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Research article

# *Ohwia caudata* aqueous extract attenuates senescence in aging adipose-derived mesenchymal stem cells

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A R T I C L E I N F O

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A B S T R A C T

Stem cells exhibit pluripotency and self-renewal abilities. Adipose-derived mesenchymal stem cells can potentially be used to reconstruct various tissues. They possess significant versatility and alleviate various aging-related diseases. Unfortunately, aging leads to senescence, apoptosis, and a decline in regenerative capacity in adipose-derived mesenchymal stem cells. These changes necessitate a strategy to mitigate the effects of aging on stem cells. *Ohwia caudata* (*O. caudata*) has therapeutic effects against several illnesses. However, studies on whether *O. caudata* has therapeutic effects against aging are lacking. In this study, we aimed to identify potential therapeutic anti-aging effects in the crude aqueous extract of *O. caudata* on adipose-derived mesenchymal

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stem cells. Using 0.1 μM doxorubicin, we induced aging in human adipose-derived mesenchymal stem cells (hADMSCs) and evaluated whether various concentrations of *O. caudata* aqueous extract exhibit anti-aging effects on them. The *O. caudata* extract exhibited significant antioxidant effects on hADMSCs without any toxicity. Furthermore, after treatment with the *O. caudata* aqueous extract, the levels of mitochondrial superoxide, DNA double-strand breaks, and telomere shortening were reduced in the hADMSCs subjected to doxorubicin-induced aging. The extract also suppressed doxorubicin-induced aging by upregulating klotho and downregulating p21 in hADMSCs. These findings indicated that the *O. caudata* extract exhibited anti-aging properties that modulated hADMSC homeostasis. Therefore, it could be a potential candidate for restoring the self-renewal ability and multipotency of aging hADMSCs.

## 1. Introduction

Stem cells are multipotent and capable of self-renewal. Stem cells in tissues typically undergo growth and regeneration under normal physiological or stress conditions [1]. Mesenchymal stem cells are adult stem cells that can be easily isolated from the tissues of animals and humans. They have self-renewal and multipotent properties with few ethical controversies [2]. Adipose-derived mesenchymal stem cells have been identified as adult stem cells and the primary source of precursor cells in postnatal tissues. Adipose-derived mesenchymal stem cells have the potential to be utilized in the reconstruction of various tissues, such as cartilage, bone, muscle, myocardium, nerve, liver, kidney, and pancreas. Furthermore, young stem cells have better regeneration and differentiation capabilities than aged stem cells [3–6]. In combination with other therapeutics, adipose-derived mesenchymal stem cells can alleviate several diseases more effectively than other strategies [7–10]. Hence, the potential effects of adipose-derived mesenchymal stem cells are considerably diverse.

Aging is an irreversible dynamic process in all animals; it involves cell senescence, apoptosis, and the loss of regenerative capability in mesenchymal stem cells [11]. In aged mesenchymal stem cells, reactive oxygen species and DNA damage accumulate. Aging exacerbates the damage to proteins and mitochondrial dysfunction in mesenchymal stem cells. All these changes contribute to stem cell loss and dysfunction during aging [11]. Given that adipose-derived mesenchymal stem cells regulate continuous cell renewal in tissues, and the loss of their normal function may be the primary cause of aging, delaying aging in these cells could be a promising avenue for anti-aging research.

Traditional Chinese herbal medicines have been used therapeutically in Asian countries for thousands of years. Various naturally occurring compounds in traditional Chinese herbal medicines have multiple targets, which contribute to their therapeutic effects of strong and diverse [12,13]. Some traditional Chinese herbal medicines exhibit pharmacological effects against aging and aging-related disorders. For instance, Huangqi (*Astragalus membranaceus*), which can limit oxidative stress and inflammation, can reduce neurodegeneration and tumor growth; the saponins of this plant are reported to have anti-aging effects [14]. Ginseng (*Panax ginseng*), traditionally used to extend lifespan, reduces oxidative stress, inflammation, and tumor growth. It can also alleviate aging-related damage to the skin, nervous system, and cardiovascular system [15]. Lingzhi (*Ganoderma lucidum*) can reduce the effects of aging by ameliorating oxidative stress and DNA damage [16,17]. These findings indicate that traditional Chinese herbal medicines have the potential efficacy to alleviate aging. *Ohwia caudata* (*O. caudata*), formerly known *as Desmodium caudatum*, belongs to the Leguminosae family and is widely found in East African countries. Recently, several studies have revealed that *O. caudata* displays a wide variety of biological effects against oxidative stress, inflammation, *respiratory* viruses, Alzheimer's disease, fever, and tumor growth [18–23]. Although *O. caudata* has therapeutic effects against several illnesses, research on the therapeutic effects of the aqueous extract of *O. caudata* is lacking.

