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Induction of mitochondrial-dependent apoptosis by essential oil of *Toona sinensis* root through Akt, mTOR and NF- κ B signalling pathways in human renal cell carcinoma cells

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Abstract

Natural products have long been considered as a kind of complementary medicine. In this study, we investigate the apoptotic effect of essential oils of *Toona sinensis* roots (TSR) on human clear cell renal cell carcinomas (ccRCC). The sesquiterpene content of TSR essential oil was determined via GC/MS analysis. TSR decreased ccRCC cell viabilities, inducing ROS generation and reduction of the mitochondrial membrane potential. Moreover, TSR inhibited Bcl-2 and Hsp90 expression but increased PARP-1 cleavage and cytochrome c release. Akt, mTOR and NF- κ B phosphorylation and HIF- α expression were all inhibited, which likely contributed to the anti-proliferative and anti-adhesive effects of TSR.

Keywords: Apoptosis, Essential oil, Renal cell carcinoma, Signalling pathway, *Toona sinensis*

1. Introduction

Renal cell carcinoma (RCC) occurs in renal tubules and is the most common type of kidney cancer [1], with 76,080 cases and 13,780 deaths predicted in America in 2021 [2]. Since RCC is resistant to traditional chemo- and radiotherapies, the rapid development of targeted drugs is important [1]. Approximately 15% of patients with RCC present with locally advanced or metastatic RCC, for which surgery is non-curative [3]. Clear cell renal cell carcinoma (ccRCC), which accounts for 80% of RCC, is the most common histologic subtype, characterised by mutations or epigenetic silencing of the Von

Hippel–Lindau (VHL) gene [3]. This leads to the stabilisation and accumulation of HIF- α , which causes the constitutive activation of several downstream oncogenic pathways, such as phosphatidylinositol 3 kinase (PI3K)/Akt/mTOR, ras/raf/mitogen-activated protein kinase and vascular endothelial growth factor pathways [3, 4].

Toona sinensis (A. Juss) M. Roem is a woody plant belonging to the Meliaceae family and is widely distributed in Asia [5]. It has a distinct flavour, especially the leaves which are very popular in vegetarian cuisine [6, 7]. Moreover, it is used in folk medicine to treat infections, diarrhoea, colds, pain, etc. [8]. Recent pharmacological studies on *T. sinensis* have demonstrated its anti-microbial, anti-oxidant, anti-inflammatory, anti-diabetic, anti-

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nociceptive and anti-tumour activities owing to its abundant phytochemical constituents (phenols, flavonoids, limonoids, phytols, etc.) [8–13]. To our knowledge, few reports focus on the effects of TSR extracts on tumour cells. In this study, we investigated whether TSR essential oil induces apoptosis in ccRCC cells by using a primary ccRCC cell line, 786-O and a metastatic line, Caki-1 cell.

2. Methods

2.1. Supercritical fluid extraction of TSR

The roots of *T. sinensis* were collected in 2016 in Yun-Lin, Taiwan, and the specimens were authenticated by Prof. Hseng-Kuang Hsu (Kaohsiung Medical University). Powdered roots (265 g) were extracted by supercritical carbon dioxide (CO₂) fluid after 95% ethanol extraction. Pure CO₂ was compressed *via* a high-pressure diaphragm pump to 299.92 bar at 49°C and allowed to flow through the extraction vessel at a flow rate of 4.2 kg of CO₂ per hour. After separation, the supercritical CO₂ fluid TSR extract was collected from the valve of the separator, and the regenerated CO₂ flowed back into the reservoir. The extraction time was in the range of 60–120 min, and it stopped when the yield reached less than 0.1% of TSR as 40 kg of CO₂ passed through the vessel. The recovery rate of supercritical CO₂ fluid extract of TSR was 1.88%.

2.2. Gas chromatography and mass spectrometry (GC–MS) analysis

Gas chromatography and mass spectrometry (GC–MS) analysis of TSR essential oil was conducted using a high resolution time-of-flight mass spectrometer (Shimadzu Corp., QP2010, Kyoto, Japan) utilising a DB-5MS UI column (30-m length, 0.25-mm diameter, 0.25-μm film thickness). Helium was used as the carrier gas at a flow rate of 1 mL/min with a pressure of 53.5 kPa. The percentage of each of the constituents was calculated by the integral area under its respectively peak in relation to the total area of all sample constituents. Essential oil (1 μL) was directly injected, and a 1:2 split ratio was applied. The temperature of the oven was set to 50°C for 2 min, followed by 85°C for 4 min and then 200°C for 1 min. Next, the oven was heated to 2.0°C/s until 230°C, where it was kept for 4 min. The injector and transfer line temperatures were both set to 230°C. The mass spectrometer was operated in an electron impact mode with the electron energy set to 1 kV. Mass spectral identification was

performed using the Shimadzu FFNSC 1.2 and FFNSC 3 library.

2.3. Cell culture

786-O and Caki-1 human ccRCC cell lines were obtained from the Bioresource Collection and Research Centre (HsinChu, Taiwan) and American Type Culture Collection (Manassas, VA, USA), respectively. The 786-O cells were cultured in RPMI1640 and Caki-1 in McCoy's 5A media supplemented with 10% foetal bovine serum and 100 μg/mL penicillin–streptomycin. The cells were maintained at 37°C in a humidified 5% CO₂/95% air incubator. 786-O cell line is defective in *VHL* expression, as it lacks wild-type pVHL and only expresses HIF-2α [14]. Caki-1 cell line harbors wild-type *VHL* gene and expresses the detectable level of HIF-1α [15].

2.4. Drug treatment

The cells were treated with serial dilutions of TSR for 24–72 h. TSR solution was prepared 1:1 TSR extract to 99.9% ethanol and then diluted 2,000-fold in a culture medium for treatment. Due to the different cytotoxicities of the cell lines, the total treatment periods for 786-O and Caki-1 cells were 24 and 48 h (except for the MTT assay), respectively. In all experiments, 0.1% ethanol was used as a vehicle control. The cells were seeded and cultured at 80%–90% confluence (96-well plates, 7,500 cells/well; 35 mm dishes, 3 × 10⁵ cells/dish; 60 mm dishes, 4 × 10⁵ cells/dish) overnight.

2.5. Cell morphology and viability assays

After stimulation with TSR at concentrations between 62.5 and 250 ppm for 24–72 h, morphological changes were investigated using an inverted phase-contrast microscope (Leica, USA). Images were also taken using a digital camera (Nikon, Japan). Proliferation of TSR-treated cells was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (BIO BASIC Inc., Markham, ON, Canada). After plating, 50 μL of MTT dye (0.5 mg/mL) was added for 2.5 h; then, the medium was discarded, and 100 μL of dimethyl sulfoxide (DMSO) was added to extract the dye. Optical density was measured at 570 nm using a microplate reader (Bio Tek Instruments Inc., Winooski, VT, USA). Survival rates (%) were normalised to the vehicle-treated control group.

2.6. Acridine orange (AO) and ethidium bromide (EB) staining

After TSR treatment, cell suspensions were prepared in culture media, 97 μ L cell suspension was mixed with 3 μ L AO and EB dyes, and then the samples were analysed *via* fluorescence microscopy (Nikon) using a cooled charge-coupled device (CCD) (NIKON, Japan) at magnifications of $200\times$ and $400\times$. AO freely permeates into the cells and stains the nucleus green, whereas EB stains the nucleus during membrane breakdown in late stage of apoptosis. Cells treated with orange or red nuclei were considered necrotic. Herein, the percentage of apoptotic cell population is the combination of early and late apoptotic cells.

