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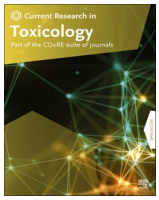

The cancer preventive activity and mechanisms of prenylated resveratrol and derivatives

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ABSTRACT

Resveratrol is regarded as neutraceuticals with multiple health benefits. The introduction of prenyl can enhance the bioactivity. In this work, the cancer preventive activities and mechanisms of 18 prenylated reseveratrol and derivatives were investigated. The results showed that prenyl increased the antiproliferative activities of resveratrol, oxyresveratrol and piceatannol against cancer cells, and their antiproliferative activities were time- and dose-dependent. 4-C-prenylation was important for the antiproliferative activity of stilbenoids. The 4-*C*-prenyl stilbenoids showed better antiproliferative activities than other prenylated stilbenoids. 4-*C*-prenyl piceatannol showed the best antiproliferative activity. Human hepatoellular carcinomas ($HepG_2$) cell was more sensitive to prenylated stilbenoids than human MCF-7 breast carcinoma cell. 4-*C*-prenyl piceatannol had high affinities to Caspase-3, Caspase-9, CDK2 and Cyclin A2. The possible amino acids involved in binding 4-*C*-prenyl piceatannol were revealed. The expression of *Caspase-3* and *Caspase-9* were upregulated by 4-*C*-prenyl piceatannol and the expression of *CDK2* and *Cyclin A2* in $HepG_2$ cells were downregulated, which contributed to apoptosis. The above results eludicated the possible antiproliferative mechanisms of prenylated stilbenoids.

1. Introduction

Stilbenoids are secondary metabolites presented in plants, which are involved in defence against injury or infection. They have also been recognized to afford positive effect to health maintenance and disease prevention (Akinwumi et al., 2018). Resveratrol is a well-known stilbenoid with an abundant level in wine (Navarro-Orcajada et al., 2022). Piceatannol and oxyresveratrol are analogues of resveratrol. All these compounds have been reported to possess a variety of biological activities, including antitumor, antiviral, anti-inflammatory, antimitotic and antidiabetic activities. Protein tyrosine phosphatase 1B is a key negative regulator in the insulin signal transduction pathway. Glucosidase is responsible for catalysing the hydrolysis of carbohydrates. Stilbenoids showed strong inhibition activity to diabetes-related receptors involving protein tyrosine phosphatase 1B and glucosidase. Therefore, these compounds show antidiabetic activity. (Biais et al., 2017; De Filippis et al., 2017; Shen et al., 2009). Furthermore, these compounds exhibit antifungal properties. Pinosylvin monomethyl ether is a stilbenoid isolated from *Cajanus cajan* which shows a broad antifungal spectrum. It can bind to the cell membrane phospholipids and cause the cell lysis (Li et al., 2022). Oral administration of piceatannol increases the number of astrocytes in the brains of adult mice (Arai et al., 2016). Furthermore, both piceatannol and resveratrol are able to induce apoptosis in cancer cells. Piceatannol, instead of resveratrol, is a more efficient inducer of apoptosis (Wieder et al., 2001). The cytochrome p450 enzyme CYP1B1, which is generally present in human tumors, can convert the chemopreventive compound resveratrol to the anticancer compound piceatannol. This observation provides a novel explanation for the cancer prevention capability of resveratrol (Chae et al., 2008).

Most polyphenols, including flavonoids and stilbenoids, have low sub-chronic toxicity. Previous studies have shown that these compounds are usually recognized as safe. Its toxicity is related to dose, and can be used to protect cells within a certain dose range (Khan et al., 2021). Previous study has suggested that daily intake of 100 mg of flavonoids play a significant role in preventing from cardiovascular diseases. This dose will not cause liver damage (Rana et al., 2022). Twenty-four

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phenolic compounds, including oxyresveratrol obtained from *Millettia erythrocalyx* and *Artocarpus lakoocha*, show no toxicities in pripheral bood mnonuclear cell, CEM cell and Vero cell at 100 μM (Likhitwitayawuid et al., 2005). The structure of oxyresveratrol is similar to resveratrol. However, the sub-chronic toxicity assessment shows that oxyresveratrol has a lower toxicity than resveratrol. Previous study has evaluated the acute and sub-chronic toxicity of oxyresveratrol in rat. No adverse effects have been observed for oxyresveratrol at 100 mg/kg per day for Wistar rats. Similarly, oxyresveratrol is not as toxic to cancer cells as resveratrol. The anticancer activity of oxyresveratrol is lower compared with that of resveratrol (Sintuyanon et al., 2017). Oxyresveratrol can effectively scavenge H_2O_2, NO and the artificial free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). Moreover, oxyresveratrol can be transported into tissues at a high rate with a bioavailability of about 50%. This makes oxyresveratrol capable of nervous and cardiovascular protection. However, the effect of this substance on liver cells should be considered, especially in the case of cirrhosis (Rahimkhani and Ghofrani, 2008).