Doxorubicin is a cytotoxic chemotherapeutic agent. However, it has also been shown to induce cellular senescence in both cancer and normal cells, acting as a promoter of aging in *in vitro* and in vivo studies [24]. In *in vitro* studies, low concentrations of doxorubicin have been found to induce aging in various cell types, including cardiomyocytes [25], human cardiac progenitor cells [26], vascular smooth muscle cells, human chondrocytes [27], fibroblasts [28], and several different types of human stem cells [29,30]. Similarly, in *in vivo* studies, doxorubicin has been validated to elevate senescence in C57BL/6 mice, result in brain aging in Fischer 344 rats and induce cellular senescence in murine ovaries [24,31,32]. Therefore, in the present study, doxorubicin was used as an aging inducer to induce stem cell senescence. To establish a foundation for investigating the beneficial effects of *O. caudata* against aging and its potential to enhance the well-being of elderly individuals, we examined the anti-aging properties of *O. caudata* aqueous extracts on human adipose-derived mesenchymal stem cells (hADMSCs). After inducing aging with doxorubicin, hADMSCs were treated with the aqueous extract of *O. caudata* to evaluate the anti-aging effects of the extract and elucidate the potential anti-aging mechanisms.

## 2. Materials and methods

### 2.1. Chemical reagents

Unless explicitly specified otherwise, all chemicals used in this study were of the highest analytical grade, meticulously sourced from reputable suppliers such as Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany). *O. caudata* was cultivated in Hualien, Taiwan, and the freshly harvested leaves were gathered in June. The mature and healthy leaves of *O. caudata* were later transported to a Chinese herbal medicine establishment located in Hualien, Taiwan, where they underwent sun drying. Doxorubicin

hydrochloride (44583) was purchased from Sigma-Aldrich.

### 2.2. Preparation of the aqueous extract from O. caudata

Approximately 50 g of *O. caudata* dry leaves were washed to remove dust and then left to dry. The leaves were ground into a powder with a blender. The powder was mixed with 500 ml of MilliQ water, and the mixture was boiled until the water volume reduced to 50 ml. Subsequently, the crude extract underwent centrifugation at 10,000 rpm for 15 min at 4 °C. Following this, the resultant aqueous extract was filtered to eliminate any lingering debris. The aqueous extract obtained was then stored at −20 °C for future use. The clear aqueous extract of *O. caudata* was quantified and found to be 50 mg/ml. It was then stored at −20 °C for future use.

### 2.3. Culture of mesenchymal stem cells derived from human adipose tissue

The StemPro™ human adipose-derived mesenchymal stem cell line (R7788115) was obtained from Thermo Fisher (Waltham, MA, USA). The human adipose-derived mesenchymal stem cells (hADMSCs) were cultured in MesenPRO RS™ Basal Medium supplemented with MesenPRO RS™ Growth Supplement (12,746,012, ThermoFisher) in a controlled incubator with a humidified atmosphere containing 5 % $CO_2$ at 37 °C. Sub-culturing was performed when the cell culture reached 70 % confluency, and cells at passage 8 were used for the experimental procedures.

### 2.4. MTT cell viability assay

Cell proliferation was quantified using the MTT assay [33]. The hADMSCs (passage 8) were seeded in 96-well plates and exposed to various concentrations of *O. caudata* aqueous extract (12.5–800 μg/ml) for 24–72 h. Then, the culture medium was replaced with 100 μl of MTT (0.5 mg/ml) and incubated at 37 °C for 4 h. Subsequently, the excess medium was removed by suction, and the resulting purple formazan was dissolved in 100 μl of dimethyl sulfoxide with gentle shaking in the dark for 15 min. The absorbance was measured at 590 nm using a spectrophotometer [34,35].

### 2.5. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH Antioxidant Assay Kit (Colorimetric) (ab289847, Abcam, Cambridge, UK) was utilized to measure the antioxidant activities of *O. caudata* aqueous extract. The procedure was executed in accordance with the guidelines provided by the manufacturer. Several concentrations of *O. caudata* aqueous extract (10, 50, 100, 200, and 400 μg/ml) were tested. The change in color was measured at 517 nm using a spectrophotometer. The radical scavenging activity was determined using the formula: $((A_{\_control} - A_{\_treatment})/A_{\_control}) \times 100$ %.

### 2.6. Mitochondrial superoxide assay

A mitochondrial superoxide assay kit (fluorometric) (ab219943, Abcam) was used to measure mitochondrial superoxide levels in hADMSCs. Following 24 h of doxorubicin stimulation and subsequent treatment with the aqueous extract of *O. caudata* for another 24 h, all cells were stained with MitoSOX red according to the manufacturer's instructions. This was followed by a 5-min incubation with 4′,6-diamidino-2-phenylindole (DAPI) to stain the cell nucleus. Mitochondrial superoxide production was measured using images captured with an OLYMPUS® BX53 microscope equipped with an image analysis system (Olympus® Corporation, Tokyo, Japan).

### 2.7. Immunofluorescence staining

The treated cells were washed with PBS, fixed with 4 % paraformaldehyde for 30 min at 25 °C, and then permeabilized with 0.1 % Triton X-100 for 30 min at 4 °C. All samples were blocked using 1 % horse serum in PBS for 60 min at 25 °C, followed by incubation with the primary antibodies against γ-H2AX (AP1267, Abclonal, Woburn, MA, USA), klotho (A12028, Abclonal), or p21 (sc-6246, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 12 h at 4 °C. The cells were then washed and incubated for 60 min at 25 °C with the secondary antibodies: Alexa Fluor® 594 goat anti-rabbit IgG (A11012, Invitrogen, Waltham, MA, USA), Alexa Fluor® 488 goat anti-rabbit IgG (A11008), or Alexa Fluor® 594 goat anti-mouse IgG (A11032). The cells were then washed with PBS and stained with DAPI for 10 min. Photographs were taken using an OLYMPUS® BX53 microscope equipped with an image analysis system.

