of hexane was added to extract seed oil. Then, hexane was dried up using a rotary vacuum evaporator and the extracted oil was kept at −30°C before analysis. Extracted oil (450 mg) was mixed with 75 ml of 0.3 N KOH/90% methanol, and the mixture was incubated at 37°C for 2 h. After cooling to room temperature, hexane was added to remove unreacted triglycerides, and 6 N HCl was added to 90% methanol layer to convert the sodium salts of fatty acids to free fatty acids. To verify the purity of the fatty acids, we performed thin layer chromatography using silica gel thin layer chromatography (Wako, Osaka, Japan). Hereinafter, fatty acids prepared from jacaranda seed oil are called “JFA.”

2.3 Analysis of fatty acid composition

The procedure for extracting cellular lipids was performed according to the method described by Folch et al. using a chloroform/methanol (2:1, by vol) mixture. After extraction, seed oil was resolved into 0.5 M KOH/methanol and kept 100°C for 5 min to saponificate triglycerides. Then, 5% HCl/methanol was added to methylate fatty acids and kept 100°C for 5 min. Methylated fatty acids were re-extracted using hexane. Fatty acid composition was analyzed by gas-liquid chromatography (GC-14B Shimadzu) equipped with a Supelcowax-10 column (0.32 mm × 60 m, film thickness 0.25 μm, Supelco Inc., Bellefonte, PA). Column temperature was kept at 220°C, and the detector and injector temperatures were set at 250°C.

2.4 Cells and cell culture

HL-60 cells were purchased from RIKEN BioResource Center (Tsukuba, Japan). Cells were maintained in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) containing 100 units/ml penicillin G and 100 μg/ml streptomycin. Cells were subcultured twice a week. For experiments, the cell number was adjusted to 1.0 × 10⁵ cells/ml. The number of viable cells was counted by the trypsin blue exclusion method.

2.5 Internucleosomal DNA fragmentation

Internucleosomal DNA fragmentation was evaluated using agarose gel electrophoresis. Briefly, cells were washed with ice-cold PBS and lysed with 10 mM Tris-HCl (pH 7.4) containing 10 mM EDTA and 0.5% Triton X-100. Cell lysate was treated with 100 μg/ml RNase A (Sigma) at 37°C for 1 h followed by 100 μg/ml proteinase K (Sigma). Then, DNA was precipitated in 0.5 M NaCl in 50% isopropanol and dissolved in Tris-EDTA buffer. DNA samples were subjected to 2% agarose gel electrophoresis and detected by SYBR Green dye staining (Takara Bio, Inc, Otsu, Japan).

2.6 Cell cycle and apoptosis analysis

For cell cycle and sub-G₁ analysis, cells were fixed in ice-cold methanol for 30 min and then treated with 10 μg/ml propidium iodide and 10 μg/ml RNase. Analysis of cell cycle and detection of sub-G₁ population were performed using a flow cytometer (Cell Lab Quanta SC MPL, Beckman Coulter Inc., Fullerton, CA) equipped with MultiCycle software (San Diego, CA).

2.7 Statistical analysis

Data, except those in Fig. 4, were analyzed using Student’s t test to evaluate differences among groups. Values marked with asterisks are significantly different from the control values at p < 0.05 (*) or p < 0.01 (**). Data in Fig. 4 were analyzed by the Tukey-Kramer test, and differences were considered significant at p < 0.05.