2.7. Mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) measurement

For fluorescent staining (rhodamine 123, Rh123 for the MMP and H₂DCFDA for ROS, the cells were washed with phosphate-buffered saline (PBS) and then stained with Rh123 (10 μ g/mL, Molecular probes, Eugene, OR, USA) and H₂DCFDA (10 μ M, AAT Bioquest Inc., Sunnyvale, CA, USA) for 15–30 min, respectively. The cells were visualised *via* fluorescence microscopy (Nikon), and images were taken using cooled CCD (Nikon) at magnifications of $200\times$ and $400\times$. The quantification of fluorescent signals (Rh123 and ROS), cells were stained with the Rh123 and ROS, then the cells were analyzed by using flow cytometry (Attune NxT Flow Cytometer; Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Clonogenic assay

The proliferative capacity of cells over a long-term period was measured *via* clonogenic assays. Dishes (35 mm) were seeded with 8,000 cells, and after 24–48 h of TSR treatment, the media was replaced with plain media and then incubated with 5% CO₂ at 37°C for 7 days. At the assay endpoint, cells were stained with 0.5% crystal violet containing 6% glutaraldehyde and photographed using an inverted microscope (Leica, Wetzlar, Germany). A group of ~50 cells was counted as one colony.

2.9. Fibronectin adhesion assay

Prior to adhesion, cells were treated with varying concentrations of TSR essential oil for 24–48 h. Subsequently, adherent cells were trypsinised and allowed to recultivate on fibronectin-coated (20 μ g/

mL) 24-well plates at a density of 10^4 cells/mL. After 2 h, non-adherent cells were removed by washing with serum free media twice, and the remaining cells were fixed with 10% formalin and stained with 2.5% Coomassie Brilliant Blue in PBS. After visualisation under an inverted microscope (Leica), the dye was extracted in 100 μ L of DMSO. The optic absorbance was read at 595 nm using an automated ELISA reader (BioTek).

2.10. Western blot analysis

RCC cells in 35-mm dishes were incubated with TSR. Cell lysates were collected in lysis buffer containing 0.15% Triton X-100, 2 mM magnesium chloride, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 60 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM β -glycerolphosphate, 2.5 mM sodium pyrophosphate, 1 μ g/mL aprotinin, 1 μ g/mL pepstatin A and 1 μ g/mL leupeptin (pH 6.9) and were sonicated for 20 times with 1-s pulses. Protein concentrations were measured using a Bradford Protein Assay Kit (Bio-Rad Life Sciences, Hercules, CA, USA), and the samples were mixed in reducing sample buffer, boiled and stored at -80°C until further analysis. Protein samples were resolved on 10%–12.5% sodium dodecyl sulphate polyacrylamide gels and transferred to a nitrocellulose membrane, as previously described. The membranes were blocked in 5% non-fat milk or 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 (TBS-T) containing 150 mM NaCl and 50 mM Tris base (pH 8.2) for 60 min at room temperature. Blots were then incubated overnight with blocking solution with the primary antibodies (Table 1) at 4°C . After washing the membranes with TBS-T for several times, blots were incubated with alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibodies (1:2,000), followed by the NBT/BCIP substrate solution (Sigma-Aldrich Inc., St. Louis, MO, USA) and/or enhanced chemiluminescence detection kit (Advansta Inc. San Jose, CA, USA). Immunoreactive bands were quantified *via* densitometry and compared with the expression of GAPDH or related total protein.

2.11. Preparation of mitochondrial and cytosolic fraction

After treatment of TSR, cells were collected and homogenized in buffer containing 0.28 M sucrose,

Table 1. List of antibodies.

Primary antibodies	dilution	Company
pAkt1 (Ser473) (ab81283) Akt1 (ab32505), mTOR (ab134903), cleaved PARP-1 (ab32064), NF- κ B (ab32536), Hsp90 (ab203126)	1:1000	Abcam Plc. (Cambridge, MA, USA)
pmTOR (ser2448) (sc-293132)	1:1000	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA)
pNF- κ B (ser536) (#3033)	1:1000	Cell Signaling Technology Inc. (Danvers, MA, USA)
HIF-1 α (2015-S)	1:1000	Epitomics, INC., a brand of Abcam (Burlingame, CA, USA)
HIF-2 α (NB-100-122)	1:1000	Novus Biologicals, LLC., a Bio-Techne brand (Centennial, CO, USA)
Bcl-2 (B3170)	1:1000	Sigma
Cytochrome c (45-6100)	1:1000	Thermo Fisher Scientific Inc. (Waltham, MA, USA)
GAPDH (60004-1-Ig)	1:2000	Proteintech Group Inc. (Rosemont, IL, USA)

20 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), 50 mM sodium chloride, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM PMSF, 100 mM sodium fluoride, 2 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 10 μ g/mL leupeptin. The pellets were discarded after centrifugation at 500g for 10 min. The supernatants were then collected and centrifuged at 12,000 r.p.m. for 60 min. After centrifugation, the supernatants were cytosolic fraction while the pellets contains mitochondria [16, 17].

2.12. Caspase-3/7 activity assay

786-O and Caki-1 cells were seeded on 35 mm dishes overnight and then treated with various concentration of TSR for 24 and 48 h, respectively. Caspase-3 activity was measured using a caspase-3/7 activity assay kit (#10009135) according to the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI, USA). One hundred microliters specific caspase-3/7 substrate N-Ac-DEVD-N'-MC-R110 was added into 90 μ L cell based assay buffer and incubated at 37°C for 90 min. Caspase-3/7 activity was analyzed at an absorbance of 485 (excitation) and 535 nm (emission) under Synergy H1 multi-mode microplates reader (BioTek).

2.13. Statistical analyses

All data are expressed as mean $\pm$ SD. Differences between the groups were evaluated *via* one-way analysis of variance, followed by correction for Dunnett's *post hoc*. The results were considered significant if the *P*-value was less than 0.05.

3. Results

3.1. Chemical composition of TSR essential oil

As presented in Fig. 1 and Table 2, the GC–MS analysis of TSR led to the identification of five

major compounds: spathulenol (area: 37.72%; retention time: 46.606 min), caryophyllene oxide (area: 14.84%; retention time: 46.799 min), pogostol (area: 17.22%; retention time: 47.018 min), viridiflorol (area: 16.39%; retention time: 47.357) and cedr-8 (15)-en-9-ol (area: 13.82% retention time: 47.95 min). In addition, some chemicals were detected using a database containing 39 common essential oil standard references, such as fenchone (0.04681 ppm), camphor (0.19215 ppm), cedrol (1.0714 ppm) and guaiol (0.43624 ppm), detected in the ppm range (Table 3).

3.2. Cytotoxic effects of TSR essential oil on ccRCC cells

To test the potency of TSR on the cell viability of ccRCC cells, 786-O and Caki-1 cells were incubated with different TSR concentrations (62.5, 93.75, 125 and 250 ppm) for 24, 48 and 72 h to record the morphological changes and measure the survival rates *via* MTT assays. At 250 ppm, 786-O cells were retracted after 24 h of treatment (Fig. 2A), whereas Caki-1 cell retraction was observed after 48 h of treatment (Fig. 2B). At 250 ppm of TSR, the cell viability of 786-O was 41.86% of the vehicle control (0 ppm, *p* < 0.001) after 24 h of treatment (Fig. 2C). In Caki-1 cells, TSR demonstrated cytotoxicity at 93.75, 125 and 250 ppm. The viabilities of TSR-treated Caki-1 cells were 89.04%, 80.52% and 44.73% at 24 h (Fig. 2D). Overall, the cytotoxicity of TSR was more prominent after 48 and 72 h at 93.75–250 ppm of TSR in both cell lines.