Both oxyresveratrol and piceatannol are the analogues of resveratrol. Hydroxylation location causes significantly different biological activity. This suggests that chemical modification is effective to change the biological activity of stilbenoids. Previous literatures have showed that the introduction of prenyl increases the lipophilicity of phenolics, enhances the interaction with biological membrane, and improves the absorption (Mukai et al., 2013; Prausova and Kollar, 2019). In this study, we synthesized 18 prenylated stilbenoids using resveratrol, piceatannol and oxyresveratrol as the initial substrates. Their proliferation inhibition activity against human $HepG_2$ hepatoma cell line and human MCF-7 breast carcinoma cell line were evaluated. The structure–activity relationship was analyzed. The possible mechanisms of action were investigated.

2. Materials and methods

2.1. Chemicals and reagents

All prenyl acceptors (resveratrol, oxyresveratrol and piceatannol) and prenyl donors (3,3-dimethyllallyl bromide, 2-methyllallyl bromide and geranyl bromide) were purchased from Macklin (Shanghai, China). All the other regents used were of analytical grade.

CCK-8 kit was purchased from Beyotime Institution of Biotechnology (Shanghai, China, lot number C0037). Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, MO, USA, lot number D1435). Williams' medium E, Dulbecco's Modifed Eagle's medium (DMEM, lot number 11885084) fetal bovine serum (FBS, lot number 10099141), penicillin–streptomycin solution (lot number 15140148), trypsin/EDTA solution (lot number R001100), phosphate buffer solution (PBS, lot number 10010001) and other cell culture reagents were provided by Thermo Fisher Scientific Co., Ltd. (Shanghai, China).

2.2. Synthesis of prenylated stilbenoids

Prenylated stilbenoids were synthesized according to the protocol described previously (Puksasook et al., 2017). Briefly, 325 mg of dry lithium carbonate were mixed with 500 mg of resveratrol in 30 mL of dry dimethylformamide (DMF). 3,3-Dimethylallyl bromide (0.76 mL) was added dropwisely. To synthesize other prenylated stilbenoids, resveratrol was replaced by piceannol or oxyresveratrol. 3,3-Dimethylallyl bromide was replaced by 2-methyllallyl bromide or geranyl bromide. The reaction was refluxed and stirred in nitrogen gas. After 3 h, the reaction mixture was filtered. The filtrate was added with water and extracted with ethyl acetate. The combined ethyl acetate extracts were dried by Na_2SO_4, and the solvent was removed under reduced pressure.

2.3. Cell viability test

Two cancer cells, Human hepatoma cancer cells ($HepG_2$) and human breast cancer cells (MCF-7), were obtained from the Laboratory of Animal Center, Sun Yat-Sen University, China. $HepG_2$ cells were cultured in William's medium E supplemented with 5% fetal bovine serum, 50 units/mL penicillin, 10 mM HEPES, 50 μg/mL streptomycin, 5 μg/mL insulin and 0.05 μg/mL hydrocortisone. MCF-7 cells were cultured in DMEM, which included 5% fetal bovine serum, 10 mM HEPES, 50 units/mL penicillin, 50 μg/mL streptomycin and 100 μg/mL gentamicin. All the cells were maintained in an incubator (37 °C, 5% CO_2 atmosphere).

