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Scandenolone from *Cudrania tricuspidata* fruit extract suppresses the viability of breast cancer cells (MCF-7) *in vitro* and *in vivo*

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Abbreviations

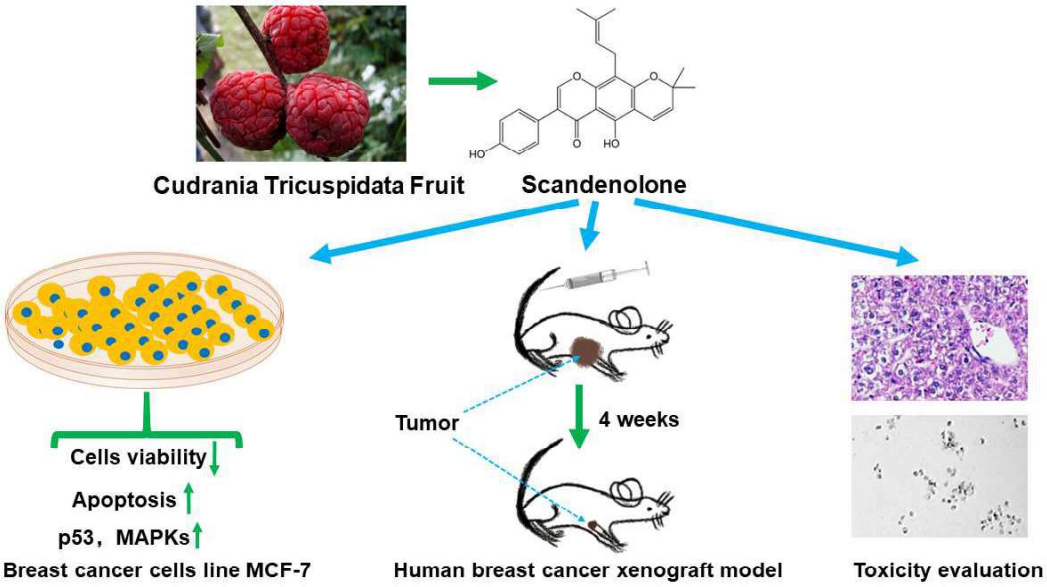
C. *tricuspidata*, *Cudrania tricuspidata*; MAPKs, mitogen-activated protein kinases; JNK, c-Jun NH(2)-terminal kinase; ERK, extracellular signal-regulated kinase; PARP, poly-ADP-ribose polymerase; MMP, mitochondrial membrane potential

ABSTRACT

Scandenolone, an isoflavone, has shown anti-cancer potential. In this study, we extracted scandenolone from *Cudrania tricuspidata* fruit and evaluated its anti-breast cancer effects as well as toxicity in cell and animal models. In cell model, scandenolone suppressed the breast cancer MCF-7 cells viability, ceased mitotic cell cycle, decreased mitochondrial membrane potential, up-regulated cleaved caspase-3 and promoted the phosphorylation of p53. Additionally, this isoflavone promoted cell apoptosis and induced a sustained activation of the phosphorylation of p38 and ERK, but not JNK and Akt. The effects were further verified in a human MCF-7 breast cancer xenograft model, where scandenolone efficiently suppressed the cancer growth and increased apoptotic cells in tumor tissue. However scandenolone has also shown certain toxicity to normal hepatocytes and breast epithelial cells. It could be concluded that scandenolone suppressed the growth of breast cancer cells, but its toxicity towards normal cells might limit its potential clinical use.

KEYWORDS

Polyphenols; Flavonoid; MCF-7 cells; Warangalone; Toxicity; Xenograft model



1. Introduction

Breast cancer is the most common malignancy in women, resulting a high rate of mortality in both developed and developing countries (Ferlay et al., 2015). In the USA, the new cases of breast cancer is the first outstanding cancer on the cancer list, which takes up to 29% of all cancer types and the death rate is about 14% of all deaths from cancer (Siegel et al., 2016). The ever-growing incidence of breast cancer, creating financial burden to the society, calls for the discovery of new efficient preventative and therapeutic measures. Chemotherapeutic drugs such as paclitaxel and anthracyclines induce apoptosis and inhibit the proliferation of the cancer cells. However, some of the patients are not sensitive to these drugs, which may also lead to undesirable side effects to normal cells (Chen et al., 2017). Furthermore, some recent studies have shown that ordinary chemotherapy might enhance tumor vascularization, which further leads to resistance of tumor removal (Zhang et al., 2016). Scientist have been continuously searching for the new ideal anticancer drugs with high efficacy, low toxicity and side effects, and phytochemicals from plant food are under active consideration (Wilsher et al., 2017).

Breast cancer appears to be closely related to the daily diet. The estrogen-related dietary pattern increases the risk of breast cancer (Guinter et al., 2018), whereas some of the polyphenol metabolites suppresses breast cancer cells proliferation (Teixeira et al., 2017). Flavonoid-enriched extracts could suppress the tumor proliferation and induce apoptosis via p53 (Vadde et al., 2016). Some flavonoids also have shown inhibition of cathepsin B in some cancer cells, which is related to the cell migration (Li et al., 2016). Moreover, flavonoid-rich extracts could also target Akt phosphorylation and inhibit angiogenesis in tumor (Zhang et al., 2018). These studies formed the basis for anticancer potential of flavonoids. Interestingly, where most of the widespread anticancer drugs have severe side effects, the reported anti-cancer flavonoids, such as anthocyanidin in berry, were safe to normal cells, but only toxic to cancer cells, (Aqil et al., 2017). However, most of these flavonoids are less effective on the cancer cells suppression, and most of the anticancer studies were limited to *in vitro* cells studies with insufficient *in vivo* tests, making the evidence for anti-breast cancer potential of flavonoids refutable.

Cudrania tricuspidata (*C. tricuspidata*) is a deciduous tree from the family Moraceae, mainly distributed in China, Korea, Japan and Africa. The entire *C. tricuspidata* plant has been used as a

traditional medicine for curing neuritis and inflammation in some parts of Asia (Kwon et al., 2016a). Notably, the wild *C. tricuspidata* fruit is a red and edible berry (Fig. 1), which contains several bioactive compounds (Hiep et al., 2015). *C. tricuspidata* extract has been reported to possess antioxidant activity and inhibitory effects on nitric oxide synthase (Xin et al., 2017). Recent studies have shown that the bioactive compounds from *C. tricuspidata* may exert anti-cancer effect. Crude extracts of the stem of *C. tricuspidata* could induce apoptosis in SiHa cervical cancer cells (Kwon et al., 2016b). Scandenolone, an isoflavone from *C. tricuspidata* fruit, could inhibit the proliferation and migration of human melanoma cells and additionally promote cells apoptosis via autophagy flux (Hu et al., 2017). However, the individual component in *C. tricuspidata* fruit has been rarely investigated with respect to the anti-breast cancer activity.

In this study, we purified and identified efficient anti-breast cancer components from *C. tricuspidata* fruit, investigated the biological effects and the related mechanism of the most efficient compound on breast cancer MCF-7 cells. Additionally, we explored the anti-breast cancer effect of scandenolone in a human cancer xenograft model.