3.3. Proapoptotic effects of TSR essential oil on 786-O and Caki-1 cells

To further elucidate the effects of TSR on ccRCC cells, we performed dual AO–EB staining to evaluate apoptosis in TSR-treated cells. ccRCC cell lines were treated with TSR for 24 h (786-O) or 48 h (Caki-1), and at concentrations of 62.5 ppm, there were

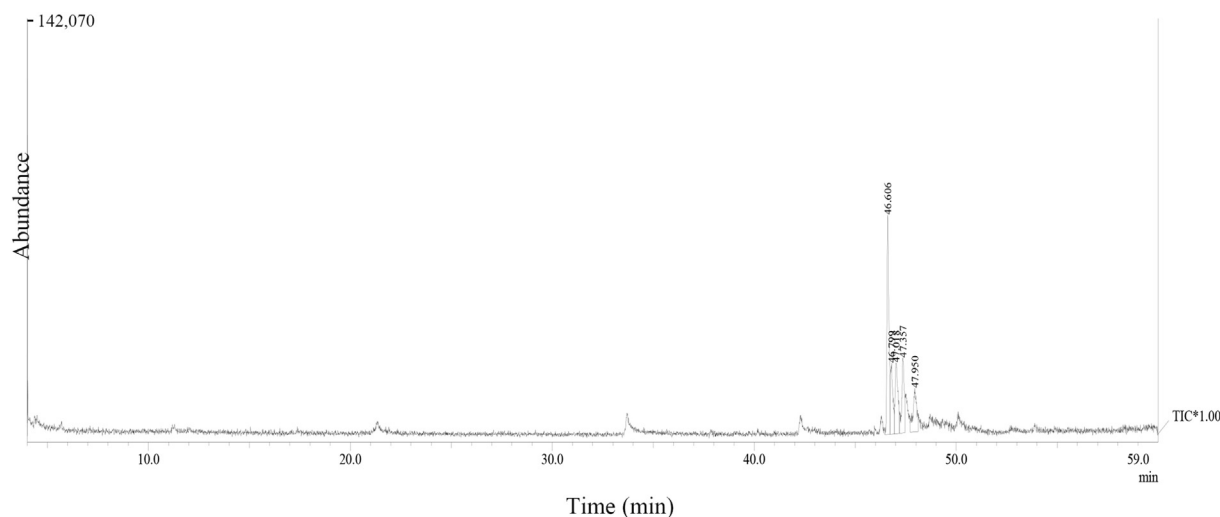


Fig. 1. GC–MS analysis of TSR essential oil. Total ion chromatogram of TSR essential oil.

obvious green dots in both TSR-treated cell lines (Fig. 3). Many yellow to orange dots in TSR-treated cells appeared due to the concentrations of over 93.75 ppm (Fig. 3). Compared with control cells, TSR-treated 786-O and Caki-1 cells exhibited high proportions of late apoptotic (19%–33% at 62.5 ppm, 33%–37% at 93.75 ppm, 33.9%–47% at 125 ppm and 54%–59% at 250 ppm; Fig. 3B and C) but low proportions of necrotic cells (<5%, data not shown) after 24 h of treatment.

3.4. TSR essential oil inhibits colony formation and adhesion

Although TSR essential oil demonstrated an effect on apoptosis, we wanted to further explore if TSR-treated cells could still proliferate and adhere to an extracellular matrix. The colony-forming ability of both 786-O and Caki-1 cell lines was eliminated by the increasing concentrations of TSR (Fig. 4A). At concentrations of 62.5 and 93.75 ppm, the inhibitory effect of TSR was more prominent in 786-O cells than in Caki-1 cells (Fig. 4B and C). Compared with controls, TSR-treated 786-O and Caki-1 cells formed

fewer colonies (the inhibition rates were 20.5%–44.4% at 62.5 ppm, 63.8%–82.4% at 93.75 ppm, 86.6%–97.4% at 125 ppm and 100% at 250 ppm; Fig. 4B and C). Conversely, both 786-O and Caki-1 cells adhered less to fibronectin after TSR treatment (Fig. 4D). At concentrations of 62.5 and 93.75 ppm, Caki-1 cell adhesion was significantly blocked by TSR (Fig. 4E and F). At higher TSR concentrations (125 and 250 ppm), both ccRCC cells adhered less (Fig. 4E and F).

3.5. TSR essential oil diminishes MMP and induces ROS generation

Disruption of the mitochondrial membrane is an important step in the mitochondria-dependent apoptotic pathway, which leads to the loss of the mitochondrial transmembrane potential [18]. MMP maintenance in both cell lines was examined *via* Rh123 staining and flow cytometry analysis. In the vehicle-treated control group, long, tubular-shaped mitochondria can be observed in both cell lines (Fig. 5A). After treatment with TSR, the numbers of mitochondria were diminished and swollen in both

Table 2. Identification of chemical composition of essential oil from TSR by GC/MS analysis.

Peak#	R.Time	I.Time	F.Time	Peak Report TIC						
				Area	Area%	Height	Height%	A/H	Mark	Name
1	46.606	46.475	46.725	467302	37.72	68626	45.70	6.81	V	Spathulenol
2	46.799	46.725	46.933	183788	14.84	22181	14.77	8.29	V	Caryophyllene oxisde
3	47.018	46.933	47.183	213356	17.22	22199	14.78	9.61	V	Pogostol
4	47.357	47.183	47.458	203081	16.39	23529	15.67	8.63	V	Viridiflorol
5	47.950	47.717	48.125	171200	13.82	13623	9.07	12.57	V	1H-3a, 7-Methanoazulen-5-ol, octahydro-3, 8
				1238727	100.00	150158	100.00			

Table 3. Quantitative results of essential oil by GC/MS using 39 essential oil standard compounds.