The assay was conducted by following the protocol of Wen et al. with some modifications (Wen et al., 2015). $HepG_2$ cells were seeded at a density of 2.5×10^3 cells/well in a 96-well microplate with 100 μL of growth medium/well. MCF-7 cells were seeded at a density of 5×10^3 cells/well in a 96-well microplate with 100 μL of growth medium/well. After the cells adhered to the wall, the growth medium was removed and the wells were washed with PBS. The growth medium (100 μL) with different concentrations of analytes were added. The concentrations of tested compounds were in the range of 0–100 μM (0, 10, 20, 40, 60, 80, 100 μM). The wells adding medium without addition of tested compounds were served as control. The cells were incubated for 48 h and 72 h at 37 °C, respectively. After incubation, 10 μL of CCK-8 were added to the medium and incubated for 2 h at 37 °C. The absorbance was measured at 450 nm by using a microplate reader (Spark, Tecan Group Ltd., Männedorf, Switzerland). The cell viability was calculated as follows: cell viability (%) $= \frac{As-A0}{Ac-A0} \times 100\%$, where As, Ac and A_0 represent the mean absorbance values of cells incubated with the tested compound, cells with vehicle medium (0.5% DMSO in growth medium) without the tested compound, and blank wells without cells, respectively. The results were expressed as the mean value of three independent determinations.

2.4. ROS measurement

The ROS production in cells was monitored by using fluorescent dyes DCFH-DA. $HepG_2$ cells were seeded at a density of 2.5×10^5 cells/well in a 6-well plate to incubate for 6 h and then incubated after corresponding treatment for 24 h. Then the cells were harvested by trypsinization and rinsed with PBS three times. Serum-free DMEM medium containing individual fluorescent dye (10 mM) were added to the cells and incubated for 25 min at 37 °C in dark. The cells were rinsed with PBS three times again and resuspended in fresh PBS. The intensity of fluorescence (Ex 485/Em 525) was measured using a microplate reader at 37 °C. The results were normalized by the vehicle control and then calculated as percentage.

2.5. Gene expression analysis

$HepG_2$ cells were seeded at a density of 2.5×10^5 cells/well in a 6-well plate for 6 h prior to treatment. Then, the cells were incubated with different concentrations of the tested compounds (0, 10, 20, 40, 60, 80, 100 μM). Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was used for detecting gene expression analysis. The gene expression levels of *Caspase-3*, *Caspase-9*, *CDK2* and *Cyclin A2* were measured after treatment for 24 h. HiPure total RNA Mini kit was used to extract the total RNA. *GADPH* was applied as the housekeeping gene.

2.6. Molecular docking

Molecular docking of 4-*C*-prenyl piceatannol with *Caspase-3* and *Caspase-9* were completed using AutoDock. The docking models with high score were selected. Figures were generated with PyMol. The interaction between 4-*C*-prenyl piceatannol and Caspase-3 and Caspase-

9 were analyzed using Ligplot. The data for 3D structures of proteins for Caspase-3 (PDB ID: 2C1E) and Caspase-9 (PDB ID: 2AR9), were used in this study.

3. Results and discussion

3.1. Cell viability test

The chemical structures of eighteen prenylated stilbenoids synthesized in this work are presented in Fig. 1. The anti-cancer activity of prenylated stilbenoids against $HepG_2$ and MCF-7 cells were examined by CCK-8 assay. The results indicated that prenylation led to an increase of the anticancer activities for most of prenylated stilbenoids. One possible mechanism is that the prenyl side chain increases the lipophilicity of stilbenoid. The pharmacological activity is modified by enhanced affinity with the lipophilic membrane and improved absorption (Mohammadhosseinpour et al., 2022). Another possible mechanism is that prenylated compounds may inhibit the ras signal transduction. Ras protein plays an important role in the intracellular protein synthesis. It can be activated by attachment of farnesyl moiety (Manne et al., 1995). Ras protein is involved in regulating cell functions, such as proliferation, differentiation and inflammation. Furthermore, the prenyl moiety may help stilbenoids to recognize specific biological targets. This mechanism has not been definitively proved. Further study about the mechanism is required.

-B shows the cell viability treated by eighteen stilbenoids, respectively. All the tested compounds showed dose-dependent behaviors. Table 1 presents the half maximal inhibitory concentration (IC_{50}) value