### 2.8. Measurement of telomere length using quantitative PCR

Telomere length analysis was performed using quantitative PCR according to a previously published protocol [36]. The GeneDirex® Genomic DNA Isolation Kit (NA026-0100, GeneDireX, Inc., Taoyuan, Taiwan) was used to isolate genomic DNA. Telomere length was assessed by calculating the ratio of telomere repeat copies to copies of a single-copy gene. The primer sequences for telomeres were 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′ and 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTTT-3′. The primer sequences for the human β-globin, a single-copy gene, were 5′-CACCAACTTCATCCACGTTCACC-3′ and 5′-GCTTCTGACACAACTGTGTTCACTAGC-3′. A two-step PCR cycling protocol was used for the PCR amplification of telomeric sequences. The settings were 95 °C for 15s and 56 °C for 60s, repeated for 40 cycles. Another two-step PCR cycling protocol was used for

PCR amplification of the human β-globin gene. The settings were 95 °C for 15s and 58 °C for 60s, repeated for 40 cycles. Melt curve analysis was performed at the end of 40 cycles of PCR to ensure the absence of primer dimers.

### 2.9. Statistical analysis

The data are expressed as the mean ± standard deviation obtained from three separate experiments. Statistical analysis was conducted using GraphPad Prism 9 statistical software (San Diego, CA, USA). To assess the statistical significance of multiple experiments, a one-way analysis of variance followed by Tukey's test was employed [37]. Statistical significance was defined as a $p$-value less than 0.05.