ID#	R.Time	m/z	Area	Height	Conc.	Name
1	-	93.00	—	—	N.D.(Peak) ppm	Pinene <alpha->
2	-	93.00	—	—	N.D.(Peak) ppm	Sabinene
3	-	93.00	—	—	N.D.(Peak) ppm	Pinene <beta->
4	-	93.00	—	—	N.D.(Peak) ppm	Phellandrene <alpha->
5	-	121.00	—	—	N.D.(Peak) ppm	Terpinene <alpha->
6	-	119.00	—	—	N.D.(Peak) ppm	Cymene <para->
7	-	68.00	—	—	N.D.(Peak) ppm	Limonene
8	-	93.00	—	—	N.D.(Peak) ppm	Terpinene <gamma->
9	-	71.00	—	—	N.D.(Peak) ppm	Sabinene hydrate <trans->
10	-	93.00	—	—	N.D.(Peak) ppm	2,2-Dimethyl-5-methylene norbornane
11	-	93.00	—	—	N.D.(Peak) ppm	Myrcene
12	-	93.00	—	—	N.D.(Peak) ppm	Carene <delta-3->
13	-	43.00	—	—	N.D.(Ref) ppm	Cineole <1,4->
14	-	43.00	—	—	N.D.(Ref) ppm	Eucalyptol
15	-	93.00	—	—	N.D.(Peak) ppm	Ocimene <(E)-, beta->
16	-	93.00	—	—	N.D.(Peak) ppm	Terpinolene
17	17.37	81.00	1497	260	0.04681 ppm	Fenchone
18	-	81.00	—	—	N.D.(Peak) ppm	Fenchyl alcohol
19	-	71.00	—	—	N.D.(Peak) ppm	Linalool
20	21.33	95.00	2597	422	0.19215 ppm	Camphor
21	-	67.00	—	—	N.D.(Ref) ppm	Isoisopulegol
22	-	95.00	—	—	N.D.(Peak) ppm	Isoborneol
23	-	71.00	—	—	N.D.(Peak) ppm	Menthol
24	-	93.00	—	—	N.D.(Peak) ppm	Caryophyllene <(E)->
25	-	161.00	—	—	N.D.(Peak) ppm	Valencene
26	-	93.00	—	—	N.D.(Peak) ppm	Selinene <alpha->
27	-	69.00	—	—	N.D.(Peak) ppm	Nerolidol <(Z)->
28	-	93.00	—	—	N.D.(Peak) ppm	Bisabolene <(Z)-, alpha->
29	-	112.00	—	—	N.D.(Peak) ppm	Isomenthone
30	-	95.00	—	—	N.D.(Peak) ppm	Borneol
31	-	59.00	—	—	N.D.(Peak) ppm	Terpineol <alpha->
32	-	121.00	—	—	N.D.(Peak) ppm	Terpineol <gamma->
33	-	81.00	—	—	N.D.(Peak) ppm	Pulegone
34	-	119.00	—	—	N.D.(Ref) ppm	Cedrene <alpha->
35	-	93.00	—	—	N.D.(Ref) ppm	Humulene <alpha->
36	-	69.00	—	—	N.D.(Ref) ppm	Nerolidol
37	47.35	161.00	4598	552	0.43624 ppm	Guaiol
38	47.91	95.00	7530	781	1.07140 ppm	Cedrol
39	-	109.00	—	—	N.D.(Peak) ppm	Bisabolol <alpha->

R. time: retention time. N.D.: non determined.

cell lines (Fig. 5A). 786-O cells treated with the mitochondrial oxidative phosphorylation uncoupler CCCP were used as the positive control. Rh123 quantification *via* flow cytometry demonstrated that TSR inhibited Rh123 signal intensities in both 786-O and Caki-1 cells (Fig. 5B and C).

Mitochondria-dependent apoptosis was preceded by ROS overproduction [18]. TSR essential oil significantly induced ROS production in a concentration-dependent manner in both cell lines (Fig. 6A–C). Quantification of ROS fluorescence intensity *via* flow cytometry demonstrated that TSR essential oil (62.5–250 ppm) significantly triggered Caki-1 ROS overproduction, whereas 786-O required a higher concentration (250 ppm) to induce similar effects (Fig. 6C).

3.6. TSR essential oil promotes the expression of pro- and anti-apoptotic proteins and caspase-3/7 activities

To further evaluate the mechanism of TSR-induced apoptosis, the levels of intrinsic apoptotic proteins were determined *via* Western blot analysis and caspase-3/7 activity assays. Accordingly, TSR essential oil increased cytosolic but blocked membranous cytochrome c expression (Fig. 7A–E). Anti-apoptotic proteins, such as Bcl-2 and heat shock protein 90, were decreased with TSR treatment (Fig. 7A–E). In addition, caspase activity assays further supported the apoptotic effects of TSR on ccRCC cells. As expected, TSR increased caspase-3/7 activities > twofold in a concentration-dependent manner in both cell lines (Fig. 7F).

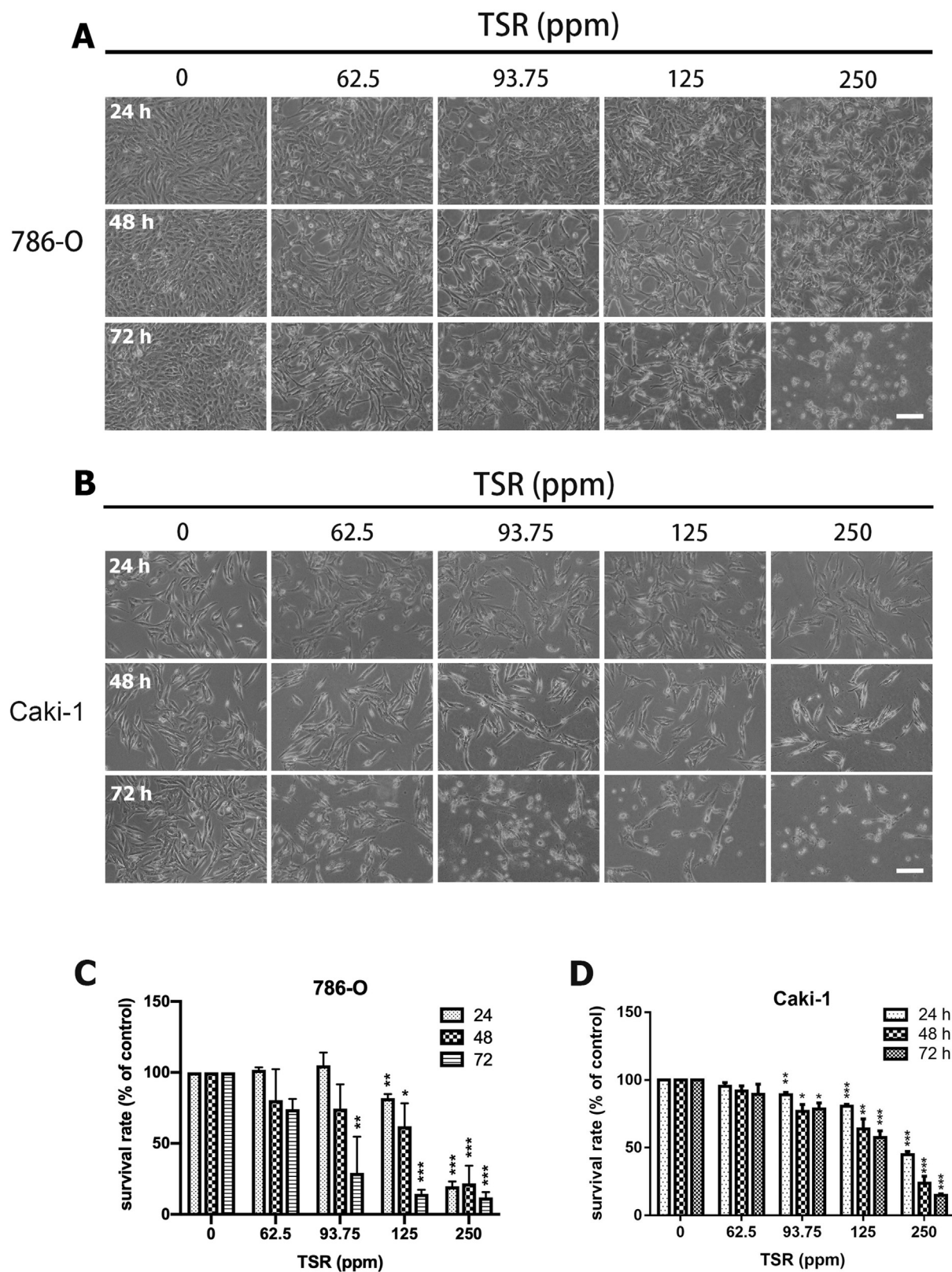


Fig. 2. Effects of TSR essential oil on cell morphology and viabilities. (A and B) Cell morphology and (C and D) cell viability assays. Scale bar = 100 μ m. Results are expressed as mean $\pm$ SD of five independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with the control group.

