

Original Article

CYTOTOXIC EFFECTS OF *ALBIZIA ZYGIA* (DC) J. F. MACBR, A GHANAIAN MEDICINAL PLANT, AGAINST HUMAN T-LYMPHOBLAST-LIKE LEUKEMIA, PROSTATE AND BREAST CANCER CELL LINES

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ABSTRACT

Objective: The objectives of this study were to investigate the cytotoxic effects of extracts and fractions of *Albizia zygia* roots (AZR) on human T-lymphoblast-like leukemia (Jurkat), prostate cancer (LNCap) and breast cancer (MCF-7) cells and the apoptotic effect in Jurkat cells.

Methods: Aqueous and hydroethanolic extracts were prepared and tested for cytotoxic effects on the cell lines using the tetrazolium-based colorimetric assay. Apoptosis induction was determined by DNA fragmentation, cell morphological changes, flow cytometric and mitochondrial membrane potential assays.

Results: Both aqueous and hydroethanolic extracts were more cytotoxic to Jurkat cells than the other cell lines, with selective index (SI) values of 104.4 and 86.6, respectively. The SI values of the extracts on LNCap cells were 9.0 and 35.4, respectively. Some of the fractions were non-cytotoxic. Nevertheless petroleum ether fraction was cytotoxic towards MCF-7 cells with SI value of 2.4. The hydroethanolic extract exhibited apoptosis via induction of DNA fragmentation in Jurkat cells. Cell morphological changes were consistent with the extract-mediated cytotoxicity and DNA fragmentation. Flow cytometric and mitochondrial membrane potential assays also showed significant apoptotic induction confirming apoptosis by the AZR extract.

Conclusion: This study has shown that AZR possesses anticancer activity by demonstrating a high selective toxicity against Jurkat cells. Furthermore, the hydroethanolic extract induced apoptosis in the Jurkat cells. *Albizia zygia* roots may be a source of novel compounds for the development of new anti-cancer agents.

Keywords: *Albizia zygia*, Cancer cells, Cytotoxicity, Apoptosis

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INTRODUCTION

Albizia zygia (DC) J. F. Macbr is a plant that belongs to the family *Leguminosae* subfamily *Mimosoideae*. It is a gum producing plant, which is commonly found in West Africa [1]. Pharmacological properties of *Albizia* species include activities against inflammatory conditions such as tumours, asthma, arthritis, boils, bronchitis, ulcers and leprosy [2, 3]. In Southern Sudan, Traditional Medicine Practitioners use the aqueous decoction of the powdered bark of the plant to treat malaria and other parasitic infections [2, 3]. Species of the genus *Albizia* have different classes of secondary metabolites including saponins, terpenes, alkaloids and flavonoids. Some bioactive compounds from genus *Albizia* are triterpenoid saponins (julibroside J₂₉, julibroside J₃₀, julibroside J₃₁), macrocyclic alkaloids (budmunchiamines A, B and C) and flavonol glycosides (quercitrin and isoquercitrin) [2]. These have shown biological activities such as antitumor, antiplatelet aggregation, and bactericidal activities. The triterpenoid saponins from *Albizia julibrissin* bark were found to have anticancer activities, and they induced apoptosis in human acute leukemia T-cells [4]. Other *Albizia* species have also been shown to have cytotoxicities towards other cancer cell lines [2]. In Ghana, there are anecdotal claims by Traditional medicine practitioners that *A. zygia* has anticancer property. These claims have, however, not been substantiated scientifically. Earlier phytochemical studies have shown that the plant contains lupen-20(30)-en-3 β -ol and its glycoside, stigmast-5-en-3 β -ol, 5 α -stigmasta-7, 22-dien-3 β -ol, albiziaprenol, phytol and flavonoids [3, 5, 6].

A ten-year review of cancer mortality cases at the Korle-Bu Teaching Hospital in Ghana showed that cancer is a major cause of deaths in

the country and hematopoietic organ, breast, and prostate cancers accounted for the highest deaths caused by cancers [7]. Treatment options available for cancer include chemotherapy, radiation therapy, surgery and biological therapy [8]. Nonetheless, challenges associated with these include resistance to therapy, numerous adverse side effects due to the treatment procedures and the high cost of medication. Thus, there is need for alternative anticancer agents which are more efficacious, less toxic and more affordable.