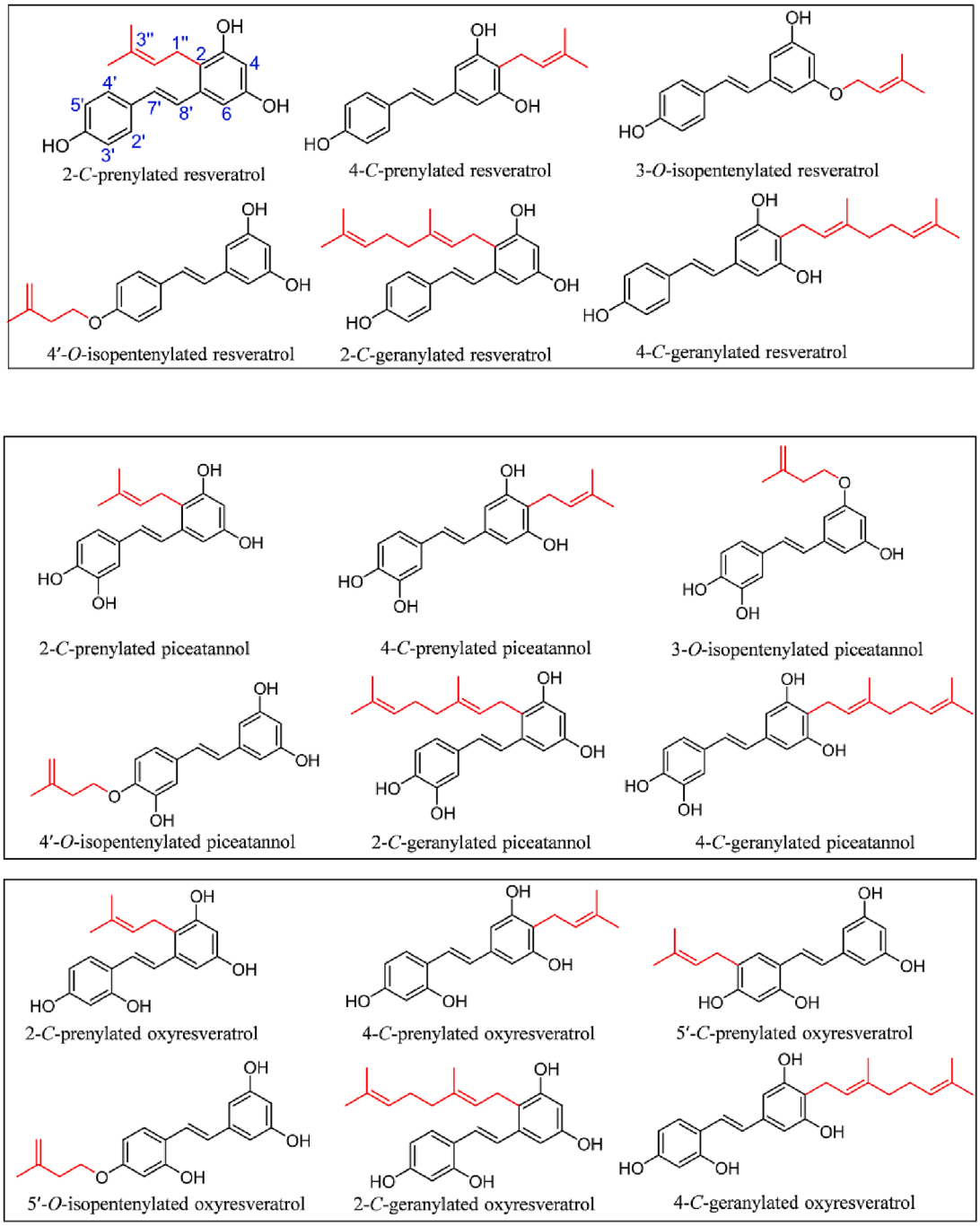


Fig. 1. Structures of 18 prenylated stilbenoids produced in this work.

Table 1
The half maximal inhibitory concentration (IC_{50}) value of 18 stilbenes.