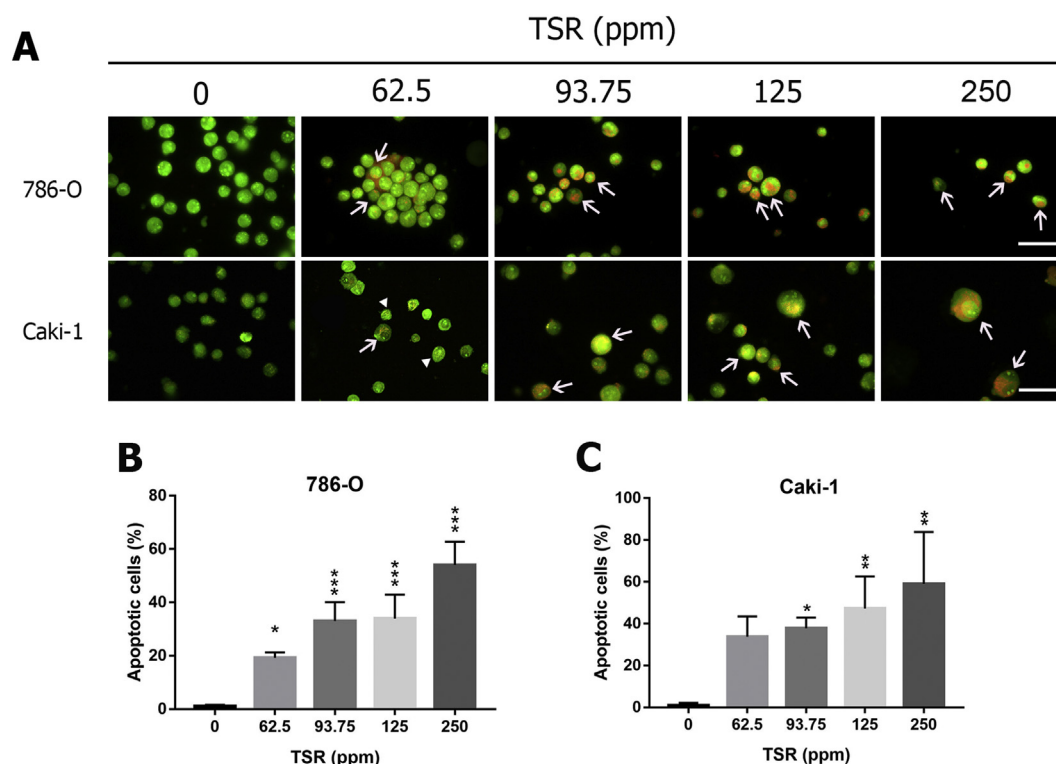


Fig. 3. Effects of TSR essential oil on apoptosis. (A) Dual AO–EB staining on TSR-treated 786-O (24 h) and Caki-1 cells (48 h). Scale bar = 50 μ m. Arrow head: early apoptotic cells; arrows: late apoptotic cells. Representative images are from one of three independent experiments. (B and C) Late apoptotic populations are expressed as mean $\pm$ SD of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with the control group.

3.7. Inhibitory effects of TSR essential oil on Akt, mTOR and NF- κ B phosphorylation and HIF-1/2 α protein expression

Under normoxic conditions, HIF- α expression can increase with growth factor signalling [19]. To determine whether TSR blocks intracellular signalling in 786-O and Caki-1 cells, Western blot analyses were employed to examine the HIF- α , pNF- κ B, pAkt and pmTOR expressions. TSR inhibited NF- κ B, Akt and mTOR phosphorylation in both cell lines (Fig. 8A–H). Furthermore, TSR reduced HIF-1 α and HIF-2 α expressions in 786-O and Caki-1 cells at 24 and 48 h, respectively (Fig. 8A, B, and I).

4. Discussion

T. sinensis has long been considered as a multi-functional plant owing to its nutraceutical and medicinal uses [8]. There are only three scientific reports focusing on the effects of *T. sinensis* roots. Methyl gallate extracted from the methanol extracts of *T. sinensis* roots exhibited an anti-oxidant activity against hydrogen peroxide-induced DNA damage

in MDCK cells [20]. Water extracts of TSR could help improve the anti-oxidant system in senescence-accelerated mouse prone 8 for clearing β -amyloid plaques [21]. Besides, Yang et al. (2013) demonstrated that ethyl acetate extracts of TSR inhibits the proliferation of MGC-803 and PC3 tumour cells [22]. However, the essential oil of TSR and its anti-tumour effects have never been reported. Our study examined the composition of TSR essential oil and identified five major compounds and some rare ingredients from essential oil standard references. Fifty six terpenoids have been isolated from this plant [8]. Two triterpenoids, betulonic acid and 3-oxours-12-en-28-oic acid are from TSR, exhibiting antitumour activity [22]. Compare to our results, specific proportions and concentrations of sesquiterpenoids (pogostol, viridiflorol, and guaicol), sesquiterpenes (spathulenol, cedr-8 (15)-en-9-ol, cedrol, and caryophyllene oxide) as well as monoterpenoids (fenchone and champhor) have been identified. Among them, spathulenol was the predominant compound (37.2%). Sesquiterpenes and their metabolites exhibit numerous pharmacological activities, including anti-tumour, anti-inflammatory,