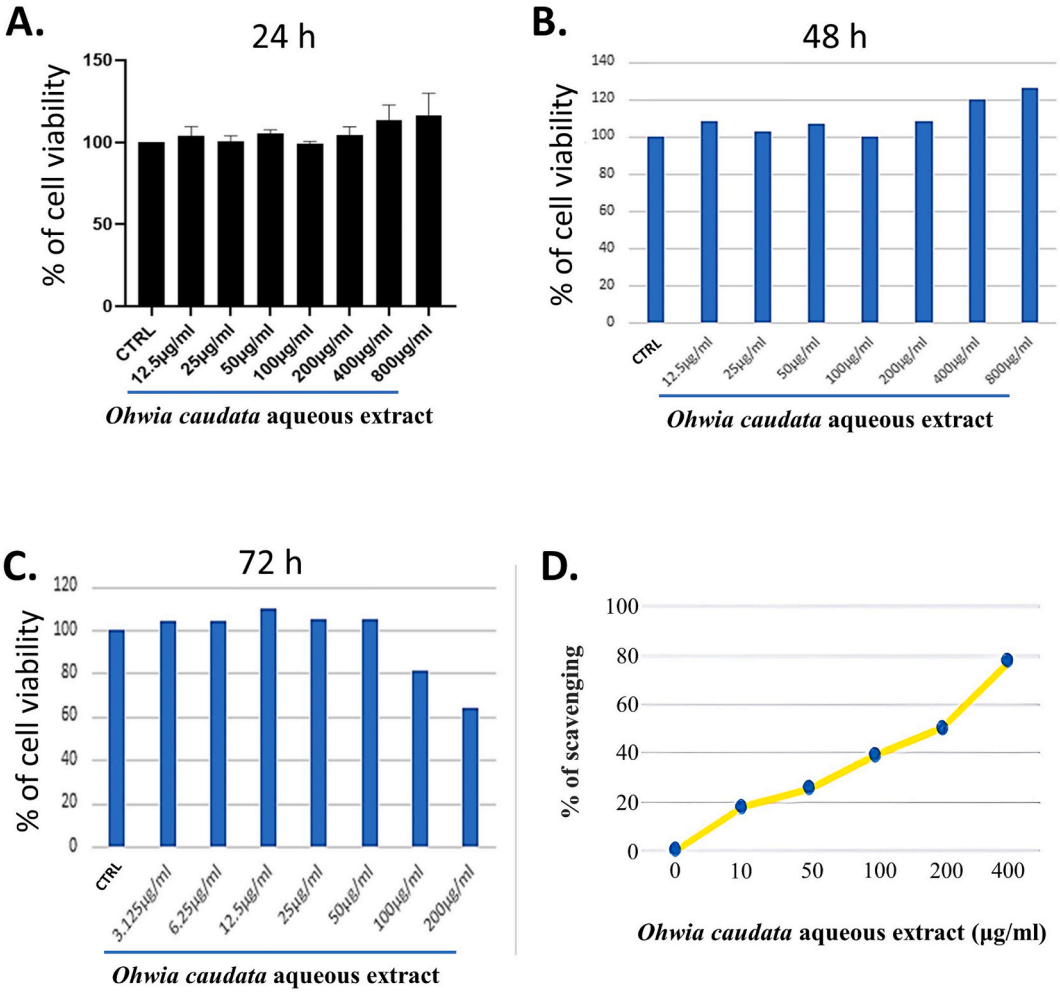


**Fig. 1.** *O. caudata* aqueous extract is non-toxic in hADMSCs after 24 and 48 h of exposure and exhibits considerable antioxidant activity via the DPPH radical scavenging assay (A) Results of the MTT cell viability assay after treating hADMSCs with various concentrations of *O. caudata* aqueous extract (12.5–800 μg/ml) for 24 h. Cell viability in the control group was set at 100 %. Error bars represent the standard deviation. (B) Results of the MTT cell viability assay after treating hADMSCs with various concentrations of *O. caudata* aqueous extract (12.5–800 μg/ml) for 48 h. Cell viability in the control group was set at 100 %. (C) Results of the MTT cell viability assay after treating hADMSCs with various concentrations of *O. caudata* aqueous extract (3.125–200 μg/ml) for 72 h. Cell viability in the control group was set as 100 %. No notable toxicity was detected in hADMSCs following exposure to the *O. caudata* aqueous extract for 24 and 48 h. However, a reduction in cell viability was noted in hADSCs after 72 h of stimulation. (D) Assessment of the in vitro antioxidant activity of *O. caudata* aqueous extract using the DPPH radical scavenging assay. The aqueous extract of *O. caudata* exhibited promising free radical scavenging activity. The experiment was repeated three times. OCAE: treatment with *O. caudata* aqueous extract. CTRL: untreated control group.

## 3. Results

### 3.1. The aqueous extract of O. caudata demonstrates significant antioxidant effects and shows no cytotoxicity in hADMSCs

First, we used the MTT assay to confirm the non-toxicity of the aqueous extract of *O. caudata* towards hADMSCs. After treating hADMSCs with various concentrations of *O. caudata* aqueous extract for 24, 48, and 72 h, the MTT cell viability assay revealed no significant cytotoxic effects within each group after 24-h and 48-h treatments (12.5–800 μg/ml; Fig. 1A–B). However, a reduction in cell viability was noted in hADSCs after 72 h of treatment, exceeding 100 μg/ml (Fig. 1C). These findings indicate that there was no significant negative impact on hADSCs occurred when treated with *O. caudata* aqueous extract between 24 and 48 h. Consequently, the 24-h treatment duration was selected for subsequent experiments.

To evaluate the antioxidant properties of the *O. caudata* aqueous extract, we conducted the DPPH radical scavenging assay. The aqueous extract of *O. caudata* exhibited promising free radical-scavenging effects. The antioxidant effects of the extract were dose-dependent (10–400 μg/ml; Fig. 1D). Together, these results demonstrate that the aqueous extract of *O. caudata* is not toxic to hADMSC after 24 and 48 h of treatment and it has remarkable antioxidant properties.

### 3.2. Treatment with O. caudata aqueous extract reduces mitochondrial superoxide levels during doxorubicin-induced aging in hADMSCs

As the aqueous extract of *O. caudata* exhibited antioxidant properties, we aimed to investigate whether superoxide levels in hADMSCs decrease after treatment with the *O. caudata* aqueous extract. The hADMSCs were incubated for 24 h with 0.1 μM doxorubicin to induce aging, followed by subsequent treatment with varying concentrations of the aqueous extract from *O. caudata* (50, 100, or 200 μg/ml) for another 24 h. The mitochondrial superoxide assay was used to measure mitochondrial superoxide levels in hADMSCs. We noted that doxorubicin significantly increased the levels of cellular mitochondrial superoxide (red color), but the