Efforts have been made to understand the mechanisms of action of anticancer agents to guide the production of alternative and more effective therapies. A preferred target mechanism for cancer treatment is apoptosis, a process of programmed cell death involving various morphological changes and eventual cell death [9]. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation [10]. Mitochondria play an essential role in apoptosis induction by a variety of death stimuli. Mitochondrial changes include the loss of the mitochondrial membrane potential ($\Delta\psi$) and cytochrome C release from the mitochondria to the cytosol, which leads to Caspase-9-dependent activation of caspase-3 resulting finally in apoptosis [11].

In the present study, we investigated the cytotoxic activity of AZR and fractions on Jurkat, LNCap and MCF-7 cells. Furthermore, we determined induction of apoptosis by AZR using Jurkat cell line.

MATERIALS AND METHODS

Materials and reagents

RPMI culture medium, Fetal Bovine Serum (FBS), Penicillin-streptomycin, Trypsin, EDTA, 3-(4,5-Dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) and 7-Aminoactinomycin D (7-AAD) were obtained from Sigma Chemical Company (St. Louis, MO, USA). RNase A and dimethyl sulfoxide (DMSO) were obtained from Wako Pure Chemical Industries (Osaka, Japan) and DNA marker and loading dye were purchased from Watson (Japan). Annexin V-phycoerythrin (PE) was obtained from Molecular Probes (Eugene, OR, U. S. A.). The roots of *Albizia zygia* (Voucher number GC DO 001) were collected from the Botanical Gardens of the University of Ghana, Ghana, (Geographical coordinates: N05.657235° W000.18751°) in September 2012 and identified by a taxonomist at the University of Ghana Herbarium, Accra, where the voucher specimen has been kept. The Jurkat, LNCap, MCF-7 and PNT2 cells were obtained from the RIKEN BioResource Center Cell Bank (Japan). All other chemicals and reagents used were of analytical grade and obtained from standard suppliers.

Preparation of plant extract

Air-dried and pulverized *Albizia zygia* root sample was cold-macerated with 70% ethanol in water (v/v) for 24 h (3X). The ethanol content of the extract was removed using a BUCHI® rotary evaporator. The remaining aqueous extract was lyophilized (LABCONCO®, USA) to obtain the hydroethanolic extract. To obtain aqueous extracts, pulverized samples were extracted with distilled water at 80 °C for 1 h and centrifuged at 4500 rpm for 20 min at 25 °C. The supernatant was frozen and lyophilized.

The petroleum ether, dichloromethane, and ethyl acetate fractions were prepared by sequential extraction from the hydroethanolic extract as previously described [12, 13]. Subsequently, the organic solvents in the fractions were removed under reduced pressure using a rotary evaporator (Buchi R-205, Switzerland).

Cell culture and cytotoxicity assay

The Jurkat, LNCap and PNT2 cells were cultured in RPMI-1640 and MCF-7 in DMEM medium. The media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cultured cells were maintained in a humidified incubator at 37 °C in the presence of 5% CO₂.

Cytotoxic effects of AZR on the cell lines were determined using the MTT assay [14]. Jurkat, LNCap, MCF-7 and PNT2 cells were plated at 1x10⁴ cells/well in a 96 well plate. The cells were treated with varying concentrations of the aqueous and hydroethanolic plant extracts (0–1000 µg/ml) and dichloromethane, ethyl acetate and petroleum ether fractions (0–100 µg/ml). Treated cells were incubated as indicated above for 72 h after which 20 µl of MTT solution (2.5 mg/ml in PBS) was added to each well and incubation was continued for 4 h. Acidified isopropanol containing 1% Triton-X was added to each well, and the plates were kept in the dark at room temperature to dissolve any formazan crystals formed. The optical densities of the wells were measured at 570 nm with a microplate reader (Tecan Infinite M200, Austria). The experiments were performed in triplicate.