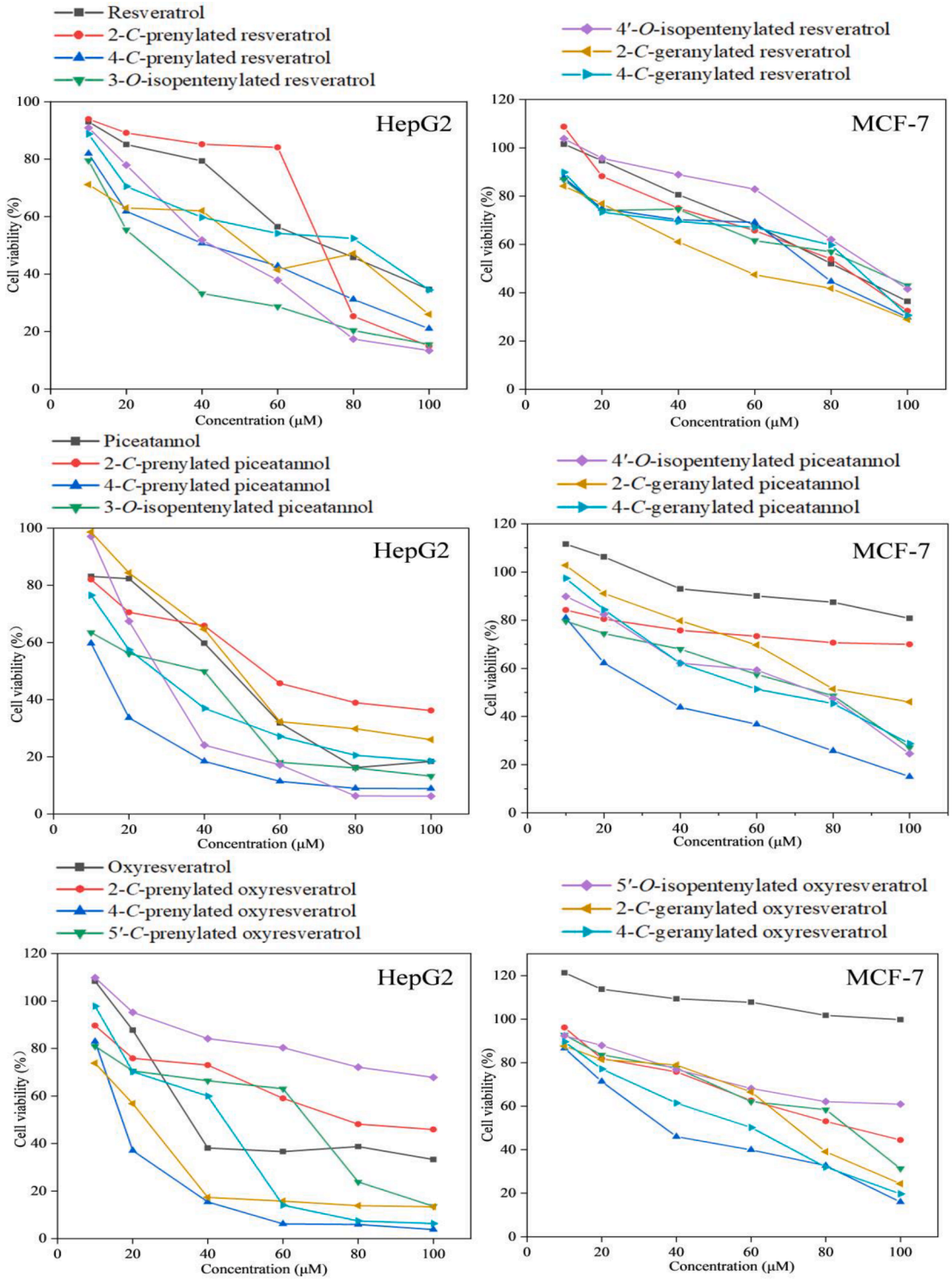
Compounds	$HepG_2$		MCF-7	
	48 h	72 h	48 h	72 h
Resveratrol	72.51 ± 0.12	43.54 ± 0.17	80.92 ± 0.26	>100
2-*C*-prenyl resveratrol	66.44 ± 0.04	38.95 ± 0.29	78.17 ± 0.31	68.50 ± 0.16
4-*C*-prenyl resveratrol	37.60 ± 0.23	32.9 ± 0.32	71.01 ± 0.44	58.69 ± 0.33
3-*O*-isopentenyl resveratrol	25.98 ± 0.33	20.58 ± 0.19	97.58 ± 0.40	93.86 ± 0.26
4′-*O*-isopentenyl resveratrol	39.72 ± 0.09	30.18 ± 0.33	95.71 ± 0.28	100.42 ± 0.28
2-*C*-geranyl resveratrol	44.62 ± 0.07	14.07 ± 0.21	54.16 ± 0.18	67.98 ± 0.25
4-*C*-geranyl resveratrol	65.39 ± 0.22	32.22 ± 0.33	82.54 ± 0.45	63.97 ± 0.33
Piceatannol	39.76 ± 0.43	47.68 ± 0.13	>100	>100
2-*C*-prenyl piceatannol	55.48 ± 0.31	39.97 ± 0.16	>100	>100
4-*C*-prenyl piceatannol	12.71 ± 0.23	10.94 ± 0.35	32.17 ± 0.31	27.30 ± 0.23
3-*O*-isopentenyl piceatannol	21.99 ± 0.36	21.19 ± 0.20	49.79 ± 0.24	50.91 ± 0.35
4′-*O*-isopentenyl piceatannol	28.47 ± 0.09	23.92 ± 0.17	62.34 ± 0.19	49.75 ± 0.26
2-*C*-geranyl piceatannol	50.84 ± 0.31	43.55 ± 0.33	90.23 ± 0.28	74.39 ± 0.40
4-*C*-geranyl piceatannol	26.30 ± 0.46	20.73 ± 0.18	61.43 ± 0.07	53.92 ± 0.42
Oxyresveratrol	44.77 ± 0.23	39.77 ± 0.44	>100	69.24 ± 0.32
2-*C*-prenyl oxyresveratrol	84.4 ± 0.35	45.71 ± 0.70	87.22 ± 0.14	78.64 ± 0.17
4-*C*-prenyl oxyresveratrol	17.99 ± 0.42	17.05 ± 0.53	39.23 ± 0.33	38.08 ± 0.28
5′-*C*-prenyl oxyresveratrol	46.10 ± 0.46	42.2 ± 0.44	80.82 ± 0.56	56.73 ± 0.34
5′-*O*-isopentenyl oxyresveratrol	>100	>100	>100	>100
2-*C*-geranyl oxyresveratrol	20.44 ± 0.32	13.7 ± 0.03	66.83 ± 0.43	52.94 ± 0.52
4-*C*-geranyl oxyresveratrol	35.02 ± 0.17	11.07 ± 0.16	54.26 ± 0.32	39.13 ± 0.09