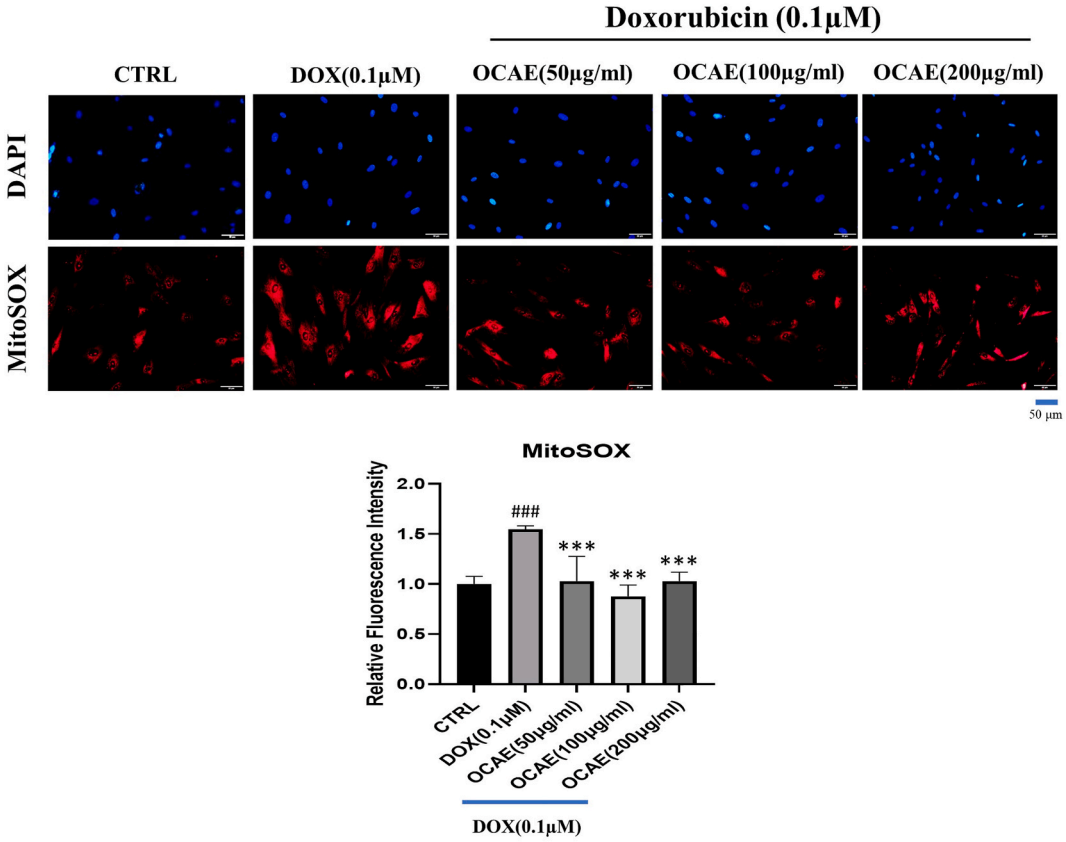


**Fig. 2.** Mitochondrial superoxide levels are increased by doxorubicin-induced aging are reduced by treatment with *O. caudata* aqueous extract. The doxorubicin-exposed hADMSCs were treated with various concentrations of the *O. caudata* aqueous extract (50, 100, and 200 μg/ml) and subsequently evaluated for mitochondrial superoxide generation using MitoSOX Red and fluorescence microscopy. Treatment with the aqueous extract reduced the cellular levels of mitochondrial superoxide radicals in a dose-dependent manner. CTRL: untreated control group. OCAE: Treatment with *O. caudata* aqueous extract. DOX: Doxorubicin. Scale bar: 50 μm ###$p < 0.001$ compared to the untreated control group. ***$p < 0.001$ compared with that in the doxorubicin-induced aging hADMSC group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

addition of the *O. caudata* aqueous extract diminished the rise in mitochondrial superoxide levels (Fig. 2). The reduction in mitochondrial superoxide levels seemed to depend on the dose. These findings indicate that the aqueous extract from *O. caudata* attenuates the rise in mitochondrial superoxide levels induced by doxorubicin.

### *3.3. Administration of the aqueous extract from O. caudata reduces the incidence of DNA double-strand breaks and alleviates the extent of telomere shortening induced by doxorubicin in hADMSCs*

To further investigate the protective effects of the *O. caudata* aqueous extract against doxorubicin-induced aging in hADMSCs, we utilized the γH2AX antibody to identify DNA double-strand breaks through immunofluorescence. Following the same hADMSC culture conditions mentioned in the previous section, doxorubicin-induced γH2AX nuclear foci (red color) were clearly detected. The aqueous extract of *O. caudata* protected against the emergence of DNA double-strand breaks in hADMSCs in a dose-dependent manner (Fig. 3A).

During aging, telomere shortening results in senescence and apoptosis [38]. To investigate whether the *O. caudata* aqueous extract protects against telomere shortening, we assessed telomere length using quantitative PCR. In this study, the relative telomere length was evaluated based on the ratio of telomeric repeat copy number to that of the human β-globin gene, which serves as a single-copy gene (T/S ratio). On average, doxorubicin treatment lowered the T/S ratios in hADMSCs relative to those in the control group. However, the ratio increased after treatment with a high-dose aqueous extract of *O. caudata* (Fig. 3B). These results illustrate that the aqueous extract of *O. caudata* could reduce the number of doxorubicin-induced DNA double-strand breaks and the degree of telomere shortening in hADMSCs.

### *3.4. The aqueous extract of O. caudata suppresses doxorubicin-induced aging in hADMSCs*

Klotho is an anti-aging enzyme. A reduction in its expression can induce the expression of p21 (CDKN1A), an aging marker in cells [39]. Immunofluorescence staining was used to detect the expression levels of klotho (green color) and p21 (red color) in hADMSCs. Under the same hADMSC culture conditions as mentioned in previous sections, doxorubicin induced a reduction in the levels of klotho in the hADMSCs, while levels of p21 increased in the nuclei of hADMSCs. By contrast, the aqueous extract of *O. caudata* successfully reversed the decline in klotho levels during doxorubicin-induced aging and reduced p21 levels in the nuclei of doxorubicin-challenged hADMSCs (Fig. 4). These molecular changes induced by the aqueous extract were also found to be dose-dependent. These findings suggest that the aqueous extract of *O. caudata* could suppress doxorubicin-induced aging in hADMSCs.

## 4. Discussion

Life expectancy has increased worldwide. Nowadays, most people are expected to live into their sixties and beyond. The global aging population is increasing, and the number of people aged 80 years and above is expected to reach approximately 400 million by 2050. Aging has contributed to a worldwide rise in morbidity and disability rates associated with aging. The most effective way to lower disease burden and control costs is to delay the aging process by extending the duration of healthy periods in an individual's lifetime before the onset of aging-related conditions. The most critical factor in increasing healthy lifespans is to alleviate the effects of aging [40]. Aging is a complex process in which homeostasis within the body is disrupted by endogenous and environmental pressures,

