Research Article

Tournefortia sarmentosa Inhibits the Hydrogen Peroxide-Induced Death of H9c2 Cardiomyocytes

Chih-Jen Liu,1 Lu-Kai Wang,2 Chan-Yen Kuo3, Mao-Liang Chen3, I-Shiang Tzeng3, and Fu-Ming Tsai3

1Division of Cardiology, Department of Internal Medicine, Taipei Tzu Chi Hospital, The Buddhist Tzu Chi Medical Foundation, New Taipei City 231, Taiwan
2Radiation Biology Core Laboratory, Institute for Radiological Research, Chang Gung University/Chang Gung Memorial Hospital, Linkou, Taoyuan 333, Taiwan
3Department of Research, Taipei Tzu Chi Hospital, The Buddhist Tzu Chi Medical Foundation, New Taipei City 231, Taiwan

Correspondence should be addressed to Fu-Ming Tsai; afu2215@gmail.com

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Tournefortia sarmentosa is a traditional Chinese medicine used to reduce tissue swelling, to exert the antioxidant effect, and to detoxify tissue. T. sarmentosa is also used to promote development in children and treat heart dysfunction. However, many of the mechanisms underlying the effects of T. sarmentosa in the treatment of disease remain unexplored. In this study, we investigated the antioxidant effect of T. sarmentosa on rat H9c2 cardiomyocytes treated with hydrogen peroxide (H₂O₂). T. sarmentosa reduced the cell death induced by H₂O₂. T. sarmentosa inhibited H₂O₂-induced changes in cell morphology, activation of cell death-related caspases, and production of reactive oxygen species. In addition, we further analyzed the potential active components of T. sarmentosa and found that the compounds present in the T. sarmentosa extract, including caffeic acid, rosmarinic acid, salvianolic acid A, and salvianolic acid B, exert effects similar to those of the T. sarmentosa extract in inhibiting H₂O₂-induced H9c2 cell death. Therefore, according to the results of this study, the ability of the T. sarmentosa extract to treat heart disease may be related to its antioxidant activity and its ability to reduce the cellular damage caused by free radicals.

1. Introduction

Oxidative stress caused by free radicals is associated with many cardiovascular diseases, including ischemic heart disease, atherosclerosis, hypertension, and cardiomyopathies [1–4]. Free radicals are generated in the human body during the process of energy production. When the body suffers from inflammation, such as inflammation elicited by infection or injury, many free radicals are produced to remove foreign substances [5]. However, if the host does not have a sufficient protective mechanism to remove excess free radicals after infection, these free radicals will cause cell damage (including lipid peroxidation of the cell membrane and DNA damage) [6, 7]. In addition, environmental factors such as ultraviolet radiation, chemical drugs, and even excessive pressure result in a substantial increase in the production of free radicals in the body [8]. When cardiac muscle cells are attacked by free radicals, the free radicals-induced injury can damage nuclear DNA and lead to permanent DNA damage. The lipids in the cell membrane can be oxidized, altering cell membrane fluidity, preventing nutrients from entering the cell, and eventually causing necrosis. Free radicals attack the side chains of amino acids, causing proteins to lose their function and disrupting the normal functions of cells [5].

Several antioxidant enzymes in the human body, such as superoxide dismutase, glutathione peroxidase, or catalase, counteract free radicals. These enzymes quickly convert the free radicals produced by the body into less toxic or nontoxic substances through redox reactions [9, 10]. However, when the activity of such antioxidant enzymes is insufficient due to insufficient intake of cofactors (superoxide dismutase
requires metal ions, such as Cu\textsuperscript{2+} or Zn\textsuperscript{2+}) or differences in genetic background, cells are unable to avoid free radicals attack and subsequent damage [11]. When a tissue is damaged and dies, it can be restored by the proliferation of stem cells present in the tissue in order to maintain the normal operation of the organ or tissue. However, the existence of cardiac stem cells remains controversial [12]. Therefore, methods to prevent cardiomyocyte death by free radicals or to reduce cells damage caused by the oxidative pressure generated by free radicals are important topics at present.

\textit{Tournefortia sarmentosa} is often mentioned in reports that evaluate its antioxidant activity in Chinese medicine [13, 14]. \textit{T. sarmentosa} also exerts the effects of relieving wind, detoxifying, reducing swelling, treating aching muscles and bones, and promoting childhood development [15–17]. In addition to being used as a specific therapeutic drug in traditional Chinese medicine, \textit{T. sarmentosa} has recently been promoted as a health food. However, although several studies have investigated the antioxidant effect of \textit{T. sarmentosa}, researchers have not determined whether \textit{T. sarmentosa} protects cardiomyocytes from oxidative stress. In this study, we investigated the protective effect of \textit{T. sarmentosa} on hydrogen peroxide- (H\textsubscript{2}O\textsubscript{2}) induced myocardial cell death. In addition to the protective effect of \textit{T. sarmentosa} on cardiomyocytes, we also analyzed the potential active components of \textit{T. sarmentosa} that protect against cardiomyocyte death.