Cell morphology examination

The morphology of cultured Jurkat cells was examined after treatment of the cells with hydroethanolic AZR extract as earlier described [15]. Intact cells were considered viable whilst the presence of fragments (apoptotic bodies) was indicative of apoptosis. Jurkat cells were seeded into Petri dishes at a density of 1x10⁶ cells/ml. The cells were treated with concentrations 2.5 and 5.0 µg/ml of the extract and incubated for 24 h at 37 °C. Ursolic acid was used as positive control. The nuclear morphology of cells was observed under a fluorescence microscope (X40) to determine the degree of cell fragmentation and blebs, using Hoechst 33258 staining.

DNA fragmentation and agarose gel electrophoresis

DNA fragmentation and agarose gel electrophoresis were performed as described by Uto *et al.* [15]. The Jurkat cells (1x10⁶ cells/ml) were treated with hydroethanolic AZR extract at concentrations of 2.5 and 5.0 µg/ml or 1% ethanolic solution (negative control) for 24 h. Ursolic acid was used as positive control. The treated cells were washed in ice-cold phosphate buffer saline (PBS) and harvested by

centrifugation. The pellets were resuspended in a lysis buffer (10 mM Tris at pH 7.4, 5 mM EDTA, 1% Triton X-100). After centrifugation, supernatants were removed, and pellets were treated with 1 mg/ml RNase A at 50 °C for 30 min. Cells were then treated with 0.1 mg/ml proteinase K at 50 °C overnight. DNA was isolated, separated on 2% agarose gel and stained with ethidium bromide. DNA band pattern was visualized under UV illumination.

Measurement of apoptosis by flow cytometry

A flow cytometer was used to measure apoptotic effect as described by Xing *et al.* [16] with slight modification. Jurkat cells were seeded into 96-well plates at a concentration of 2 x 10⁵ cell/ml and cultured for 24 h and treated with hydroethanolic AZR extract, ursolic acid (positive control) and 1% ethanolic solution (negative control) for 24 h. The cells were treated with equal volumes of guava nexin reagent (containing Annexin V-phycoerythrin (PE) and 7-Aminoactinomycin D (7-AAD)) and incubated for 20 min in the dark at room temperature and analyzed immediately after incubation with a Guava easy Cyte HT flow cytometer (Millipore Corporation, U. S. A.).

Effect of hydroethanolic AZR on mitochondrial membrane potential

Jurkat cells, seeded at a density of 1x 10⁶ cells/6 ml into 6 cm petri dishes, were treated with vehicle (negative control), 2.5 µg/ml and 5 µg/ml of hydroethanolic AZR extract for 16 h. Ursolic acid was used as positive control [15]. The cells were stained with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) solution (a sensitive mitochondrial membrane potential probe) and analyzed using a microplate reader (Tecan Infinite M200) at wavelength 1: 560 nm (excitation) and 595 nm (emission) for red J-aggregate; wavelength 2: 485 nm (excitation) and 535 nm (emission) for green JC-1 monomers.

Phytochemical analysis of extracts

The presence of alkaloids, saponins, tannins, anthraquinones, flavonoids, terpenoids and cardiac glycosides present in the AZR were determined using established procedures which have been previously described [17].

Statistical analysis

The percent cell viability was calculated using the formula of Ayisi *et al.* [18]. These values were plotted against concentrations of extracts, and the concentrations at which 50% inhibitory activities were attained (IC₅₀) were determined. The selectivity indices of the extracts were also computed as the ratio of the IC₅₀ values in cancer cells to that in normal cells. All data are expressed as means of three experiments+SD. Significant differences between the control and test experiments were determined using Student's t-test from Microsoft Excel 2007 version. P values less than 0.05 was considered statistically significant.

RESULTS

In the present study, we investigated the cytotoxic activities of aqueous and hydroethanolic extracts as well as ethyl acetate, dichloromethane and petroleum ether fractions of AZR towards Jurkat, LNCap and MCF-7 cells. We also evaluated the potential of hydroethanolic AZR extract to induce apoptosis in Jurkat cells.

Results of the MTT assay (table 1) showed that AZR extracts suppressed proliferation of all the cancer cell lines tested. However, suppression of cell growth after 72 h of treatment, which was in a dose-dependent fashion, was strongest in Jurkat cells followed by LNCap cells. The extract showed weaker suppressive effects in MCF-7 cells. The AZR extracts had rather weak inhibitory effects on the proliferation of PNT2 thus translating into very good SI values of AZR for both Jurkat and LNCap cells (table 1).