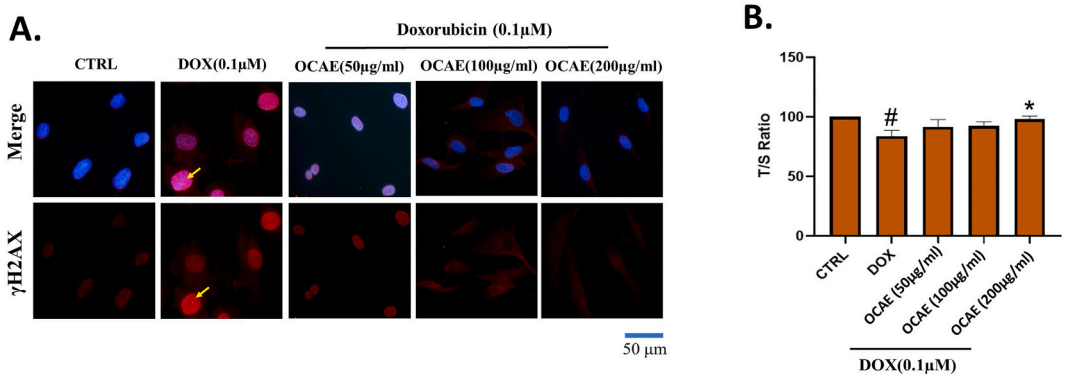


**Fig. 3.** The aqueous extract of *O. caudata* reduces the extent of DNA double-strand breaks and telomere shortening induced by doxorubicin in hADMSCs. (A) Effects of the *O. caudata* aqueous extract on DNA double-strand damage were assessed using γ-H2AX staining. DNA double-strand breaks (red; marked with yellow arrows) were observed during doxorubicin-induced aging in hADMSCs; however, treatment with *O. caudata* aqueous extract reduced the number of DNA double-strand breaks caused by doxorubicin in a dose-dependent manner. Scale bar: 50 μm. (B) Telomere length was analysed using qPCR. Average relative telomere length was represented by the T/S ratios in doxorubicin-induced senescent hADMSCs before and after treatment with *O. caudata* aqueous extract. Error bars represent the standard deviation. #$p < 0.05$ compared with that in the untreated control group. *$p < 0.05$ compared with that in the doxorubicin-induced aging hADMSC group. T: telomere. S: human β-globin. CTRL: untreated control group. OCAE: treatment with *O. caudata* aqueous extract. DOX: doxorubicin. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