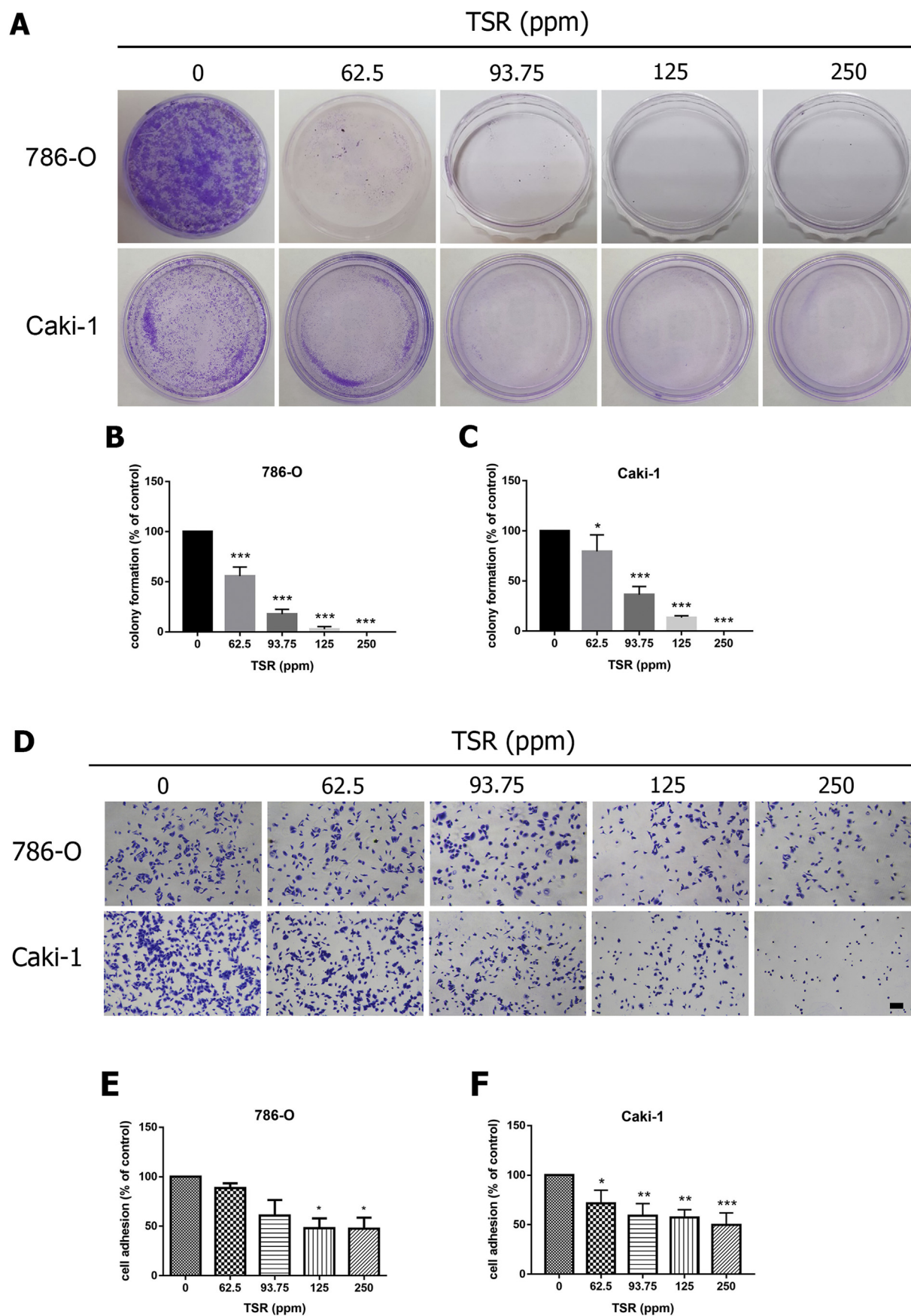


Fig. 4. Effects of TSR essential oil on colony formation and cell adhesion. Cells were treated with TSR at concentrations of 0, 62.5, 93.75, 125 and 250 ppm for 24 h (786-O) and 48 h (Caki-1), respectively. (A) Colony-forming assay, (B) quantification of colonies and (C) fibronectin adhesion assay. Scale bar = 50 μ m; magnification: 100 $\times$. Representative images are from three independent experiments. Data were quantified for three independent experiments and expressed as mean $\pm$ SD. * p < 0.05 compared with the control group. Scale bar = 100 μ m.

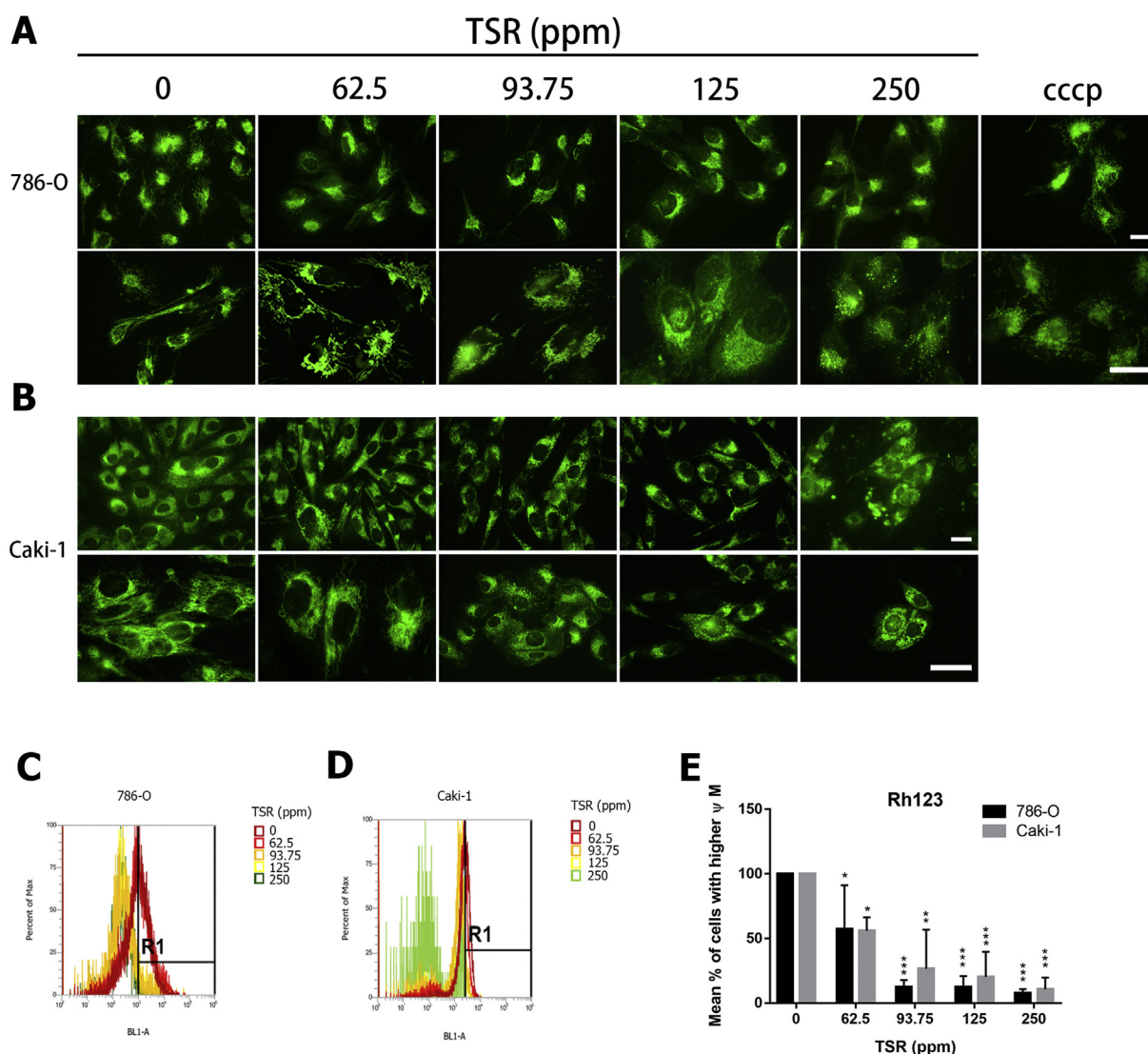


Fig. 5. Effects of TSR essential oil on MMP. Cells were treated with TSR at concentrations of 0, 62.5, 93.75, 125 and 250 ppm for 24 h (786-O) and 48 h (Caki-1), respectively. CCCP (50 μ M) was used as a positive control. (A and B) Rhodamine 123 staining of 786-O and Caki-1 cells. (C and D) Flow cytometry histogram of Rh123 staining. (E) The bar graph shows Rh123 intensities of 786-O and Caki-1 cells examined via flow cytometry. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the control group.

anti-microbial, cytotoxic, immunosuppressive and other effects [23]. Wang et al. (2014) reported that trace amounts (<0.1%) of spathulenol can be found in the essential oil of *T. sinensis* leaves, whereas caryophyllene oxide accounts for 0.82% [24]. Interestingly, these two compounds are the major ingredients in the essential oil of TSR. Sesquiterpene oxides, such as caryophyllene oxide, are toxic to ants and inhibits the growth of ant-associated fungi [25]. According to the RIFM fragrance ingredient safety assessment, caryophyllene oxide and cedr-8(15)-en-9-ol demonstrate no observed genotoxicity,

phototoxicity or photoallergic effects [26, 27]. The LD50 of caryophyllene oxide and cedr-8(15)-en-9-ol for acute oral toxicity are more than 5 gram/kg in rats [26, 27]. The NOAEL of caryophyllene oxide for fertility was considered to be 21000 ppm (equivalent to 1398 mg/kg/day for males and 1660 mg/kg/day for females) [26]. Briefly, the five major compounds identified in TSR essential oil, only spathulenol had been reported to exhibit cytotoxicity on human cancer cell lines [28, 29]. However, the influence of other trace constituents and their synergetic effects should be considered when used *in vivo*.