2. Materials and methods

2.1 Materials

Hematoxylin, eosin, loading buffer, TUNEL assay kit and CCK8 were purchased from Beyotime Biotechnology (Shanghai, China). Fetal bovine serum (FBS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against pro-caspase-3, PARP, Bcl-2, Bax, Bcl-xL, Bad, p53, p-p53 were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-GAPDH, ERK, p-ERK, Akt, p-Akt, p38, p-p38, JNK, p-JNK and secondary anti-rabbit, anti-mice antibody were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Paclitaxel was purchased from MedChemExpress (MCE, New Jersey, USA). All the rest of the chemicals used in the study, including DMF, Tween 80 are analytically pure and supplied from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Scandenolone purification and identification

Frozen *C. tricuspidata* fruits were smashed and soaked in methanol (1:20 w/v) in triplicate. The extract was concentrated in a rotary evaporator, resuspended in petroleum ether, ethyl acetate or n-butyl alcohol separately. After the primary test of CCK8 on breast cancer MCF-7 cells, the most efficient ethyl acetate extract was mixed with petroleum ether and subsequently subjected to silica column chromatography. A mixture of petroleum ether and ethyl acetate with a ratio ranging from 100:3 to 0:100 was used for gradient elutions. Different fractions were collected, subsequently separated and combined by thin-layer chromatography using vanillin in sulfuric acid spray to locate the spots. The combined fractions were subjected to a Sephadex LH-20 gel column (25 mm× 1200 mm) chromatography using mobile phase comprising chloroform and methanol (1:1), the fractions were collected and recrystallized (Hu et al., 2017). The different crystals were analyzed and identified by liquid chromatography-mass spectrometry (LC-MS) and X-ray single crystal diffraction. Total Antioxidant Capacity Assay Kit with ABTS method (T-AOC) was adopted to primarily evaluate the bioactivities of the purified crystals. Briefly, antioxidants inhibited the oxidation of ABTS, which was changed to be $ABTS^{+}$ and detected under the wavelength of 405 nm. The antioxidative abilities of different chemicals were measured and compared with the standard curve of Trolox.

2.3 Cell culture

The human breast tumor line MCF-7 was used for the experiments. Cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher, MA, USA) with 10% FBS and 1% penicillin/streptomycin, at 37℃ under 5% CO_2. Normal liver cell line LO-2 and normal breast cell line MCF-10A were sustained in RPMI-1640 culture medium (Gibco, Thermo Fisher, MA, USA) with 10% FBS and 1% penicillin/streptomycin, at 37℃ under 5% CO_2.

2.4 Cell viability and proliferation

The MCF-7 cells were planted in 96 well plate with a concentration of 3×10^4/mL, 200 μL per well. Twelve hour later, cells were treated with scandenolone at different concentrations (5, 10, 15, 25 μg/mL) for 12-72 h. At the end of the incubation, to each well was added 20 μL CCK8 solution and kept in 37°C for another 4 h. Then the plate was shaken and measured at 570 nm using a BIO-RAD Microplate Reader. Cells viability of LO-2 and MCF-10A cells were also evaluated with the treatment of scandenolone. LO-2 and MCF-10A cells with a total number of 1×10^4/mL per well were planted in a 96 well plate. After 12 h to let the cells adhere in the well, scandenolone or paclitaxel were used to treat the cells for another 48 h (Afrin et al., 2016). Cells morphology were studied under an inverted microscope, and subsequently CCK8 were added for the evaluation of cells viability.

2.5 Cell migration test

The MCF-7 cells were firstly cultured in a 6 well dish with a total number of 5×10^5 cells per well for 12 h. Subsequently, the cells at the bottom of each well was scratched with a 200 μL tip, and the culture dish was washed by PBS for twice to remove the loose cells. The cells were then cultured with high glucose DMEM, and treated with scandenolone or vehicle for 1 h, 24 h, and 48 h. At each time point, cells were observed under microscope of 40 × magnification, and typical pictures were taken (Jiang et al., 2017a).

2.6 Cell-cycle analysis

The MCF-7 cells were treated with scandenolone at 10-15 μM for 48 h. Then, cells were harvested and fixed with ice-cold ethanol for 2 h. Cells were subsequently washed by PBS for twice and stained with propidium iodide medium, and finally examined by FACSort flow cytometer and analyzed by FCS Express 4 software (Zafar et al., 2017).

2.7 Mitochondrial membrane potential detection

MCF-7 cells were planted into 6 well plates with an amount of 4×10^4 per well and cultured for 24 h. Then cells were treated with scandenolone (5, 10, 15 μg/mL) for another 48 h. Thereafter, cells were harvested and subsequently incubated with JC-1 working solution for 20 min, at 37°C. Following, cells were washed with staining buffer for once and then detected by flow cytometry, and the results were analyzed by Flowjo software (Kwon et al., 2016b).

2.8 Western blotting analysis

The MCF-7 cells were cultured and intervened by scandenolone as mentioned above. Western blotting was used following the previous studies (Jiang et al., 2017b), briefly as bellow: cells were harvested and disrupted with RIPA lysis, which was added with 1% PMSF and cocktail protease inhibitor. Subsequently, cells solutions were centrifuged at 12000 g for 15 min at 4°C. The supernatant was collected and the protein concentration was quantified by BCA protein assay kit. Samples were mixed with loading buffer and boiled at 100°C for 5 min. SDS-PAGE was used to separate proteins with different molecular weights, then the protein bands were transferred onto a polyvinylidene fluoride membrane (PVDF). Membranes with protein were then blocked by 5% skim milk in TBST for 1.5 h, followed by incubation with primary antibody over night at 4°C on a rocker. Secondary antibody were then incubated with the membranes for another 1.5 h at room temperature, and the bands were incubated with ECL assay and visualized by a chemiluminescence system.

2.9 Animals raising

The protocol of the animal test was approved by the Animal Care and Protection Committee of Jinan University (Guangzhou, PR China). Additionally, all the animals were fed and intervened following the guidance of the Care and Use of Laboratory Animals. Female athymic nude mice (nu/nu), 5 weeks old,

were purchased from Ling Chang biotechnology (Shanghai, China), and kept in a SPF environment of Animal Center of Jinan University. Mice were housed at 23-27°C and under 40%-70% humidity, with a 12-h light/dark cycle, *ad libitum* access to water and chow. After a week of accommodation, mice were randomly divided into 4 groups, and received different treatments.

2.10 Human cancer xenograft models

The MCF-7 human breast tumor cells were harvested with Trypsin EDTA, washed three times with PBS and suspended at a cell density of 1×10^7 in saline medium. Animals were anaesthetized with isoflurane and placed in a supine position. 100 μL Tumor cells medium were slowly injected into the right axillary subcutaneous of each mouse (Khaled et al., 2016). After 12 days of establishing the animal model, succeeded subjects with a tumor of 75-80 mm^3 were randomly divided into three groups, 10 mice per group. Sandenolone were dissolved in a mixed vehicle of normal saline: DMF: Tween 80 = 88:10:2, and injected through the tail vein with a dose of 5 mg/kg·bw or 7.5 mg/kg·bw, once alternate days. The control group was received with vehicle only. Mice were intervened for 28 days and then sacrificed for further analysis.