3 RESULTS

We first examined the fatty acid composition of jacaranda seed oil to determine its JA content. As shown in Table 1, LA is the most common fatty acid (41.4 wt.%), and JA accounts for 30.9 wt.% of total fatty acid. In addition, small percentages of JA isomers were detected. We harvested jacaranda seeds in October, December, and February in 2010 and 2011, and found that the JA level was comparable among all seasons (data not shown). After preparation of JFA, we examined the cytotoxic effect of JFA on HL-60 cells. As shown in Fig. 1, JFA dose dependently increased cell death ratio; further, a significant difference in cell death ratio was detected between 2.5, 5, and 10 μg/ml JFA and the control group. In addition, commercially available pure JA decreased viable cell number and increased cell death ratio; further, a significant difference in cell death ratio was detected between 2.5, 5, and 10 μg/ml JFA and the control group. In addition, commercially available pure JA decreased viable cell number and increased cell death ratio; further, a significant difference in cell death ratio was detected between 2.5, 5, and 10 μg/ml JFA and the control group. In addition, commercially available pure JA decreased viable cell number and increased cell death ratio; further, a significant difference in cell death ratio was detected between 2.5, 5, and 10 μg/ml JFA and the control group. In addition, commercially available pure JA decreased viable cell number and increased cell death ratio; further, a significant difference in cell death ratio was detected between 2.5, 5, and 10 μg/ml JFA and the control group. In addition, commercially available pure JA decreased viable cell number and increased cell death ratio; further, a significant difference in cell death ratio was detected between 2.5, 5, and 10 μg/ml JFA and the control group. In addition, commercially available pure JA decreased viable cell number and increased cell death ratio; further, a significant difference in cell death ratio was detected between 2.5, 5, and 10 μg/ml JFA and the control group. In addition, commercially available pure JA decreased viable cell number and increased cell death ratio; further, a significant difference in cell death ratio was detected between 2.5, 5, and 10 μg/ml JFA and the control group. In addition, commercially available pure JA decreased viable cell number and increased cell death ratio; further, a significant difference in cell death ratio was detected between 2.5, 5, and 10 μg/ml JFA and the control group. In addition, commercially available pure JA decreased viable cell number and increased cell death ratio; further, a significant difference in cell death ratio was detected between 2.5, 5, and 10 μg/ml JFA and the control group. In addition, commercially available pure JA decreased viable cell number and increased cell death ratio; further, a significant difference in cell death ratio was detected between 2.5, 5, and 10 μg/ml JFA and the control group. In addition, commercially available pure JA decreased viable cell number and increased cell death ratio; further, a significant difference in cell death ratio was detected between 2.5, 5, and 10 μg/ml JFA and the control group. In addition, commercially available pure JA decreased viable cell number and increased cell death ratio; further, a significant difference in cell death ratio was detected between 2.5, 5, and 10 μg/ml JFA and the control group. In addition, commercially available pure JA decreased viable cell number and increased cell death ratio; further, a significant difference in cell death ratio was detected between 2.5, 5, and 10 μg/ml JFA and the control group. In addition, commercially available pure JA decreased viable cell number and increased cell death

Table 1 Fatty acid composition of jacaranda seed oil.

<table>
<thead>
<tr>
<th>Fatty acids (wt.%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>18:0</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>18:1 (n-9)</td>
<td>11.1 ± 0.1</td>
</tr>
<tr>
<td>18:2 (n-6)</td>
<td>41.4 ± 0.2</td>
</tr>
<tr>
<td>18:3 (8c, 10t, 12c)</td>
<td>30.9 ± 0.8</td>
</tr>
<tr>
<td>18:3 (8c, 10t, 12c)</td>
<td>1.1 ± 1.1</td>
</tr>
<tr>
<td>18:3 (8t, 10t, 12c)</td>
<td>1.9 ± 0.0</td>
</tr>
<tr>
<td>Others</td>
<td>5.7 ± 0.4</td>
</tr>
</tbody>
</table>

Data are mean ± SE of 3 measurements.
death ratio in a dose-dependent fashion.

Next, we evaluated the cytotoxic effect of LA, because it was the most common fatty acid in JFA. LA at concentrations of up to 10 μM did not affect cell viability and proliferation (Fig. 2). To evaluate the cytotoxicity of JA and JFA, we analyzed the effect of t10, c12-CLA, which exerts potent cytotoxic effects on various cancer cells. As shown in Fig. 2, the effect of t10, c12-CLA was weak, and no significant difference in viable cell number and cell death ratio was detected.