of eighteen stilbenoids. According to the data in Table 1, the IC_{50} values of 6 prenylated resveratrol, 4 prenylated piceatannol and 3 prenylated oxyresveratrol are lower than their nonprenylated precursors. This indicated that the prenylation substitution had an enhancement on the activity of the compound. Furthermore, the results demonstrated that the size and location of prenyl group affected the bioactivity of stilbenoids to a large extent. Among all the tested compounds, 4-C-prenyl piceatannol was found to be the most potent chemical (IC_{50} = 12.71 ± 0.23 μM). 5′-O-isopentenyl oxyresveratrol had the least activity with IC_{50} > 100 μM for both $HepG_2$ and MCF-7. This is consistent with previous studies. It has been documented that piceatannol has better bioactivity than resveratrol and oxyresveratrol (Choi et al., 2016; Hosoda et al., 2021). 4-C-prenyl piceatannol were the most active compound and reduced growth of both tumor cell lines by 50% at less than 35 μM and almost completely inhibited $HepG_2$ growth at 80 μM. The cytotoxicity is not strictly correlated with prenyl side chain. For example, 2-C-geranyl piceatannol showed a stronger cytotoxicity than 2-C-prenyl piceatannol. However, 4-C-prenyl piceatannol showed a higher cytotoxicity than 4-C-geranyl piceatannol. This indicated that substitution site played an important role in anti-cancer cell proliferation activity for stilbenoids.

Prenylation substitution is a common modification in aromatic natural products. Prenylated phenolics have higher bioaccumulation and bioactivity than their phenolics precursors. Flavonoids, for example, can be found prenylated at various sites. Most of prenylated flavonoids with 8-*C*-prenylation have potent antibacterial activity. 8-Prenylnaringenin could inhibit methicillin-resistant *Staphylococcus aureus*, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 (Mun et al., 2014; Ng et al., 2019). Furthermore, 8-prenylnaringenin could inhibit fish pathogenic strains *Streptococcus iniae* and *Vibrio vulnificus* (Li et al., 2022). Previous research has indicated that prenylated flavonoids with hydroxyl groups at *C*-3 and *C*-6 and prenyl moieties at *C*-8 are able to inhibit human carboxylesterase 2, which plays an important role in the metabolic activation of ester drugs and lipid metabolism (Li et al., 2015). Several prenylated flavonoids isolated from *Sophora flavescens* have been investigated for their inhibitory effects on human myeloid leukemia HL-60 cells and human hepatocarcinoma $HepG_2$ cells. The prenylated flavonoids without 3-OH showed stronger inhibition than those with 3-OH. Futhermore, the lavandulyl side chain at *C*-8 in flavanone skeleton is essential for the cytotoxic activity of the prenylated flavonoids isolated from *Sophora flavescens* (Ko et al., 2000). Kurarinone is a flavone prenylated at *C*-8. It shows a strong antibacterial effect, like the effect of antibiotics on recurrent urinary infections caused by Gram-negative bacteria and candida (Rahimkhani et al., 2014; Rahimkhani et al., 2015). Kurarinone reduces the fluidity of outer and inner layers of bacterial membranes. An additional 8-lavandulyl substition on naringenin enables it to have more affinity with bacterial cellular membrane (Chen et al., 2005). The flavonoids with prenylation group at *C*-8, such as icariin, show strong estrogen receptor regulate activity (Zhou et al., 2020). The flavonoids with prenylation group at *C*-6, such as bavachin, can significantly inhibit melanin synthesis and tyrosinase activity. It inhibits the expression of tyrosinase and c-Jun N-terminal kinases, and the expression of several extracellular signal-regulated kinase mRNA in A375 cells, leading to inhibition of melanin synthesis (Wang et al., 2016).