2.11 Tumor growth evaluation

The tumor size was evaluated by measuring two perpendicular tumor diameters: length and width, where they represent the longer and shorter tumor diameters, respectively. Diameters were measured by a slide caliper. Tumor volumes were calculated using the formula: $V=length\times width^2\times0.5$ (de Souza et al., 2014). Based on the results of tumor volume measurement, relative tumor volume (RTV) were calculated according to the following formula: $RTV=V_t/V_0$. V_0 stands for the initial volume of tumor before intervention, V_t stands for the tumor volume during or after the treatment. Then relative tumor proliferation rate T/C (%) is gained according the following formula: $T/C= T_{RTV}/C_{RTV}\times100\%$, T_{RTV} means the relative tumor volume of the group treated with sandenolone, C_{RTV} means the relative tumor volume of

control group received vehicle only. After the mice were sacrificed, tumor growth inhibition rate (IR) was adopted following the calculation: IR (%) = (tumor weight of control group - tumor weight of treatment group)/tumor weight of control group×100%. Generally, the IR≥60% and $p < 0.05$ are considered as statistically significant (Khaled et al., 2016).

2.12 Histological analysis and immunofluorescence staining

Tumor tissues were fixed in 4% paraformaldehyde for 48 h and thereafter embedded in paraffin. The blocks were cut into 4 μm thickness slides and stained by hematoxylin and eosin (H&E) for pathological analysis. Based on Nottingham grading system, the Nottingham Histologic Score were calculated, which is a strong and independent predictor of outcome (Elston and Ellis, 1991; Rakha et al., 2012). TUNEL staining was following the guidance of assay kit. Briefly, tissue slides were treated by proteinase K for 20 min, and washed by PBS for three times. Slides were incubated with TUNEL assay for 1 h in a dark and wet box, then stained with DAPI for 15 min. The slides were examined under fluorescence microscope using Cy3 and DAPI channel. The other immunofluorescence tests about pro-caspase-3, E-cadherin, MMP-9, Ki-67, p-p38 were operated similarly, but with the specific primary antibodies and fluorescent secondary antibodies. The fluorescence intensity was measured by Image J for the quantitative analysis. Additionally, heart, liver, kidney and spleen tissue was collected and operated same as the tumor tissue for the evaluation of toxic effects of the scandenolone treatment.

2.13 Statistical analysis

All the data are presented as mean ± SEM. The significances of different groups were calculated by one-way ANOVA, and was followed by Bonferroni test for further test of the two groups. The Kruskal–Wallis rank sum test was used for the evaluation of the Nottingham histological score. $p < 0.05$ was considered the significant difference. Graph Pad Prism 5.0 software (San Diego, CA, USA) was used for graphing and statistical analysis.

3. Results

3.1 The identification of the extracts from C. tricuspidata fruit and the primary test

The methanol extract of *C. tricuspidata* fruits was concentrated with a rotary evaporator, then resuspended in petroleum ether, ethyl acetate and *n*-butyl alcohol separately. Different fractions were tested for their anti-cancer ability by the CCK8 assay on MCF-7 breast cancer cells. As shown in Fig. 2, the ethyl acetate extract has shown the most significant suppression on the viability of MCF-7 cells. Subsequently, the ethyl acetate extract was purified by silica column chromatography and Sephadex LH-20 gel column, thereafter three components were obtained. The first one was identified as scandenolone by the analysis of single crystal diffraction and LC-MS (Fig. 3A-C), and its purity of was higher than 97% based on HPLC measurement (Fig. 3D). The other two molecules were identified as 4'-*O*-methyl-alpinum-isoflavone and alpinum-isoflavone (Fig. S1). All three compounds were examined for their antioxidant potential. As shown in Table 1, scandenolone was the most active compound, and the antioxidative ability of which was equal to 2.76 fold of Trolox. Additionally, 1 M alpinum-isoflavone was equal to 0.64 M Trolox, but 4'-*O*-Methyl-alpinum-isoflavone showed week antioxidant activity. All three compounds were then evaluated for the anti-breast cancer potential in MCF-7 cells. After 24 h of incubation, scandenolone showed the best inhibition of the viability of the cells, and the IC_{50} was 38.5 μmol/L. As a comparison, the IC_{50} for alpinum-isoflavone was 76.2 μmol/L, and that for 4'-*O*-methyl-alpinum-isoflavone was higher than 200 μmol/L. Thus, scandenolone was the compound of interest in *C. tricuspidata* fruit for favorable anti-breast cancer effect, it was subsequently prepared from the ethyl acetate crude extract for the further studies.

3.2 Scandenolone inhibited the growth of breast cancer cell line MCF-7

The inhibitory effect of scandenolone was further investigated based on the dose and time dependent test. As shown in Fig. 4A, MCF-7 cells viability decreased dramatically after the incubation of

scandenolone for 12-72 h. Above the dose of 5 μg/mL, scandenolone inhibited the cell viability in a dose-dependent manner, the effects magnified with longer time of incubation. Scandenolone at the concentration of 17.5 and 20 μg/mL treated the MCF-7 cells for longer than 48 h, led to extreme low survival rate (8% and 4% separately). According to the morphology examination under microscope (Fig. 4B), scandenolone suppressed the cell growth, weaken the cell adherence, and caused much vesicle in cytoplasm. With higher dose of scandenolone, most of the cells shape changed, disintegrated and generated massive cell debris.

MCF-7 cells were incubated with different concentration of scandenolone for 48 h, and cell cycle was detected by flow cytometry. As shown in Fig. 4C, scandenolone significantly increased Sub-G1 ratio in a dose-dependent manner, and above 99% of cells were in the Sub-G1 phase when the treatment reached the concentration of 15 μg/mL. However, the G0/G1, S, and G2/M phases were not changed much by scandenolone. The results implicated that the treatment of scandenolone stimulated the cells into the process of apoptosis.

Mitochondrial membrane potential (MMP) indicated the capability of oxidative phosphorylation and generate ATP to sustain the regular function of mitochondrial. The damage of mitochondrial membrane potential happened in the infancy of cells apoptosis which could be detected by JC-1. As shown in Fig. 4D, normal MCF-7 cells kept high level of red fluorescence, which indicated the high integrity of mitochondrial membrane, however scandenolone treatment caused the increase of green fluorescence ratio with a dose-dependent manner, which revealed the increase of JC-1 monomer and cells apoptosis.

3.3 Scandenolone induced the MCF-7 cells apoptosis and suppressed the cells migration

Whole cell lysis was further evaluated for cell apoptosis by western blot (Fig. 5A), scandenolone activated pro-caspase-3 and reduced the level of PARP. Bcl-2 family mainly adheres to mitochondrial and endoplasmic reticulum, which adjust cell apoptosis. Notably, scandenolone showed no significant effect on Bcl-xL, and Bad levels, and slightly increased Bcl-2, Bax level, but the Bax/Bcl-2 level was not changed (Fig. 5B).