To investigate the mechanisms behind the decrease in viable cell number and the increase in cell death ratio caused by JA and JFA, we performed cell cycle and apoptosis analysis. Table 2 shows the effect of JA and JFA on the cell cycle of HL-60 cells. Neither JFA nor JA affected the cell cycle distribution of HL-60 cells at any concentration. Because the cytotoxicity was too strong to recover the cells for cell cycle and apoptosis analysis, it was difficult to analyze the effects of JFA and JA at concentrations above 2.5 μg/ml and 2.5 μM, respectively. Apparently, 2.5 μM JA and 2.5 μg/ml JFA increased sub-G1 cell populations, and the electrophoresis data show internucleosomal fragmentation of DNA in cells treated with 2.5 μM JA and 2.5 μg/ml JFA (Fig. 3).

To investigate the effect of JFA on the fatty acid composition, cells were treated with 0 or 2.5 μg/ml JFA for 24 h and cellular lipids were extracted. Although LA level in cells treated with JFA was significantly higher than that of control cells, JFA did not affect other major fatty acid compositions shown (Table 2). Moreover, JA was not detected at this condition.

Finally, to determine the involvement of reactive oxygen species in apoptosis induction, we cotreated the cells with JA, JFA, and Toc. Treatment with 10 μM JA and 10 μg/ml JFA killed most of the cells and markedly increased cell death ratio. On the other hand, Toc completely prevented the reduction in viable cell number and the increase in cell death ratio (Fig. 4).
M. Yamasaki, C. Motonaga, M. Yokoyama et al.


928

4 DISCUSSION
In this experiment, we extracted jacaranda seed oil from *Jacaranda mimosifolia*, which is grown in the southern part of Miyazaki in Japan, because jacaranda seed oil has been reported to contain 32% JA\textsuperscript{14}. The levels of JA in the JFA used in this experiment were comparable with previ-

Table 2  Effects of jacaranda seed oil and jacaric acid on the cell cycle distribution of HL-60 cells.

<table>
<thead>
<tr>
<th></th>
<th>G\textsubscript{i}</th>
<th>S</th>
<th>G\textsubscript{2}/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>34.9 ± 3.4</td>
<td>52.7 ± 2.5</td>
<td>12.4 ± 1.6</td>
</tr>
<tr>
<td>JFA 1.25 μg/ml</td>
<td>33.5 ± 3.1</td>
<td>53.5 ± 2.6</td>
<td>12.9 ± 1.4</td>
</tr>
<tr>
<td>JFA 2.5 μg/ml</td>
<td>34.8 ± 3.3</td>
<td>51.3 ± 2.3</td>
<td>13.9 ± 2.9</td>
</tr>
<tr>
<td>JA 1.25 μM</td>
<td>34.5 ± 3.1</td>
<td>53.2 ± 2.0</td>
<td>12.3 ± 1.8</td>
</tr>
<tr>
<td>JA 2.5 μM</td>
<td>33.4 ± 4.0</td>
<td>52.2 ± 2.0</td>
<td>14.5 ± 1.9</td>
</tr>
</tbody>
</table>

Data are mean ± SE of 3 measurements. Cells were plated at a density of 1.0 × 10\textsuperscript{5} cells/ml in 10% FBS-RPMI, cultured for 24 h, and then treated with jacaranda seed oil and jacaric acid for 24 h. After incubation, cell cycle analysis was performed using flow cytometry.