The data in table 2 shows that treatment of the cell lines with ethyl acetate, dichloromethane, and petroleum ether fractions generally resulted in the weak or non-inhibitory effect on proliferation of Jurkat and LNCap cells. In contrast, the proliferation of MCF-7 cell was significantly inhibited following treatment with petroleum ether fraction of AZR, resulting in a selectivity index of 2.4.

Table 1: Cytotoxicity and selectivity indices of AZR extracts in cancer cell lines

Plant extracts	IC ₅₀ values (µg/ml)		
	Jurkat	LNCap	MCF-7
Aqueous	3.09±0.05 (104.4)	35.8±0.08*(9.0)	445.5±39.39* (0.72)
Hydroethanolic	3.37±0.112 (83.6)	6.08±0.91 (46.4)	377.5±3.81* (0.75)
Curcumin	1.42±0.11 (3.35)	4.02±1.42 (1.18)	2.56±0.82 (1.86)

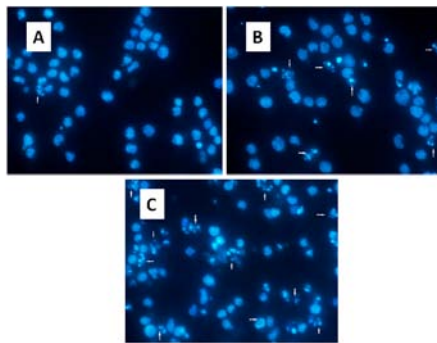
IC₅₀ values are means±SD Experiments in triplicate (n=3). Curcumin was used as positive control. Values in parenthesis are SI values. *P<0.05 shows a significant difference in IC₅₀ values compared to values obtained for curcumin.

Table 2: Cytotoxicity and selectivity indices of AZR fractions in cancer cell lines

Plant fractions	IC ₅₀ values (µg/ml)		
	Jurkat	LNCap	MCF-7
Ethyl acetate	91.4±4.25* (1.09)	>100.0	92.2±10.82* (1.09)
Dichloromethane	>100.0	>100.0	>100.0
Petroleum ether	>100.0	>100.0	41.5±2.4* (2.41)
Curcumin	1.42±0.11 (3.35)	4.02±1.42 (1.18)	2.56±0.82 (1.86)

IC₅₀ values are means±SD Experiments in triplicate (n=3). Curcumin was used as positive control. Values in parenthesis are SI values. *P<0.05 shows a significant difference in IC₅₀ values compared to values obtained for curcumin.

Results obtained for apoptosis are presented in fig. 1-4. Jurkat cells treated with hydroethanolic AZR extract had marked nuclear condensation and more apoptotic bodies compared to the negative control cells which had normal nuclear morphology with very few apoptotic bodies (fig. 1A-C).

**Fig. 1: Apoptotic effect of hydroethanolic extract of AZR in Jurkat cells**

Cells were cultured in the presence of the negative control (1% ethanolic solution, A), 2.5 µg/ml (B) and 5.0 µg/ml (C) of the extract, respectively. Magnification-X40.

Further studies showed the presence of significant DNA fragmentation in Jurkat cells when treated with the extract for 24 h (fig. 2).

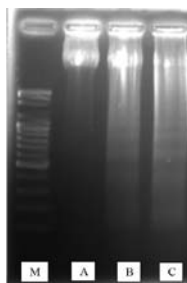
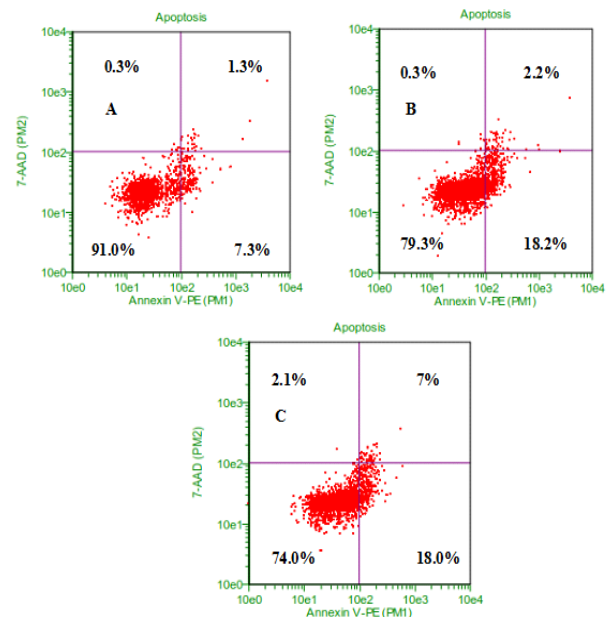
**Fig. 2: DNA fragmentation in Jurkat cells**