Furthermore, the effect of scandenolone on cells migration was examined. After the MCF-7 cells

246 planted and scratched with a tip, cells were treated either with vehicle or 5 μg/mL scandenolone for 1 h, 24
247 h and 48 h. As shown in Fig. 5C, scandenolone suppressed the migration of MCF-7 cells after 24 h
248 treatment, which indicated the suppression on the MCF-7 cells invasion.
249
250 ***3.4 Scandenolone mediated p53 and MAPKs pathway***
251
252 Many of the cancers are associated with the mutation of mitogen-activated protein kinases (MAPKs).
253 MAPKs are serine-threonine kinases that mediate various intracellular signaling including cell
254 proliferation, differentiation and apoptosis. In our study, MCF-7 cells were treated with scandenolone at
255 different concentrations for 24 h, afterwards the cell lysate was collected and analyzed by immunoblotting.
256 The treatment of scandenolone induced the phosphorylation of p53, but there was no influence on total
257 p53 level (Fig. 6). Scandenolone remarkably increased the level of p-p38 and p-ERK in a dose-dependent
258 manner. In addition, scandenolone slightly upregulated the level of p-JNK and showed no effect on p-Akt.
259 Total p38, AKT and JNK were not changed significantly by the treatment with scandenolone.
260
261 ***3.5 Scandenolone inhibited breast tumor xenografts growth***
262
263 Human breast cancer xenografts were generated by subcutaneously transplanting of MCF-7 cells into
264 the nude mice. The initial weights of the mice in different groups were indistinctive before intervention.
265 After the treatment, as is shown in Fig. 7A, body weights of the mice in the vehicle group were kept
266 increasing in the following days. However, in scandenolone treated groups, the body weights did not shift
267 significantly. The body weight change was mainly due to the growing tumor volume. Tumor volume were
268 kept enlarging, but scandenolone treatment of both dose repressed the tumor growth (Fig. 7B). At the end
269 of tumor intervention, mice were anaesthetized and the volume of xenografts were much smaller in the
270 scandenolone treated group (Fig. 7C). Both of the scandenolone treated group showed lighter tumor
271 weight compared with vehicle group (Fig. 7D). Additionally, 7.5 μg/kg of scandenolone treatment was
272 more efficient than 5 μg/kg, although showed no significance. As shown in Table 2, scandenolone
273 treatment showed lower relative tumor proliferation rate, and both doses of scandenolone treatment led to

274 significant inhibition rate (63.5% and 69.1% separately).

275 Tumor tissue slides were stained with hematoxylin and eosin stain (H&E), and viewed under a
276 microscope with the magnifying power of 200× (Fig. 7E). Under the treatment of scandenolone of 5
277 mg/kg, tumor cells still showed activated growing condition, but blood vessel and fibrosis significantly
278 reduced compared to control group. Additionally, less infiltrating inflammatory cells were found. With a
279 higher dose of scandenolone (7.5 mg/kg) treatment, the growing condition of tumor cells were
280 significantly suppressed. Local tumor cells necrosis increased, blood vessels fibrosis significantly reduced
281 compared with scandenolone 5 mg/kg group. Additionally, no inflammatory cells were detected. All the
282 histological analysis were carried on by senior pathologists, and pathological score were calculated. Both
283 of scandenolone treated group reduced the pathological score, which indicated the less malignant tumor
284 growth.

285

286 ***3.6 Scandenolone induced cancer tissue cells apoptosis***

287

288 Apoptosis in the tumor tissue were examined by the TUNEL assay (Fig. 8A). More apoptotic cells
289 were shown in scandenolone treated group compared with control group, and higher dose of treatment
290 caused more severe cells apoptosis. Similarly, we measured the level of pro-caspase 3 (Fig. 8B) and found
291 a significant decrease after the treatment of scandenolone, which eventually indicated the activation of
292 caspase cascade and the cells apoptosis. Additionally, we evaluated the condition of cell invasion and
293 proliferation. E-cadherin downregulation decreased the strength of cellular adhesion within a tissue,
294 resulting in an increase of cellular dedifferentiation and invasiveness. This in turn might allow cancer cells
295 to cross the basement membrane and invade surrounding tissues. As shown in Fig. 2S, the level of E-
296 cadherin showed a slight increase after the treatment of scandenolone, which showed an alleviation of
297 tumor aggressive condition. The level of MMP-9, Ki-67 and p-P38 did not changed much, which indicated
298 the cells proliferation might not be influenced by the treatment of scandenolone.

299

300 ***3.7 Scandenolone showed toxicity to normal hepatocytes and breast epithelial cells***

301

Since scandenolone inhibited the proliferation of MCF-7 cells and caused apoptosis, it was important to examine its toxicity to normal cells. Normal hepatocyte cell lines LO-2 and normal mammary epithelial cell MCF-10A were then tested. As shown in Fig. 9A and B, after the cells were treated with scandenolone for 48 h, cells viability significantly decreased when the drug dose was higher than 12.5 μg/mL. Additionally, the toxicity of scandenolone and paclitaxel on normal cells was compared. Reassuringly, at the same concentration, scandenolone was much less toxic to cells compared with paclitaxel (Fig. 9C and D). Meanwhile, the damage of sandenolone treatment on the normal organs in the mice model was also examined. According to the histological analysis, scandenolone at the dose of 7.5 mg/kg has shown no obvious damage to heart, kidney and spleen, but obvious hepatocytes damage were detected in liver tissue (Fig. 9E).

4. Discussion

Breast cancer is the major cause of cancer-related mortality in women worldwide. Scandenolone from *C. tricuspidata* fruit showed extraordinary anticancer effects, and the IC_{50} was less than 12.5 μg/mL, whereas some of the other flavonoids might reach to 1000-5000 μg/mL (Giampieri et al., 2018; Lu et al., 2018). Cancer cell apoptosis is a main target for the therapy. Apoptotic cells can be processed through the extrinsic or the intrinsic signaling pathways. The extrinsic pathway is activated at the cell surface when a specific ligand binds to its corresponding cell surface death receptor (Zhao et al., 2012). The apoptosis programs start off and sequentially activates caspase-8 and -3, which cleaves target proteins that leading to apoptosis (Ashkenazi and Dixit, 1998). In our study, scandenolone caused MCF-7 cells apoptosis and a decrease of pro-caspase-3, which indicated that the caspase-3 was activated and cleaved into subunits. Apoptosis triggered protein PARP, and the increasing of cleaved PARP was shown after the incubation of scandenolone. We also investigated the intrinsic apoptosis signaling pathways. Intrinsic death activates the mitochondrial pathway by inducing release of cytochrome *c* and formation of the apoptosome. This death pathway is mainly controlled by the proapoptotic proteins including Bax, Bak and Bid, and anti-apoptotic proteins Bcl-2, Bcl-xL (Beesoo et al., 2014). In our study, the integrality of mitochondrial membrane was destroyed by the treatment of scandenolone, but anti-apoptotic protein Bcl-2 and Bcl-xL was not

decreased, meanwhile the level of pro-apoptotic protein Bax and Bad was not increased. Therefore, scandenolone mainly mediated cell apoptosis through extrinsic apoptosis signaling pathway, not the intrinsic pathways.