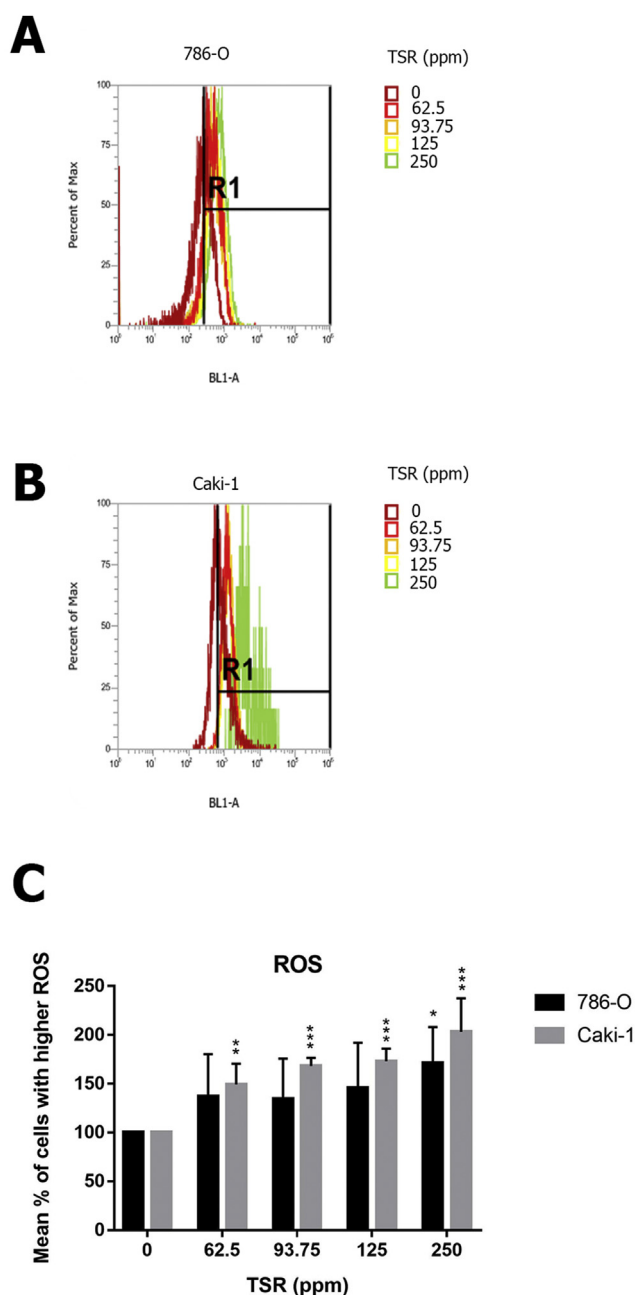


Fig. 6. Effects of TSR essential oil on ROS production. Cells were treated with TSR at concentrations of 0, 62.5, 93.75, 125 and 250 ppm for 24 h (786-O) and 48 h (Caki-1), respectively. (A and B) Flow cytometry histogram of ROS staining. (C) Bar graph showing the quantification of ROS intensities in 786-O and Cak-1 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the control group.

Akt, mTOR and NF- κ B phosphorylation has been demonstrated in numerous tumours, indicating poor prognoses for patients [30]. Natural products or compounds modulating multiple signalling

pathways to suppress tumour progression is current trends for developing anti-cancer drugs. Akt/mTOR and Akt/mTOR/NF- κ B are two main mutated pathways constitutively activated in human cancers [30, 31]. Studies have been shown that Akt/mTOR blocks apoptosis by inhibition of the tumour suppressor protein phosphatase and tensin homolog and/or transactivation of the transcription factor NF- κ B [31]. In our data, inhibition of pAkt, pmTOR, and pNF- κ B were concurrent with induction of apoptosis. Whether TSR essential oil inhibits Akt, mTOR, and NF- κ B concomitantly or blocks them in a series signalling cascade, needs more experiments to clarify.

The Akt/mTOR/NF- κ B signalling network regulates cell growth and modulates HIF- α activity, translation, expression and stability in RCC, and the accumulation of HIF- α protein is a key feature of ccRCC due to the loss of the functional VHL protein [32–34]. Deletion of either HIF-1 α or HIF-2 α is sufficient to restrict the formation of tumour, suggesting the pro-tumourigenic activities of HIF-1 α /2 α in ccRCC [19]. Due to the lack of HIF-1 α in 786-O cells and an undetectable level of HIF-2 α in Caki-1 cells, we examined the expressions of HIF-2 α and HIF-1 α in 786-O and Caki-1 cells, respectively, following TSR treatment. We found that TSR inhibited the phosphorylation of Akt, mTOR and NF- κ B and simultaneously decreased the downstream expression of HIF- α . Although the exact mechanism of how TSR inhibits the expression of HIF- α in these cells remains unclear, one possibility may be through the inactivation of Akt, mTOR or NF- κ B pathways. Furthermore, whether TSR induces HIF- α degradation needs further clarification.

Hsps can inhibit or aid the apoptotic machinery through their chaperone functions by modulating protein assembly and folding, ubiquitin-dependent degradation and protein translocation [35]. The inhibition of hsp90-Akt binding increases the sensitivity of cells to apoptotic signals, indicating a direct interaction between hsp90 and Akt [36]. Hsp90 expression and Akt phosphorylation were abrogated by TSR essential oil in both 786-O and Caki-1 cell lines. Several lines of evidence demonstrated that blocking Hsp90 not only reduces TNF α -triggered NF- κ B activity and increases cancerous cell death [37] but also downregulates acidosis-induced HIF-1 α /2 α expression in glioma cells [38]. As a multifunctional protein, Hsp90 might be another key regulator in TSR-induced effects. Together, these findings indicate that TSR essential oil-