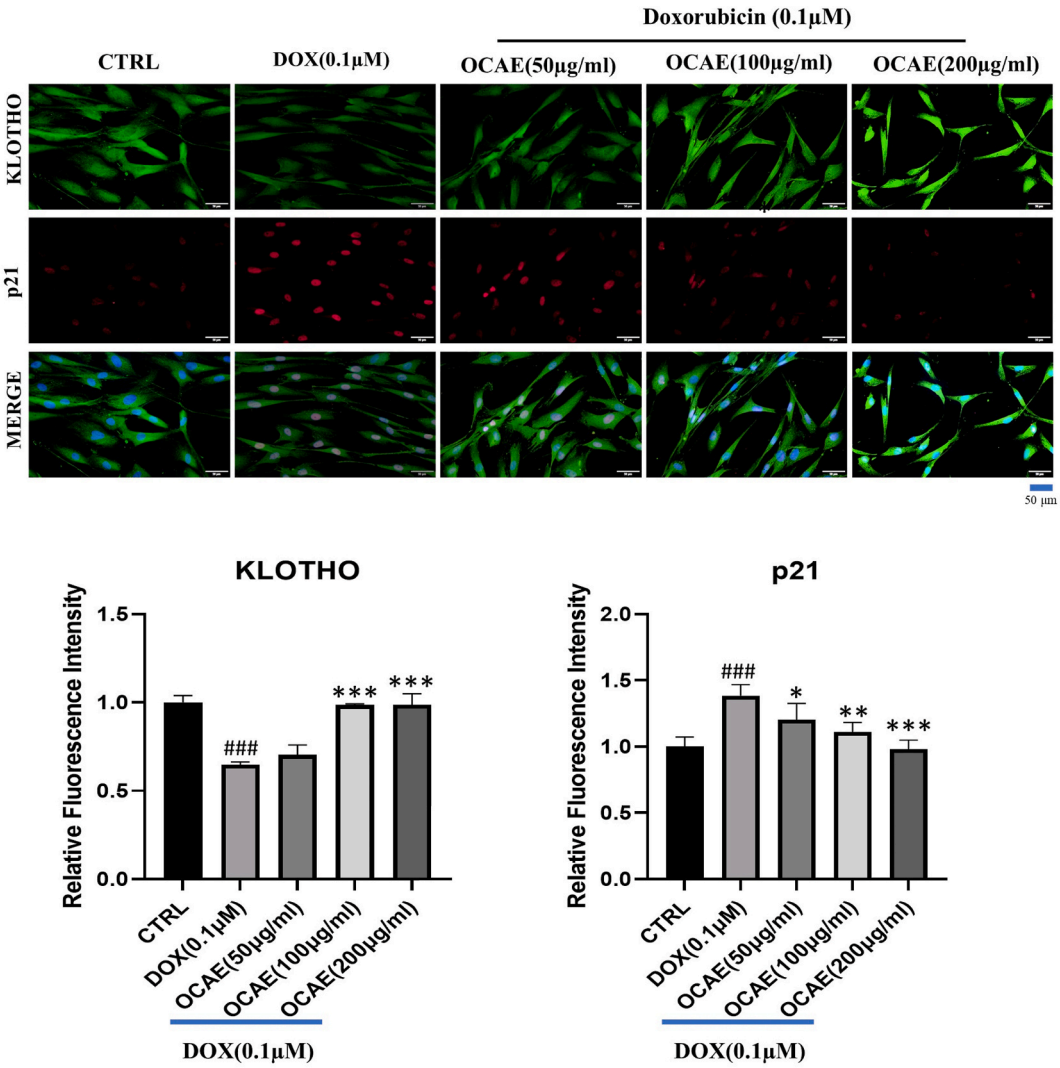


**Fig. 4.** The aqueous extract of *O. caudata* suppresses doxorubicin-induced aging in hADMSCs. After doxorubicin treatment, the hADMSCs were further treated with varying concentrations (50, 100 and 200 μg/ml) of *O. caudata* aqueous extract for 24 h. The cells were stained with anti-klotho (green) and anti-p21 (red) antibodies. CTRL: untreated control group. OCAE: treatment with *O. caudata* aqueous extract. DOX: doxorubicin. Scale bar: 50 μm. Error bars represent the standard deviation. ###$p < 0.001$ compared with that in the untreated control group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with that in the doxorubicin-induced aging hADMSC group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

resulting in morbidity, mortality, and the gradual degeneration of tissues and organs [41]. Adipose-derived mesenchymal stem cells can aid in the reconstruction of various tissues, including cartilage, bone, muscle, myocardium, nerve, liver, kidney, and pancreas. Younger stem cells have better regeneration and differentiation capabilities than mature cells and are potential targets for slowing the aging process [3–6]. However, stem cells can undergo senescence, which reduces their functional capacities. In this study, we investigated the potential anti-aging effects of *O. caudata* aqueous extract on hADMSCs. The study induced senescence in hADMSCs using doxorubicin and examined the effect of different concentrations of *O. caudata* aqueous extract on mitigating these senescence effects. The results showed that the aqueous extract of *O. caudata* exhibited significant antioxidant effects. Furthermore, treatment with the extract resulted in a decrease in mitochondrial superoxide levels, DNA double-strand breaks, and telomere shortening in doxorubicin-induced aging hADMSCs. Notably, the aqueous extract of *O. caudata* demonstrated its ability to suppress doxorubicin-induced aging by upregulating the expression of klotho and downregulating p21 in hADMSCs. These results suggest that the aqueous extract of *O. caudata* has anti-aging properties by maintaining the homeostasis of hADMSCs.

The phenomenon of oxidative stress has been widely acknowledged and established as a primary factor contributing significantly to the complex process of aging [42]. In the current study, we demonstrated that the aqueous extract of *O. caudata* showed promising free radical-scavenging activity in a dose-dependent manner. Additionally, considering the pivotal role of mitochondrial dysfunction in aging regulation, a previous study demonstrated the protective effects of the *O. caudata* aqueous extract against doxorubicin-induced

mitochondrial dysfunction in Wharton's jelly-derived mesenchymal stem cells. Doxorubicin leads to mitochondrial impairment, reduces stemness, and induces apoptosis in these cells. However, the *O. caudata* aqueous extract mitigates these effects by promoting Tid1 and Tom20 expression, reducing reactive oxygen species production, and maintaining mitochondrial membrane potential in Wharton's jelly-derived mesenchymal stem cells [43]. Similarly, in the current study, after inducing cellular aging with doxorubicin, the levels of mitochondrial superoxide increased in hADMSCs. However, this increase was successfully countered by treatment with the aqueous extract of *O. caudata*. This result implies that the anti-aging effect of *O. caudata* could be attributed to its ability to maintain mitochondrial function.

DNA damage is another vital factor contributing to aging by interfering with transcription or replication, leading to the incorrect activation of mechanisms that alter normal cell physiology. This phenomenon leads to metabolic dysregulation, mitochondrial dysfunction, impaired autophagy, and cellular senescence. Eventually, apoptosis occurs, depleting important cell populations, such as neurons and stem cells [44]. DNA double-strand breaks can trigger a severe loss of genomic stability and are recognized as one of the most lethal forms of DNA damage. A rapid cellular response to the induction of DNA double-strand breaks includes the immediate phosphorylation of the histone variant H2AX at the Ser-139 residue in mammals. The existence of phosphorylated H2AX, referred to as γH2AX, has become a notably sensitive and specific molecular indicator in cells for monitoring genomic instability within the nuclei [45]. Nuclear γH2AX can be visualized as foci through immunofluorescence staining using phospho-specific antibodies [46]. Previous studies have confirmed that doxorubicin successfully induces γH2AX nuclear foci [47,48]. Present observations were consistent with these findings, as we also detected increasing levels of γH2AX in the nuclei of hADMSCs after doxorubicin-mediated induction of aging. However, treatment with the aqueous extract of *O. caudata* reversed this increase. In addition, the antioxidant activity and protective effect of the extract on DNA were dose-dependent. Thus, the aqueous extract of *O. caudata* could reduce oxidative stress and DNA damage, two important factors responsible for cellular aging, to maintain the normal functionality of hADSCs.

The shortening of telomeres is another biomarker of cellular aging. Telomeres are non-coding, repetitive short DNA sequences (5′-TTAGGG-3′) located at the ends of all chromosomes in most eukaryotic cells. These sequences play an essential role in preventing the loss of genetic information. After birth, the telomeric sequences deplete by 50–200 base pairs with each round of DNA replication and cell division. When telomere length shortens to a critical limit, cells begin to undergo senescence, apoptosis, and organismal aging [38,49]. In the current study, we used the method published by Joglekar et al. [36] in 2020 to assess the relative telomere length in hADMSCs. Telomere length was markedly shortened during doxorubicin-induced aging of hADMSCs. However, the high-dose aqueous extract of *O. caudata* restricted the shortening of telomeres in these hADMSCs. This finding demonstrated the anti-aging potential of the aqueous extract of *O. caudata*.