Many studies have shown that MAPK signaling pathways involving JNK, p38 and ERK mediates cells apoptosis (Ravindran et al., 2011). Upon activation, p38 proteins can translocate from the cytosol to the nucleus where it orchestrates cellular responses through mediating phosphorylation of its downstream transcription factors such as caspase family (Sui et al., 2014). It is estimated that p38 may regulate mitochondrial function, thereafter cause the release of cytochrome *c* and activate caspases (Park et al., 2011). The protein p53 plays a critical role of modulating transformation, cell growth, DNA synthesis and repair, differentiation and apoptosis (Woods and Vousden, 2001). As a transcription factor, p53 targets multiple elements involved in the apoptotic pathway, including p53-regulated apoptosis-inducing protein 1 (p53AIP1) and pro-apoptotic Bcl-2 family members (Chipuk et al., 2004). Additionally, p53 is upregulated by the phosphorylation of p38 MAPK (Dewanjee et al., 2017), elevated p53 is related to the mediation of apoptosis (Zheng et al., 2018). The activated JNK can regulate a variety of transcription factors and mitochondrial proteins like Bcl-2 and Bcl-xL (Radogna et al., 2015). Our data has shown that scandenolone did not affect the level of p-JNK, and thus Bcl-2 and Bcl-xL was neither changed. Endoplasmic reticulum (ER) is essential to cellular homeostasis, strong ER stress induces apoptosis. Some stress responses require p38 MAPK but not ERK1/2 and others both p38 MAPK and ERK (Hamamura et al., 2009). In our study, p38 and ERK were both activated by the treatment with scandenolone, which indicated the activation of ER stress mediated cell apoptosis. These results were consistent with the previous studies that upregulated p38 MAPK and p-ERK in cancer cells would induce cell apoptosis (Cao et al., 2010; Deng et al., 2010).

C. tricuspidata is a valuable resource of bioactive compounds to protect against some chronic diseases. Extract from *C. tricuspidata* leaves were proven to prevent ethanol induced and db/db obesity mice liver injury (Kim et al., 2015; You et al., 2017). *C. tricuspidata* root extracts also protected drugs induced HepG2 damage (An et al., 2006; Tian et al., 2005). However, our study firstly revealed that scandenolone was toxic to normal hepatocytes and breast epithelial cells. To our knowledge most of the efficient natural compounds are not specific target to cancer cells, but can induce cells apoptosis, oncosis, DNA binding,

antimitosis, mediate ATP-binding cassette transporters, MAPK, p53, NF-κB, tubulin polymerization inhibition or some other pathways, which mostly exist in normal cells as well (Paier et al., 2018; Tewari et al., 2019). The compounds with lower toxicity usually have shown less efficiency of curing cancer, whereas the efficient ones may also show high toxicity to the normal cells. Firstly, to screen the natural compounds with higher efficiency for the cancer cells meanwhile less toxicity to the normal cells. However, it is difficult to find an ideal compound, thus some of the following solutions are needed. Appropriate dosage should be chosen for a suitable therapy effect and mild side effects. Additionally, the chemical structure of the natural compounds could be chemically modified for a better bioactivity. Docetaxel and cabazitaxel are the derivatives of paclitaxel, which have shown much better inhibition of cancer cells but less side effects (Galsky et al., 2010; Lyseng-Williamson and Fenton, 2005). Last but not least, drug combination has a bright future. The combination using of the drugs may have more efficient and broader application in cancer treatment, meanwhile reduce the toxicity. Curcumin could enhance the chemotherapy effect meanwhile reverse the drug resistance (Mehta et al., 2014), which indicated a bright future for the drugs combination using on cancer therapy.

5. Conclusions

In summary, scandenolone, purified from *C. tricuspidata* fruit exerted strong anti-breast cancer effect. Scandenolone decreased the cell viability of breast cancer cells MCF-7, and induced p53 and MAPKs mediated cells apoptosis. In addition, scandenolone efficiently suppressed breast tumor growth in human cancer xenograft model. However, an attention should be paid to the latent toxicity of scandenolone on the normal cells. For a better development of scandenolone for clinical purposes, further studies on detoxification of scandenolone are essentially needed.

Conflict of interest

The authors declare no conflict of interest.

384

385 **Supplementary material**

386 The Supporting information of Fig. S1, Fig. S2 are available on line.

387

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Table 1. The anti-oxidant capability and MCF-7 inhibition efficiency of pure compounds isolated from *C. tricuspidata* fruit extract[a].

	ABTS[b]	MCF-7 IC_{50} (μmol/L)
Scandenolone	2.76±0.52	38.5±2.3
Alpinum-isoflavone	0.64±0.25	76.2±4.1
4'-*O*-Methyl-alpinum-isoflavone	<0.025	>200.0

[a]Values are expressed as mean ± SEM

[b]The relative reactive oxygen species scavenging capability compared with Trolox.

Table 2. Tumor growth parameters of scandenolone treatment on the breast cancer xenografts model.

	Animal weight		Tumor volume		Tumor weight (g)	Relative tumor proliferation rate (T/C)	Inhibition rate (IR)
	Initial (g)	Sacrificed (g)	Initial (cm^3)	Sacrificed (cm^3)			
Vehicle	18.3±0.1	22.4±0.3	0.08±0.01	3.58±0.20	3.14±0.23	100%	0%
Scandenolone 5 mg/kg	18.4±0.1	18.2±0.1*	0.08±0.01	1.31±0.12**	1.15±0.16**	37.11%	63.5%
Scandenolone 7.5mg/kg	18.5±0.1	18.2±0.3**	0.08±0.01	1.15±0.07**	0.97±0.09**	33.47%	69.1%

$^{*}p < 0.05$, $^{**}p < 0.01$ compared with the vehicle group with significant differences.

Fig. 1. *C. tricuspidata* fruit

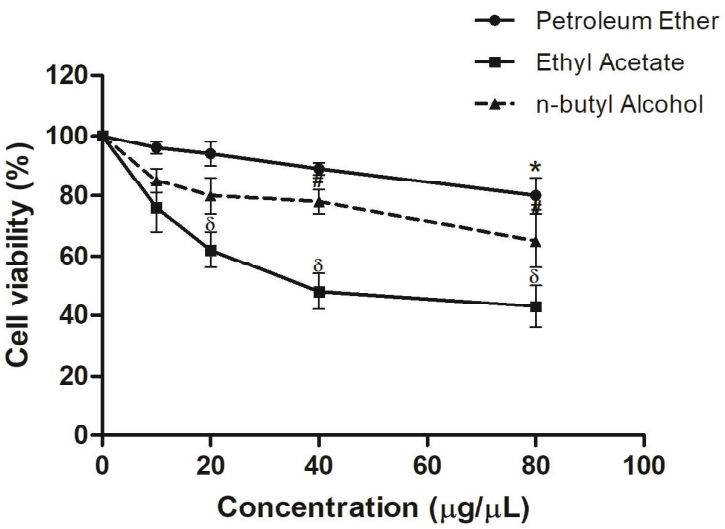

Fig. 2. The inhibition rate of different fractions from *C. tricuspidata* fruit on the MCF-7 cells. MCF-7 cells were cultured and treated by the DMSO dissolved different *C. tricuspidata* fruit extracts with different concentrations for 24 h, and the inhibition rate compared with control group was measured by CCK8 assay. The results were expressed as mean ± SEM from three independent experiments (n=3). $^{*}p < 0.05$, $^{\#}p < 0.05$, $^{\delta}p < 0.05$ significantly different compared with the relative control group.