\section{2. Materials and Methods}

\subsection*{2.1. Preparation of an Aqueous Extract of \textit{T. sarmentosa} and Reagents.}

The preparation of the aqueous extract of \textit{T. sarmentosa} was described in a previous study [14]. The various components of the \textit{T. sarmentosa} extract, including caffeic acid, rosmarinic acid, salvianolic acid A, and salvianolic acid B, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

\subsection*{2.2. Cell Culture.}

Rat H9c2 (2-1) cardiomyocytes were purchased from Bioresource Collection and Research Center (Hsinchu city, Taiwan). The cells were cultured in 90% Dulbecco’s modified Eagle’s medium (DMEM) containing 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 10% fetal bovine serum at 37°C in a 5% carbon dioxide environment. During cell culture, mycoplasma contamination was regularly evaluated, and mycoplasma contamination was not detected.

\subsection*{2.3. Detection of Cell Viability and Cell Death.}

WST-1 reagent (Roche Diagnostics, Mannheim, Germany) and a cytotoxicity detection kit (Roche Diagnostics) were used to measure cell viability and cell death, respectively [18]. H9c2 cells were plated in a 6-well plate in triplicate at a density of 10\textsuperscript{4} cells/well and cultured overnight. The medium was replaced with serum-free DMEM, and various doses of \textit{T. sarmentosa} or 100 \textmu m solutions of the components present in \textit{T. sarmentosa} were added and incubated for another 24 h.

After incubation with WST-1 for 3 h and 100 \textmu M H\textsubscript{2}O\textsubscript{2} for another 1 h, the supernatants were collected, and the absorbance values were measured at 450 nm and 650 nm using a multifunctional microplate instrument (Infinite F200, Tecan, Durham, NC, USA). Alternatively, cells growing in the serum-free medium were cultured with various concentrations of \textit{T. sarmentosa} or 100 \textmu m solutions of the components present in \textit{T. sarmentosa} for 24 h, followed by incubation with 100 \textmu M H\textsubscript{2}O\textsubscript{2} for 1 h. The samples were then centrifuged, and the supernatant of each sample was collected. The amounts of lactate dehydrogenase present in the supernatants were detected with a cytotoxicity detection kit.

\subsection*{2.4. Western Blotting.}

H9c2 cells were plated in a 10 cm dish at a density of 10\textsuperscript{6} cells/dish and cultured overnight. After the cells were cultured in the serum-free medium supplemented with various doses of \textit{T. sarmentosa} or 100 \textmu m components present in \textit{T. sarmentosa} for 24 h, the cells were treated with 100 \textmu M H\textsubscript{2}O\textsubscript{2} for 1 h, and the cell extracts were collected with mammalian protein extraction buffer (GE Healthcare Bio-Sciences, MJ, USA). The expression of protein in each sample was analyzed using Western blotting, as described in the previous literature [19]. Caspase-8, caspase-9, and caspase-3 antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). The actin antibody was purchased from Sigma-Aldrich.

\subsection*{2.5. Reactive Oxygen Species (ROS) Measurement.}

Dihydrorhodamine 123 (DHR123) dye was used to detect ROS levels in cells as previously described [14]. Briefly, H9c2 cells were plated in a 10 cm dish at a density of 10\textsuperscript{6} cells/dish and cultured overnight. After the cells were cultured in serum-free medium supplemented with various doses of \textit{T. sarmentosa} or 100 \textmu m components present in \textit{T. sarmentosa} for 24 h, the cells were treated with 100 \textmu M H\textsubscript{2}O\textsubscript{2} and 2.5 \textmu M DHR123 for 1 h. The cells were then trypsinized and passed through a 30 \textmu m filter, and the ROS contents in the cells were analyzed with a flow cytometer (FACScan, Becton Dickinson, Franklin Lakes, NJ, USA).

\subsection*{2.6. Statistical Analysis.}

The experimental data are presented as the average value ± SD of triplicate samples from each independent biological sample. Statistical analysis was analyzed using Student’s \textit{t}-test, and \textit{p} values less than 0.05 were considered significant.