To elucidate the molecular mechanisms underlying the anti-aging effects of *O. caudata*, we investigated the expression of klotho and p21. The enzyme klotho was identified as a potential age-suppressing protein. Its deficiency in mice causes premature aging, multiple organ failures, and shortened lifespan, mirroring events of premature aging in humans. Moreover, a defect in klotho activity leads to stem cell senescence and depletion [50]. Additionally, p21, a cyclin-dependent kinase inhibitor, plays an important role in regulating the functions of various senescent stem cells. The expression of p21 increases during the late passage of multipotent stromal cells, reducing their proliferative capacity. Suppression of p21 through RNA interference in human bone marrow-derived multipotent stromal cells leads to an accelerated proliferation rate and enhances the expression of stem cell markers and osteogenic potential [51]. Furthermore, the expression of p21 in bone marrow mesenchymal stem cells of aged rhesus monkeys increases compared to that in cells of younger rhesus monkeys [52]. Hence, downregulation of klotho and upregulation of p21 lead to stem cell senescence and aging. Our immunofluorescence staining showed elevated klotho expression and reduced p21 expression following the induction of aging by doxorubicin in hADMSCs. The effect was reversed by administering the aqueous extract of *O. caudata*. Taken together, these results indicate that the aqueous extract of *O. caudata* has anti-aging effects, making it a promising candidate for maintaining the self-renewal capacity and multipotency of aging hADMSCs.

Although the possible anti-aging effects of *O. caudata* on stem cells were investigated in this study, several limitations need to be considered. Firstly, a decrease in cell viability was observed in hADSCs after 72 h of treatment. This highlights the importance of careful dose selection for clinical trials due to potential side effects. It is worth noting that *O. caudata* is a traditional Chinese medicine, and the existing literature does not mention its toxicity in the human body. Hence, it is also important to consider that in vitro experimental techniques may affect cell viability in hADSCs during long-term treatment. Secondly, evaluating cell viability and aging effects under *O. caudata* and doxorubicin treatments is crucial. Without such assessment, the observed beneficial effects in this study might solely stem from the protective activity of *O. caudata* against cytotoxicity rather than manifesting true anti-aging properties in doxorubicin-challenged hADSCs. Thirdly, investigating the delay or arrest in cell cycle progression and the expression of other genes associated with the aging process, such as β-galactosidase, p16INK4a, and p53 [53–56], would enhance the comprehensiveness of the results in the current study. Lastly, an in vivo study will be essential to substantiate the in vitro findings. Therefore, further investigations are warranted to delve into the detailed anti-aging effects of *O. caudata* on stem cells in subsequent in vivo studies.

In conclusion, we have demonstrated the anti-aging benefits of *O. caudata* aqueous extracts in aging hADMSCs. The aqueous extract of *O. caudata* was not toxic to hADMSCs after 24 and 48 h of treatment and it also exhibited significant levels of antioxidant activity. The levels of mitochondrial superoxide, DNA double-strand breaks, and telomere shortening induced by doxorubicin during aging were reduced following treatment with the aqueous extract. The aqueous extract further suppressed doxorubicin-induced aging by upregulating klotho and downregulating p21. These findings suggest that the aqueous extract of *O. caudata* has anti-aging effects and could help preserve the self-renewal ability and multipotency of aging hADMSCs. This study primarily emphasized the therapeutic application of the crude aqueous extract of *O. caudata* and provided novel evidence to justify further explorations of its health benefits against aging. Finally, due to its classification as a traditional Chinese medicine, the aqueous extract of *O. caudata* can be consumed in liquid form, enabling direct ingestion and physiological impact. Future advancements in the preparation of the *O. caudata* aqueous

extract are anticipated to improve its accessibility, turning it into a convenient formulation similar to herbal tea for daily consumption by the elderly. The efficacy of *O. caudata* is expected to have a direct impact on the human body, thus helping to mitigate the aging process.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

The authors agree the publication.

## Data availability statement

Data will be made available on request. The data that support the findings of this study are available from the corresponding author.

## CRediT authorship contribution statement

**Tsung-Jung Ho:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Bruce Chi-Kang Tsai:** Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Goswami Debakshee:** Writing – original draft, Formal analysis, Data curation. **Marthandam Asokan Shibu:** Writing – review & editing, Project administration, Methodology. **Chia-Hua Kuo:** Writing – review & editing, Investigation, Formal analysis. **Chih-Hsueh Lin:** Resources, Methodology, Conceptualization. **Pi-Yu Lin:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Shinn-Zong Lin:** Writing – review & editing, Supervision, Conceptualization. **Wei-Wen Kuo:** Writing – review & editing, Supervision, Investigation. **Chih-Yang Huang:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Abbreviations

hADMSC human adipose-derived mesenchymal stem cells
DPPH - 1,1-diphenyl-2-picrylhydrazyl
MTT - 3-(4,5-dimethylthiazol)-2-yl-2,5-diphenyltetrazolium bromide
*O. caudata* - *Ohwia caudata*

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