Fig. shows DNA from untreated cells, negative control (A), cells treated with AZR extract 2.5 mg/ml (B) and 5.0 µg/ml of (C) for 24 h, and analyzed by agarose gel electrophoresis. Lane (M) is the 100-bp DNA marker; 0, negative control.

Flow cytometric assays following Annexin V-PE and 7-AAD dual staining showed that the sum of early and late apoptosis following treatment with 2.5 µg/ml (B) and 5.0 µg/ml (C) of the extract were 20.4% and 25%, respectively i.e.>2 fold the level of apoptosis in the negative control (A) (fig. 3). Results of the effect of the extract on mitochondrial membrane potential presented in fig. 4 shows significant suppression of J-aggregates and increase in JC-1 monomeric form.

**Fig. 3: Flow cytometric plots showing apoptotic effects of hydroethanolic extract of AZR in Jurkat cells**

The fig. shows plots for untreated cells (A), cells treated with 2.5 µg/ml (B), and 5.0 µg/ml (C) of the extract for 24 h.

Cells were treated with 2.5 µg/ml, 5 µg/ml of AZR and 1% ethanolic solution (negative control). '*' represents a significant difference (p<0.05) compared to the control experiment.

Results on phytochemical analysis of the hydroethanolic AZR extract showed the presence of alkaloids, saponins, tannins, flavonoids, cardiac glycosides and terpenoids. Anthraquinones were not detected in the extract.

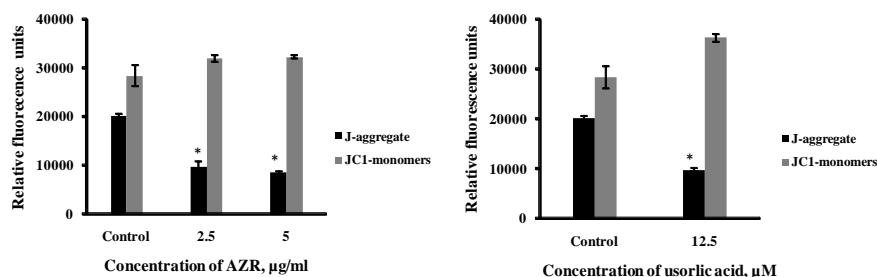


Fig. 4: Mitochondrial membrane potential of Jurkat cells treated with hydroethanolic extract of AZR

Jurkat cells were cultured in the presence of 2.5 µg/ml, 5 µg/ml of AZR and 1% ethanolic solution (negative control) for 16 h, stained with JC-1 solution and analyzed using fluorescence spectrophotometry. RFU, relative fluorescence units; *, $p < 0.05$ compared to control experiment.

DISCUSSION

Earlier studies have reported antitumor and cytotoxic activities of some secondary metabolites in *Albizia* species and their derivatives [2, 19]. Aqueous and ethanolic leaf extracts of *Albizia amara* was found to be cytotoxic to MCF-7 cells with IC_{50} values of 83.16 µg/ml and 57.54 µg/ml, respectively, while ethyl acetate extract showed stronger cytotoxicity (36.31 µg/ml) [19]. Methanolic leaf extract of *Albizia lebbek* also showed nearly 50% inhibition of MCF-7 cells at a concentration of 200 µg/ml [20]. In the present study, we investigated the cytotoxic effect of aqueous and hydroethanolic extracts as well as the ethyl acetate, dichloromethane and petroleum ether fractions of AZR towards Jurkat, LNCap and MCF-7 cells. AZR extracts exhibited a cytotoxic effect on the cell lines investigated. The effects were, however, more pronounced on Jurkat cells followed by LNCap cells suggesting some level of selectivity. Determination of *in vitro* cytotoxicity and selectivity is very important in identifying new medicinal products with potential as anticancer agents. We also evaluated the potential of hydroethanolic AZR extract to induce apoptosis in the cells.