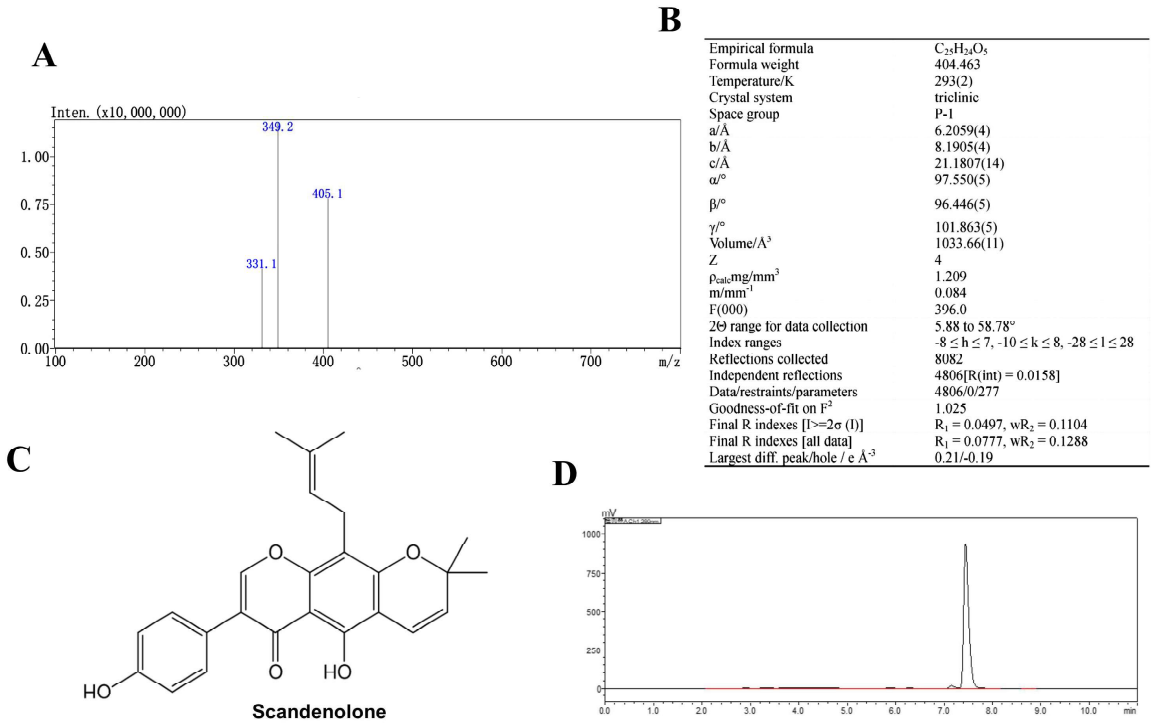

Fig. 3. Identification of the main compound extracted from *C. tricuspidata* fruit. (A) The secondary mass spectrometry of the substance. (B) Single crystal diffraction of the extracted compound was confirmed as scandenolone, and the (C) chemical structure of scandenolone. (D) The purity of scandenolone was measured by HPLC.

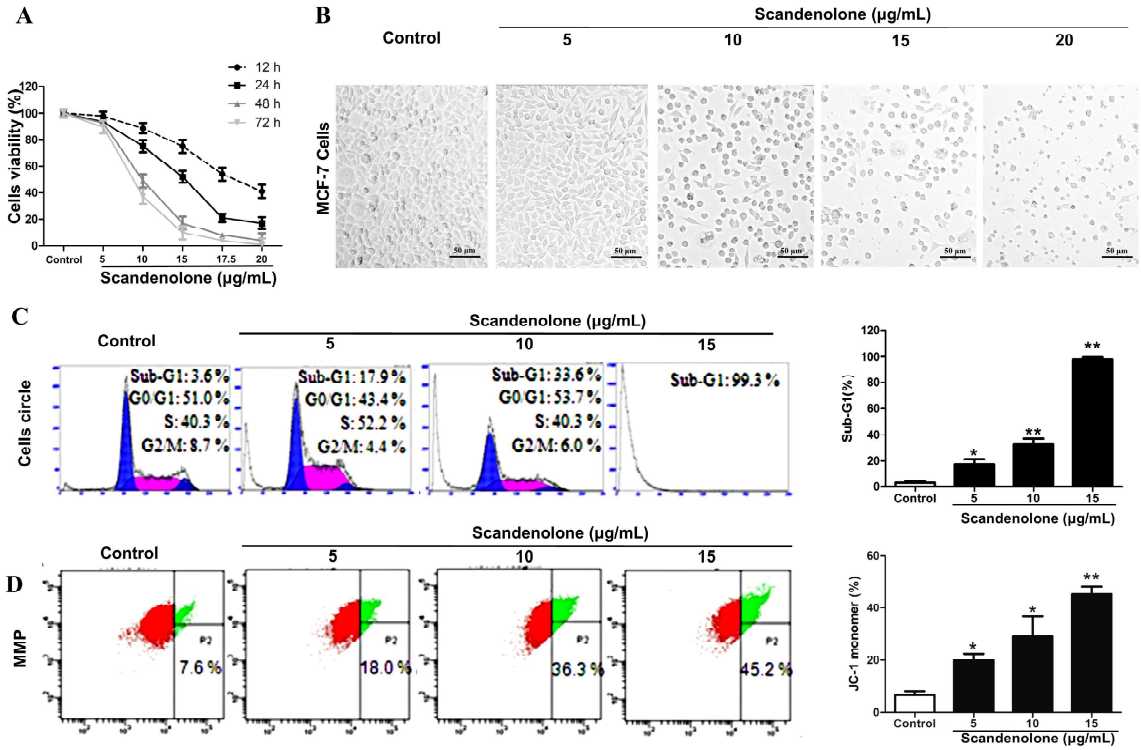

Fig. 4. Scandenolone inhibited the proliferation of MCF-7 cells and induced the cells apoptosis. (A) Breast cancer MCF-7 cells were incubated with scandenolone for 12-72 h, the cells viability were tested by CCK8 assay. (B) Cells were cultured 48 h and typical views of cells morphology are shown (100×). (C) Cell cycle distribution was examined by flow cytometry and Sub-G1 ratio was calculated. (D) Mitochondrial membrane potential was detected by fluorescence probe JC-1 and green fluorescence ratio which indicated the mitochondrial membrane damage was shown.