\section{3. Results}

\subsection*{3.1. \textit{T. sarmentosa} Prevented the H\textsubscript{2}O\textsubscript{2}-Induced Death of H9c2 Cardiomyocytes.}

H9c2 cells were selected as the experimental material and treated with 100 \textmu M H\textsubscript{2}O\textsubscript{2} for one hour to induce cell death as described in a previously published study in order to determine whether ROS caused cardiomyocyte death [20]. As shown in Figures 1(a) and 1(b), H\textsubscript{2}O\textsubscript{2} significantly reduced the cell survival rate and increased the number of dead H9c2 cells. However, when the cells were treated with increasing doses of \textit{T. sarmentosa}, the
H₂O₂-induced decrease in the cell survival rate and increase in the cell death rate were significantly inhibited (Figures 1(a) and 1(b)). When the cells were treated with different doses of *T. sarmentosa* alone, no significant changes in cell survival or cell death were observed, suggesting that *T. sarmentosa* is not cytotoxic to H9c2 cells. In addition, the morphology of the cells was observed directly under a microscope. When the cells were treated with H₂O₂, the spindle-like morphology of the cells was not maintained, and the cells detached. When the cells were pretreated with *T. sarmentosa*, especially at a high dose of 50–100 μg/mL, the cell morphology recovered noticeably. Based on these results, *T. sarmentosa* inhibited the changes in cell morphology and cell death induced by H₂O₂ (Figure 1(c)).

3.2. *T. sarmentosa* Reduced the Levels of Apoptosis-Related Caspases and Increased the Production of ROS Induced by H₂O₂. Since *T. sarmentosa* inhibited the H₂O₂-induced death of H9c2 cells, we further explored whether *T. sarmentosa* altered the H₂O₂-induced activation of cell death-related signal transduction pathways. In the cells treated with different doses of *T. sarmentosa*, no caspase-8, caspase-9, or caspase-3 expression was detected. However, when the cells were treated with H₂O₂, the levels of caspase-8, caspase-9, and caspase-3 significantly increased. When the cells were pretreated with high doses of 50–100 μg/mL *T. sarmentosa*, the H₂O₂-induced increases in the levels of the death-related caspases mentioned above were significantly reduced (Figure 2(a)).

Cell death caused by H₂O₂ is closely related to ROS production. Therefore, we further verified whether the inhibition of H₂O₂-induced cell death by *T. sarmentosa* is related to ROS production. As shown in Figure 2(b), H₂O₂ significantly increased the intracellular ROS levels, and the H₂O₂-induced increase in the intracellular ROS content was significantly decreased by treatment with increasing doses of *T. sarmentosa*.

3.3. All the Compounds Present in the *T. sarmentosa* Extract Inhibited H₂O₂-Induced H9c2 Cell Death. We selected several compounds present in the *T. sarmentosa* extract that are commercially available to further explore the components of *T. sarmentosa* that effectively inhibit H₂O₂-induced H9c2 cell death [21]. As shown in Figures 3(a) and 3(b), caffeic acid, rosmarinic acid, salvianolic acid A, and salvianolic acid B exhibited effects similar to those of *T. sarmentosa*, as they significantly reversed the H₂O₂-induced decrease in the H9c2 cell survival rate and increase in the cell death rate.

3.4. All Compounds Present in the *T. sarmentosa* Extract Decreased the H₂O₂-Induced Increases in the Levels of Apoptosis-Related Caspases and Production of ROS. We further analyzed whether the compounds present in the *T. sarmentosa* extract also altered the H₂O₂-induced expression of caspases and production of ROS. H₂O₂ induced the expression of caspases, and except for caffeic acid, which had only a slight effect on the expression of caspase-3, the
H₂O₂-induced expression of caspase-8, caspase-9, and caspase-3 was inhibited by all the compounds found in *T. sarmentosa* extract (Figure 4(a)). In addition, we also analyzed the effects of these compounds on the H₂O₂-induced intracellular ROS production. As shown in Figure 4(b), all the compounds present in the *T. sarmentosa* extract significantly inhibited the H₂O₂-induced ROS production.

### 4. Discussion

*T. sarmentosa* is widely distributed and is found in Taiwan, Vietnam, Malaysia, the Philippines, Indonesia, and many other regions. *T. sarmentosa* is also widely used as a medicinal material. For example, the stems and leaves are mashed and taken orally with wine to promote blood circulation and treat old and new injuries. The medical efficacy of this plant extract has consistently been reported; however, few studies have explored its potential efficacy because the use of traditional Chinese medicine is not often investigated in many scientific studies.