Aqueous and hydroethanolic extracts of AZR showed rather weak cytotoxicities towards MCF-7 cells compared to the activity reported on *Albizia amara*, however, petroleum ether and ethyl acetate fractions showed stronger and comparable activities. The SI of 2.4 recorded for MCF-7 cells treated with petroleum ether was significant. The aqueous and hydroethanolic extracts exhibited the strongest cytotoxicities in Jurkat cells comparable with the positive control (curcumin) with SI values greater than 80. Both extracts also showed strong cytotoxicity towards LNCap cells, but the hydroethanolic extracts showed 6 times stronger activity than the aqueous extract. With the exception of the ethyl acetate fraction that showed cytotoxicity towards both Jurkat and MCF-7 cells and petroleum ether fraction that was cytotoxic towards the latter, no other significant inhibition by fractions on the cells was recorded. Medicinal products with SI values of 2 or greater than 2 are considered to have significant therapeutic effects [21]. These results suggest that the cytotoxic constituents of AZR extracted into aqueous and hydroethanolic extracts, petroleum ether and ethyl acetate fractions and not the dichloromethane fraction. Isolation and characterization of the active component(s) from the hydroethanolic extract and the petroleum ether fraction are currently underway.

Available evidence shows that a number of the cytotoxic anticancer drugs generally induce apoptosis in susceptible cells via interaction with a number of targets and activate genes and processes that modulate 'programmed' cell death [22]. The marked nuclear condensation and observed apoptotic bodies in the AZR extract treated Jurkat cells compared to control cells suggested that hydroethanolic extract of AZR mediated the induction of apoptosis in the cells. Results on the DNA fragmentation/laddering (fig. 2) also showed that the extract is induced apoptosis in the Jurkat cells.

Interestingly, the flow cytometric analysis (fig. 3) confirmed that apoptosis was induced in the cancer cells after treatment with the extract. Studies on *Albizia julibrissin* bark also suggested that it has

anticancer activities and induces apoptosis in human acute leukemia T-cells [4].

Our study has shown that AZR extract significantly caused mitochondrial dysfunction as shown by the reduction in J-aggregates formation and increases in JC-1 monomeric forms in the presence of the extract (fig. 4). Mitochondrial dysfunction is characteristic of the intrinsic apoptosis pathway [11]. These results are consistent with other reports that show that apoptosis is associated with mitochondrial dysfunction, nuclear morphological changes and DNA fragmentation [10, 11]. Indeed, a recent study involving systems-level analysis has revealed that anticancer agents produce dynamic rewiring of oncogenic signaling pathways to make cells less tumorigenic and more susceptible to DNA damage-induced cell death via the reactivation of a suppressed extrinsic apoptotic pathway [23].

In this study, the hydroethanolic AZR extract was shown to contain alkaloids, saponins and flavonoids and other secondary metabolites previously reported to be present leaves of the plant and also in other *Albizia* species that have exhibited cytotoxicity [3, 24]. It is significant to note that some flavonoids, alkaloids and saponins have been reported to possess anticancer activities among others [25-27]. The presence of these components in AZR may account for the cytotoxic activity of AZR.

CONCLUSION

In conclusion, this study has shown that AZRE possesses potential anticancer activity and has demonstrated a high selective toxicity against Jurkat cells. Furthermore, the extract induced apoptosis in the Jurkat cells. *Albizia zygia* roots, therefore, may be a source of novel compounds for the development of new anti-cancer agents. Further studies are underway to identify the compound(s) in the extract that are responsible for the cytotoxic activity and establishes their mechanism(s) of action.

ABBREVIATION

MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AZRE, *Albizia zygia* root hydroethanolic extract; EDTA, ethylenediaminetetraacetic acid; Annexin V-PE, Annexin V-Phycoerythrin; 7-AAD, 7-Aminoactinomycin D; PBS, phosphate buffer saline; DMSO, dimethyl sulfoxide; FBS, Fetal Bovine Serum; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide

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CONFLICT OF INTERESTS

The authors declare no competing financial interests.

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