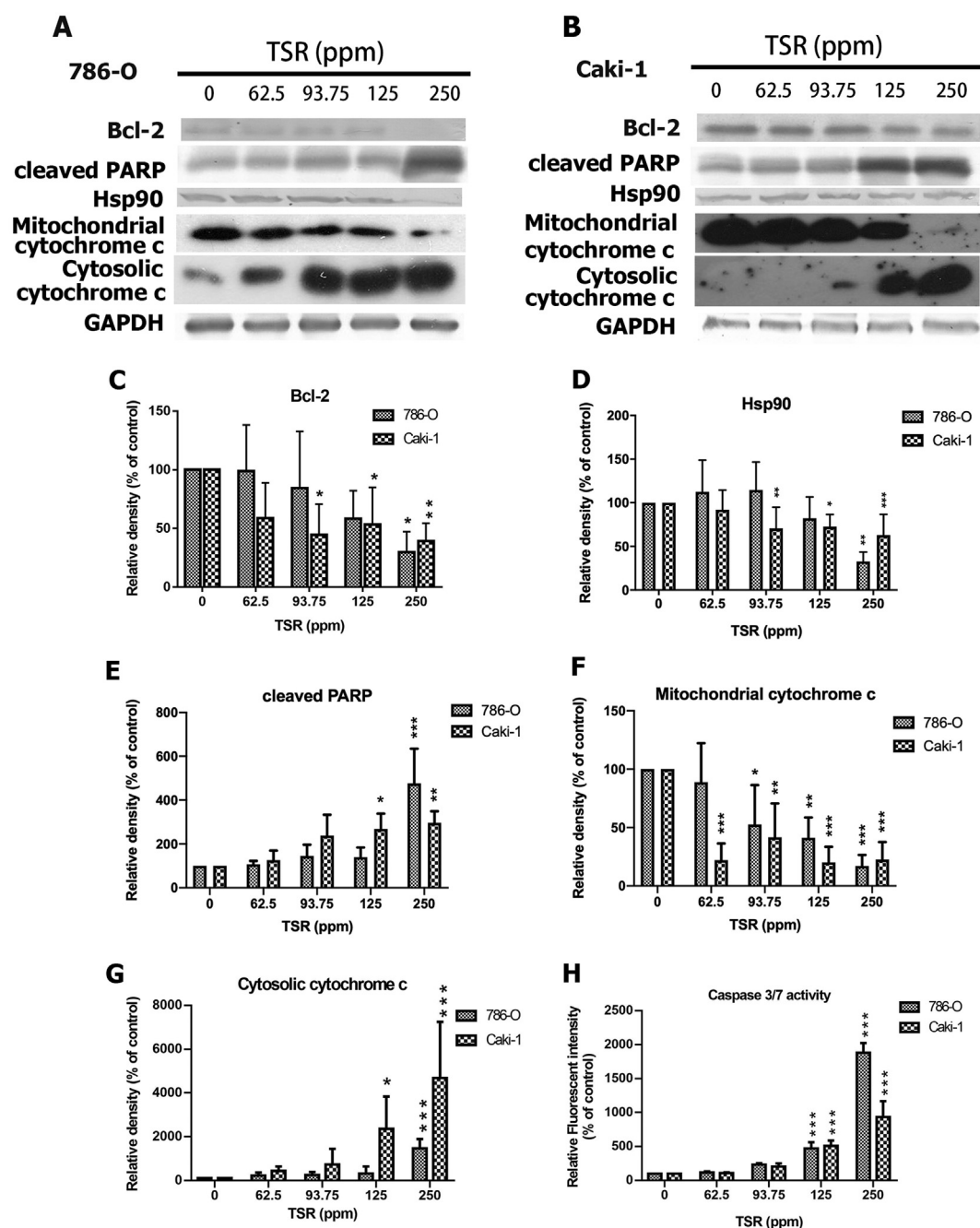


Fig. 7. Effects of TSR essential oil on cytochrome c, Bcl-2, Hsp90, cleaved PARP-1 and caspase activities. Cells were treated with TSR at concentrations of 0, 62.5, 93.75, 125 and 250 ppm for 24 h (786-O) and 48 h (Caki-1), respectively. (A and B) Protein expression of Bcl-2, hsp90, cleaved PARP-1 and cytochrome c (cytosolic and membranous fractions). GAPDH was used as the internal control. (C–G) Bar graphs showing the densitometric analysis (mean $\pm$ SD) for three to five independent experiments. (H) Caspase activity assay. Results (mean $\pm$ SD) were from three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with the control group.

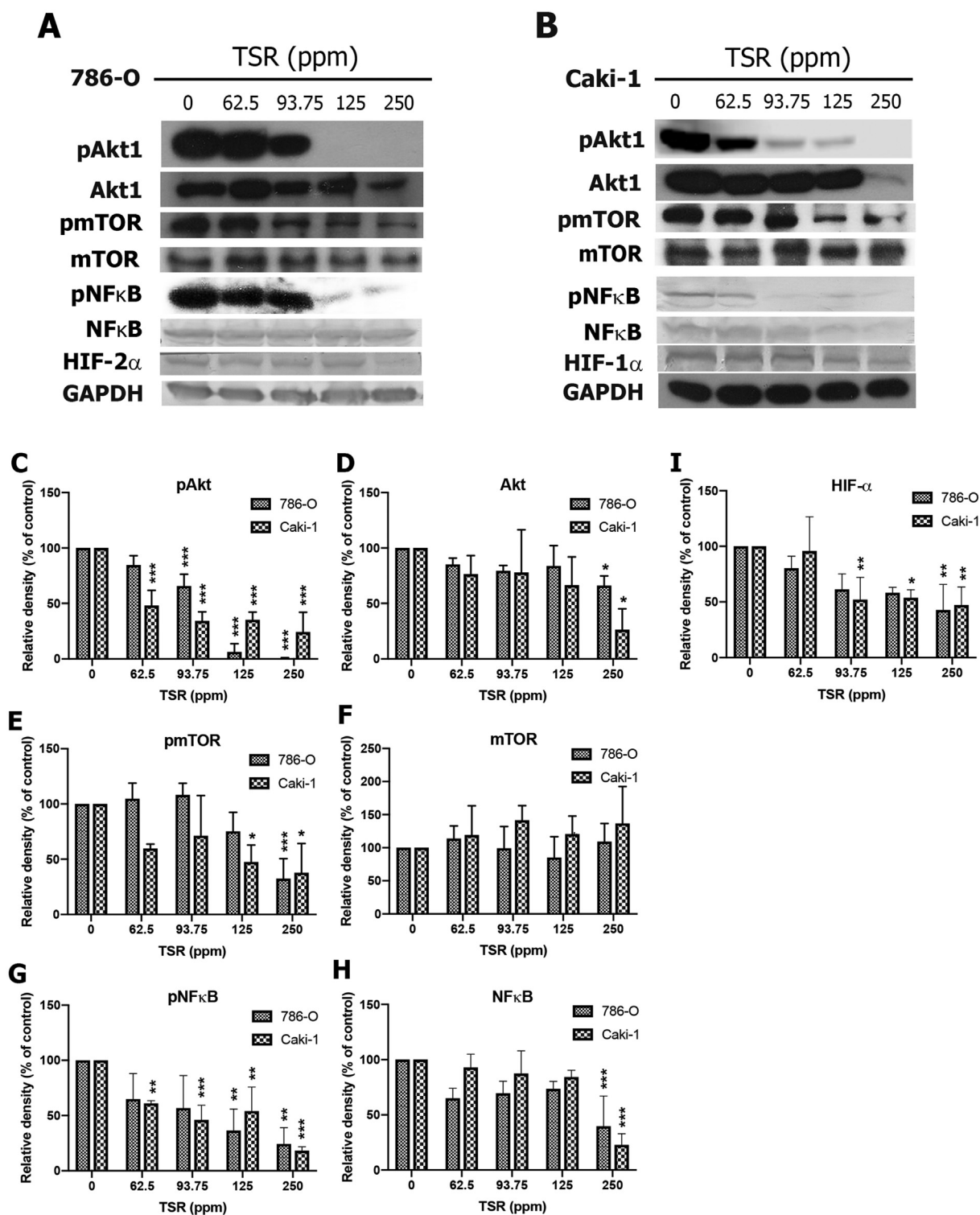


Fig. 8. The effects of TSR on oncogenic signalling proteins (pAkt, Akt, pmTOR, mTOR and HIF-1 α /2 α). Cells were treated with TSR at concentrations of 0, 62.5, 93.75, 125 and 250 ppm for 24 h (786-O) and 48 h (Caki-1), respectively. (A and B) Phosphorylation of Akt, mTOR and NF- κ B as well as protein expression of total Akt, total mTOR, total NF- κ B and HIF-1/2 α . GAPDH was used as the internal control. The figures shown are representative of one experiment. (C–I) Bar graphs showing the densitometric analysis for three to five independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the control group.

inhibited HIF-1 α /2 α expression might occur with the assistance of Hsp90.

In conclusion, TSR essential oil exhibited various anti-proliferative and cytotoxic effects, including the induction of mitochondrial-dependent apoptosis associated with the downregulation of several oncogenic signalling pathways in ccRCC. Altogether, this suggests that TSR essential oil may be a potential cancer treatment.

Conflict of interest

The authors declare no conflict of interest.

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