Fig. 2  Effect of linoleic acid and t\textsuperscript{10}, c\textsubscript{12}-conjugated linoleic acid on the proliferation of HL-60 cells. Cells were plated at a density of 1.0 × 10\textsuperscript{5} cells/ml in 10% FBS-RPMI and cultured for 24 h, and then treated with jacaranda seed oil and jacaric acid for 24 h. After that, viable cells were counted by the trypan blue dye exclusion method. Data are shown as mean ± SE. LA, linoleic acid; CLA, conjugated linoleic acid.
Previously reported values. In the present study, both JFA and JA reduced viable cell number and increased cell death ratio, suggesting the potent induction of cell death in HL-60 cells (Fig. 1). In our sample, the most common fatty acid was LA, followed by JA and oleic acid. As shown in Fig. 2, LA at concentrations of up to 10 μM did not have any cytotoxic effect on HL-60 cells. Because 10 μM LA is equivalent to 8.6 μg/ml JFA, which is highly toxic, LA is not a contributing factor in the observed effects. In addition, 10 μg/ml JFA contains 3.6 μM oleic acid. Considering that a previous study showed that 25 μM oleic acid did not affect the cell viability of HL-60 cells, we deemed it unlikely that oleic acid in JFA was responsible for the cytotoxic effects on HL-60 cells. As shown in Fig. 1, given that 2.5 μM oleic acid did not affect the cell viability of HL-60 cells, we did not consider oleic acid as a factor in the observed cytotoxic effects.

Fig. 3
Jacaranda seed oil and jacaric acid induce apoptotic cell death in HL-60 cells. Cells were plated at a density of 1.0 × 10⁵ cells/ml in 10% FBS-RPMI and cultured for 24 h, and then treated with jacaranda seed oil and jacaric acid for 24 h. After incubation, the sub-G₁ population was analyzed using flow cytometry (A), and internucleosomal DNA fragmentation was evaluated by agarose gel electrophoresis (B). Data in (A) are shown as mean ± SE, and significant differences were evaluated using Student’s t test. *p < 0.05, **p < 0.01, versus the control.

Fig. 4
The cytotoxic effects of jacaranda seed oil and jacaric acid are abolished by α-tocopherol. Cells were plated at a density of 1.0 × 10⁵ cells/ml in 10% FBS-RPMI and cultured for 24 h, and then treated with jacaranda seed oil (10 μg/mL), jacaric acid (10 μM), and 100 μM α-tocopherol for 24 h. After that, viable cells were counted by the trypan blue dye exclusion method. Data are shown as mean ± SE, and significant differences were evaluated using the Tukey-Kramer test. Values not sharing common letter are significantly different from each other at p < 0.05. JA: jacaric acid, JFA: fatty acid prepared from jacaranda seed oil, Toc; α-tocopherol.
μg/ml JFA is equivalent to 2.9 μM JA and the cytotoxic activity of 2.5 μg/ml JFA was similar to that of 2.5 μM JA, the cytotoxic activity of JFA was mainly attributed to JA. As shown in Table 1, JFA contains small percentages of CLN isomers. They are so small that we believe that they are not active components in this experiment. There are only few naturally occurring CLNs; however, α-linolenic acid can be converted into a mixture of various CLNs through alkaline isomerization. Further studies are needed to clarify the relationship between the cytotoxic activity and structure of CLN(37).

CLA, especially t10, c12-CLA, has been shown to exert cytotoxic effects on various cancer cell lines(4-6). Tsuzuki et al. reported that 5 μg/ml t10, c12-CLA did not affect the number of viable HL-60 cells, which corresponds to 18 μM(7). Therefore, the t10, c12-CLA concentration used in this experiment was too low to exert cytotoxic effects (Fig. 2) and it is obvious that the cytotoxic activity of JA is much greater than the effect of CLA. We propose the marked reduction of intracellular oleic acid and palmitoleic acid by inhibition of SCD as one of the cytotoxic mechanisms of t10, c12-CLA against cancer cells. Previous studies showed that exogenous oleic acid or palmitoleic acid completely abrogated the action of t10, c12-CLA(11-13). Interestingly, dietary jacaranda seed oil decreased the delta-9 desaturation index in the liver and adipose tissue and showed inhibitory effects on SCD-1 mRNA expression in the liver of ICR mice(19). As already described, JFA contains 11.1% oleic acid; however, the cytotoxic effect of JFA was not inferior to that of pure JA. Moreover, LA, the most common fatty acid in JFA, did not affect the cytotoxicity of JA. As shown in Table 3, although JFA increased LA level in cellular fatty acid composition, JFA did not affect the other fatty acid compositions. Altogether, the cytotoxic effect of JA on HL-60 seems to be not induced by the change in fatty acid composition. Interestingly, as we could not detect any JA in the cells treated with 2.5 μg/ml JFA, it is considered that detectable level of JA is not essential for inducing apoptotic cell death.