In this study, we confirmed the antioxidant effect of *T. sarmentosa* on cardiomyocytes, but it only inhibited H₂O₂-induced cell death at higher doses. Although *T. sarmentosa* extract has been used as a traditional Chinese medicine in humans for years, no studies have reported its concentration in human blood after consumption; thus, researchers have not determined whether the dose exerts a cardioprotective antioxidant effect. However, *T. sarmentosa* was orally administered at doses of 2,000 mg/kg bodyweight in in vivo studies and at doses of 10–100 mg/mL in cell-based experiments [15]. Therefore, the concentration of the *T. sarmentosa* extract tested in this study should be within a reasonable range. Magnolol is the most popular Chinese medicine used to protect the myocardium and possesses antioxidant activity. The oral dose of *Magnolia officinalis* extract in live animals ranges from 0.48 to 2.5 g/kg/day [22, 23], which was not significantly different from the *T. sarmentosa* dose. In addition, the half-life of magnolol in vivo and the organs to which the drug is distributed after injection have been reported in a review [24]. Therefore, compared with magnolol, the cardioprotective effects of *T. sarmentosa* still need to be verified in additional experiments.

The efficacy of *T. sarmentosa* is related to its individual components. The individual compounds present in the *T. sarmentosa* extract used in this study, including caffeic acid, rosmarinic acid, and salvianolic acid, have a benzene
Figure 3: The compounds present in the T. sarmentosa extract inhibiting H$_2$O$_2$-induced H9c2 cell death. H9c2 cells are treated with 100 µM solutions of the indicated compounds present in the T. sarmentosa extract for 24 h and then treated with 100 µM H$_2$O$_2$ for 1 h. Cell viability (a) and cell death (b) are detected using the WST-1 reagent or by measuring the amount of LDH release, respectively. *P < 0.05 compared with the control group. #P < 0.05 compared with the control group treated with H$_2$O$_2$.

Figure 4: T. sarmentosa extract inhibiting the H$_2$O$_2$-induced expression of cell death-related caspases and production of ROS. H9c2 cells treated with 100 µM solutions of the indicated compounds present in the T. sarmentosa extract for 24 h and then treated with 100 µM H$_2$O$_2$ for 1 h; the level of cell death-related caspases is analyzed by Western blotting (a). The production of ROS is measured with the DHR123 dye and analyzed by flow cytometry (b). *P < 0.05 compared with the control group. #P < 0.05 compared with the control group treated with H$_2$O$_2$. 
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inflammation and myocardial cell apoptosis during myo-
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[35, 36]. Rosmarinic acid has been shown to protect against
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cardial ischemia/reperfusion injury by activating peroxi-
some proliferator-activated receptor γ and inhibiting the
production of inflammatory cytokines, such as IL-6, TNF-α,
and C-reactive protein [37]. In addition, rosmarinic acid has
been shown to improve cardiac dysfunction and mito-
chondrial damage in mice with diabetic cardiomyopathy by
activating the SIRT1/PGC-1α signaling pathway [38]. The
pathway by which salvianolic acid A exerts its protective
effects was similar to that of rosmarinic acid. Arsenic tri-
oxide induced the production of ROS and decreased the
expression level of PGC-1α in cardiomyocytes. The addition
of salvianolic acid A could restore the above phenomena to
reduce cardiac mitochondrial damage and improve the
cardiotoxicity induced by arsenic trioxide [39]. The pro-
tective potential of compounds found in the T. sarmentosa
extract is closely related to the benzene ring structure of
these compounds.

In addition to traditional Chinese medicine, coenzyme
Q10 (CoQ10) is widely used in the market as a health food.
CoQ10 is a coenzyme that is indispensable in the human
body. CoQ10 exists in cells and is mainly distributed in the
heart, kidneys, liver, and muscles. CoQ10 functions to
stimulate mitochondrial energy production. In addition,
CoQ10 functions as an antioxidant in the mitochondria,
is involved in the energy production process, and reduces free
radicals. Damage to mitochondria maintains the integrity
and stability of cells, slows the oxidation of bad cholesterol,
delays aging, and activates the immune system [40]. The
effective dose of CoQ10 for the prevention of cardiovascular
disease is approximately 100–300 mg [41–43]. However, due
to the regulatory limitations of many local laws and regu-
lations, high doses of CoQ10 are not available to many
people, and thus, its efficacy is controversial. From the
perspective of the structures of the compounds, CoQ10 and
T. sarmentosa extracts contain similar antioxidant compo-
nents that have benzene ring structures and unsaturated
hydrocarbon chains, indicating the potential use of
T. sarmentosa as a health food for heart protection.

5. Conclusions

In summary, the T. sarmentosa extract and several com-
ponents present in the T. sarmentosa extract inhibit the
H2O2-induced death of H9c2 cardiomyocytes. Furthermore,
the T. sarmentosa extract and several of its compounds
inhibit the H2O2-induced expression of cell death-related
caspases and production of ROS. Therefore, the
T. sarmentosa extract may have potential for use as a car-
dioprotective health food.

Data Availability

The data used to support the results of this study are in-
cluded within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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