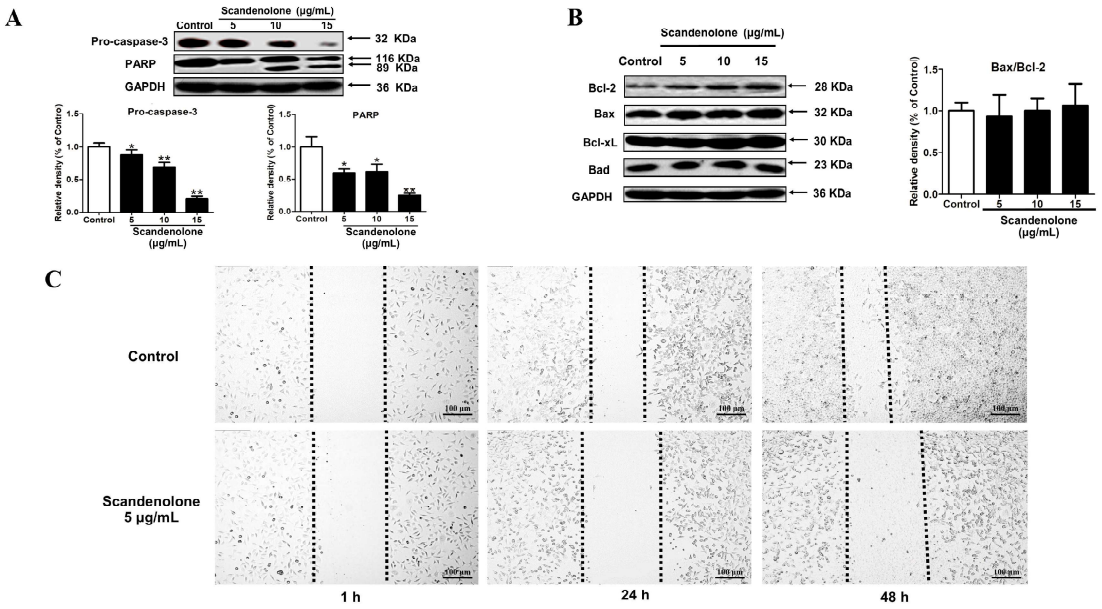

Fig. 5. Scandenolone inhibited apoptosis related protein expression and inhibited the cells migration. (A and B) MCF-7 cells were treated with scandenolone for 24 h, apoptotic proteins were detected by western blot. (C) Cells wound was made and treated with scandenolone for 1-48 h, then the cells migration capability was evaluated (40×). All data are presented as the mean ± SEM from three independent experiments (n=3). $^{*}p < 0.05$ $^{**}p < 0.01$ significantly different compared with the control group.

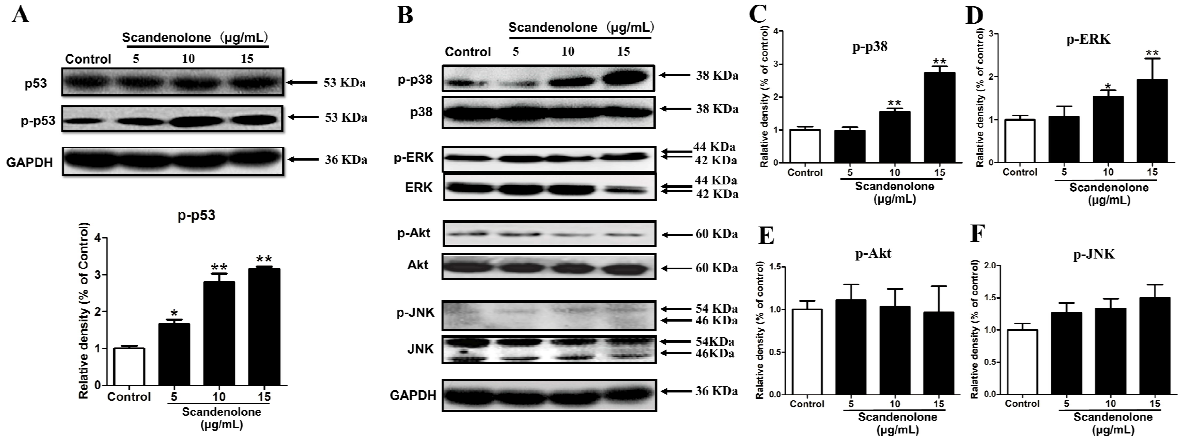

Fig. 6. Evaluation of scandenolone on the MAPKs signaling transduction pathway. MCF-7 cells were treated with scandenolone for 24 h, and the target proteins were measured by western blot. (A) Measurement of the protein expression p53 and (B-F) MAPKs including p-p38, p-ERK, p-Akt and JNK, which were detected and analyzed the density of gray bands by Quality One. All data are presented as the mean ± SEM from three independent experiments (n=3). $^{*}p < 0.05$ $^{**}p < 0.01$ significantly different compared with the control group.

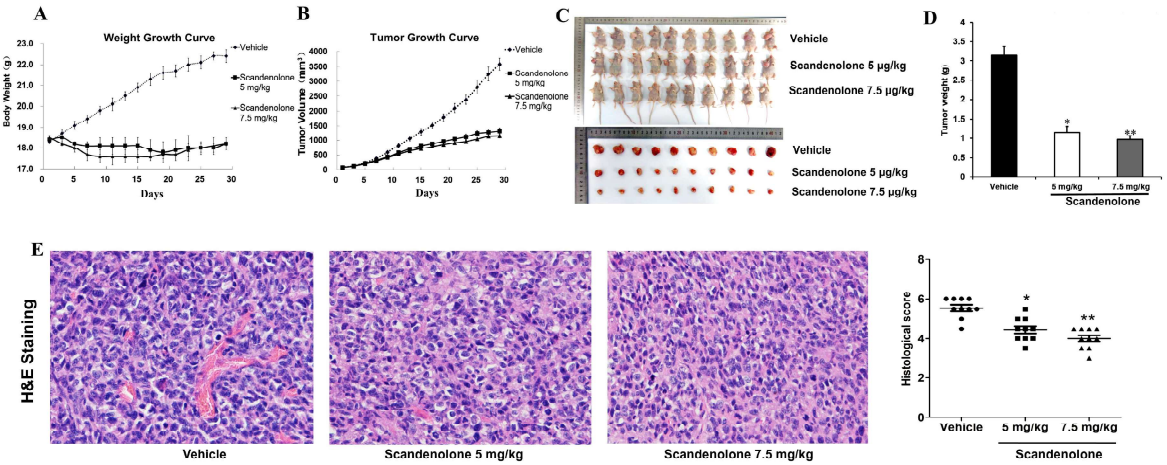

Fig. 7. Scandenolone inhibited the cancer tumor growth on the human breast cancer xenografts model. Mice models were established by the injection of MCF-7 cells to the right axillary subcutaneous and treated by scandenolone for 28 days. (A) Body weights were recorded, (B) tumor volume were measured by vernier caliper. (C) After mice were sacrificed, tumor tissues were separated carefully and (D) weighted. (E) Tumor tissue were fixed and cut into 4 μm slides, H&E staining were carried out for histological analysis (200×) and the Nottingham Histologic Score was calculated. The results were expressed as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with vehicle group. n=10.