Oxidative stress and resulting DNA damage are among the causative factors of carcinogenesis; however, excessive oxidative stress induces apoptotic cell death of cancer cells. For instance, hydrogen peroxide and peroxynitrite induced apoptotic cell death in HL-60 cells(20,21). Tsuzuki et al. have revealed that CLN induced oxidative stress-dependent cell death in cancer cells(7,8). In the present study, the cytotoxic effects of both JFA and JA were completely abolished by Toc, indicating an oxidative stress-dependent mechanism. On the other hand, we have shown that Toc failed to abolish the cytotoxic activity of t10, c12-CLA(19). These observations suggest that CLA and JA have different mechanisms of apoptotic activity.

It has been reported that excess oxidative stress induces not only necrotic cell death but also apoptotic cell death(22). Internucleosomal DNA fragmentation and the appearance of a sub-G1 population are the characteristic phenomena observed in apoptotic cells. Our previous data showed internucleosomal DNA fragmentation as one of the features of apoptosis in HL-60 cells(23). In the present study, an increase in the sub-G1 population was observed (Fig. 3), and the electrophoresis data showed an induction of DNA fragmentation at the JFA and JA concentrations that induced cell death. In the case of arachidonic acid, it induces oxidative stress-related apoptotic cell death at physiological concentrations and induces necrotic cell death at supra-physiological concentrations(24). These data suggest that JA

<table>
<thead>
<tr>
<th>Fatty acids (wt.%)</th>
<th>Control</th>
<th>JFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>8.5 ± 0.5</td>
<td>13.4 ± 2.3</td>
</tr>
<tr>
<td>16:1 (n-7)</td>
<td>3.5 ± 0.5</td>
<td>6.5 ± 1.8</td>
</tr>
<tr>
<td>18:0</td>
<td>5.4 ± 0.8</td>
<td>8.4 ± 2.3</td>
</tr>
<tr>
<td>18:1 (n-9)</td>
<td>15.2 ± 2.0</td>
<td>22.5 ± 8.0</td>
</tr>
<tr>
<td>18:2 (n-6)</td>
<td>1.5 ± 0.3</td>
<td>5.1 ± 1.4*</td>
</tr>
<tr>
<td>20:4 (n-6)</td>
<td>25.0 ± 4.0</td>
<td>15.2 ± 2.0</td>
</tr>
<tr>
<td>20:5 (n-3)</td>
<td>7.3 ± 1.2</td>
<td>16.1 ± 5.4</td>
</tr>
<tr>
<td>22:5 (n-6)</td>
<td>1.3 ± 0.5</td>
<td>4.6 ± 2.4</td>
</tr>
<tr>
<td>22:6 (n-3)</td>
<td>13.0 ± 4.1</td>
<td>7.6 ± 1.9</td>
</tr>
<tr>
<td>others</td>
<td>19.2 ± 6.9</td>
<td>15.3 ± 4.9</td>
</tr>
</tbody>
</table>

*Significantly different from the control group at p < 0.05
and JFA induce apoptotic cell death via oxidative stress-related pathways at least at concentrations used in this experiment.

5 CONCLUSION
We revealed that JA and JFA cause apoptotic cell death in HL-60 cells. JA and JFA showed strong cytotoxic effects, and thus are promising agents for leukemia chemoprevention. Their anticancer effects have been reported; however, further studies that detail their molecular mechanisms and evaluate their effects in other cancers are required. Unfortunately, to our knowledge, jacaranda seed oil has not been used as edible oil, and therefore, substantial evidence of its safety must be established.

References
8) Tsuzuki, T.; Kawakami, Y. Tumor angiogenesis suppression by alpha-eleostearic acid, a linolenic acid isomer with a conjugated triene system, via peroxisome proliferator-activated receptor gamma. Carcinogenesis 29, 797-806 (2008).