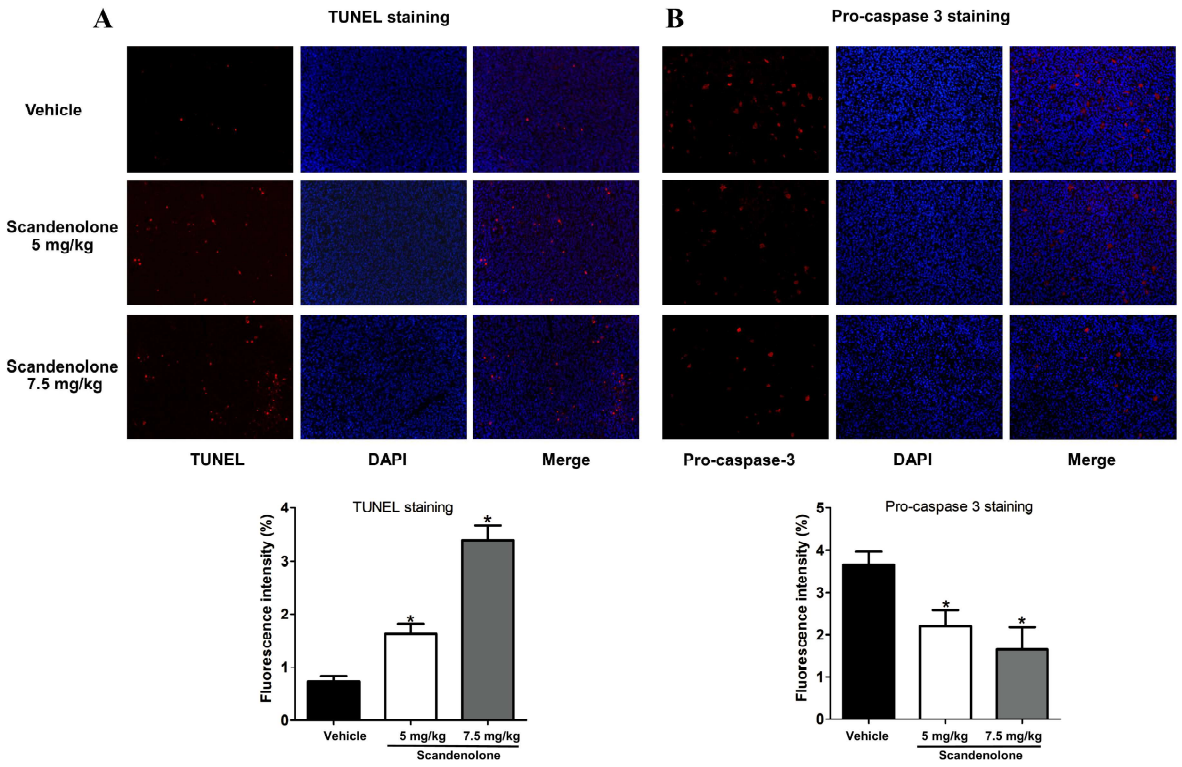

Fig. 8. Tumor tissue slides were performed with immunofluorescence staining. TUNEL (A), pro-caspase 3 (B) were carried for the detection of apoptotic cells, DAPI were used to mark the cell nucleus. The fluorescence was captured by a fluorescence inversion microscope system. Five random fields of the tissue slides were captured and typical pictures were shown in this figure. The fluorescence intensity was measured by Image J. n=4, 200×

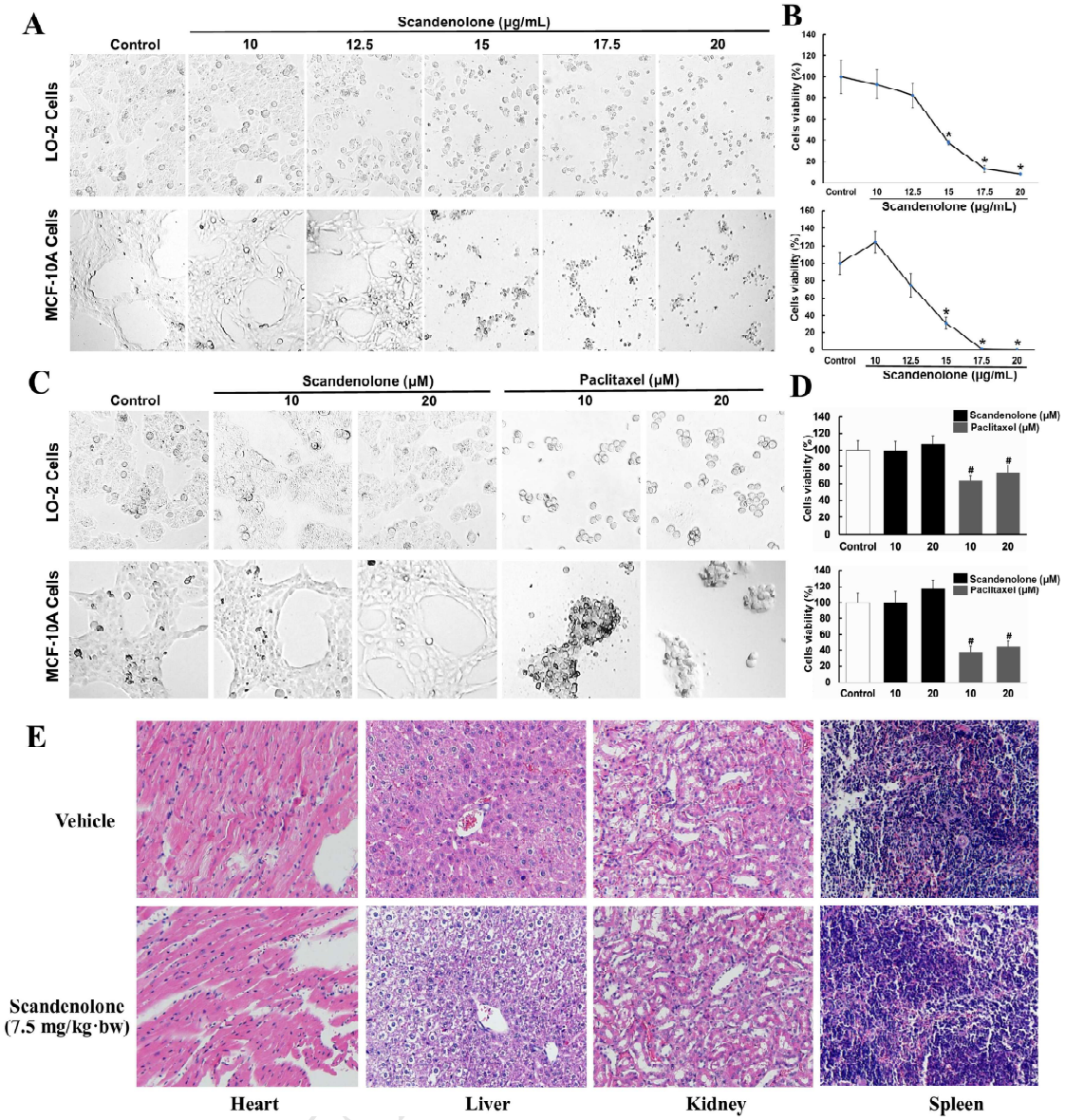

Fig. 9. Toxicity evaluation of scandenolone on normal cells and organs. Normal cells line LO-2 and MCF-10A were cultured for 48 h, (A) cells morphology (100×) and (B) cells viability were detected. (C-D) LO-2 and MCF-10A cells were cultured and treated for 48 h, and the toxicity of scandenolone and paclitaxel was compared (400×). (E) In animal models, histological analysis of heart, liver, kidney and spleen in vehicle and scandenolone (7.5 mg/kg) group, typical pictures were chosen in five random view fields, 200×. Results were expressed as mean mean ± SEM, $^{*}p < 0.05$ compared with the control group, $^{\#}p<0.05$ compared with the scandenolone group.

- Scandenolone, as an isoflavone, was prominent to inhibit the growth of MCF-7 cells.
- Scandenolone mediated p53 and MAPKs pathway, and thereafter induced apoptosis.
- Scandenolone suppressed the tumor growth in a human breast cancer xenograft model.
- Scandenolone appeared to be toxic to normal cells and caused liver injury in mice model.