Polyphenols are abundant in a diverse range of plant resources and show various bioactivities. They can significantly reduce oxidative damage, apoptosis, and neurodegeneration on nerve cell (Güzelad et al., 2021). Furthermore, polyphenols, including flavonoids and stilbenoids, show potent antioxidant activity, anti-tumor activity and immunomodulatory activity. Structurally diverse polyphenols can be considered as an interesting target for drug design and discovery (Ogut et al., 2022b). Prenyl side chain on polyphenols affects their pharmacological activities (Chen et al., 2014). These prenylated metabolites are presented in some edible plants, like mulberry leaf and *Citrus* genus of *Rutaceae* family (Marin and Manez, 2013). Compounds having the prenyl and geranyl side chains are more abundant than the other terpenoid side chains in nature (Brezani et al., 2018). This study showed that the length of prenyl side chain was important to anti-cancer cell proliferation activity. For oxyresveratrol, 6-*C*-geranyl oxyresveratrol showed a better anti-proliferation activity against human hepatocellular carcinomas cell $HepG_2$ than 6-*C*-iospentenyl oxyresveratrol. 4-*C*-isopentenyl piceatannol showed a better anti-proliferation activity against $HepG_2$ cell than 4-*C*-geranyl piceatannol. However, the effect of prenyl chain length on the activity of compounds remained unclear. Antioxidant activity is the most basic biological activity of prenylated phenolics (Chang et al., 2021). Both prenylated and geranylated phenolics show good antioxidant activities due to the hydroxyl in the aromatic ring. The prenyl side chain does not affect activity significantly. However, it can modify the solubility of compounds and eventually affect their reaction kinetics (Šmejkal et al., 2007). Different substitutions brought different cellular cytoprotective effects. 3, 3-Dimethylallyl group is the most common pattern. Xanthohumol is a hop-derived prenylated flavonoid which exerted substantial antiproliferative effects in colorectal cancer cell lines (IC_{50} ranging from 3.6 to 7.3 μM) (Ambrož et al., 2019). Geranylated flavonoids isolated from *P. tomentosa* and *M. alba* also show antiproliferative effects against human breast carcinoma cell MCF-7, human myeloma cell line U266 and Hela cell lines (IC_{50} less than 10 μM) (Šmejkal et al., 2010). $HepG_2$ cell was more sensitive to prenylated stilbenoids than MCF-7 cells. When the same concentration was used, the inhibitory effects of piceatannol, oxyresveratrol, resveratrol and

their derivatives against $HepG_2$ cell were higher than against MCF-7 cell. This indicated that $HepG_2$ cell was more sensitive to prenylated stilbenoids than MCF-7 cell. The same compound has different effects on different cells. Low-molecular citrus pectin can dose-dependently inhibit the proliferation of $HepG_2$ hepatocellular carcinoma cells and MCF-7 breast cancer cells. The half-inhibitory concentration of the low-molecular citrus pectin on $HepG_2$ cells is 1.46 mg/mL and the cell cycle is arrested in the S phase. The gene expressions of *CDK1* and *Cyclin A1* are downregulated. However, the half-inhibitory concentration on MCF-7 cells is 1.82 mg/mL and the cell cycle is arrested in the G2/M phase. The gene expressions of *CDK2* and *Cyclin B2* are downregulated (Wu et al., 2022). It is speculated that prenylated stilbenoids have



A

Fig. 2. The cell viability after 48 h (A) and 72 h (B) of treatment.

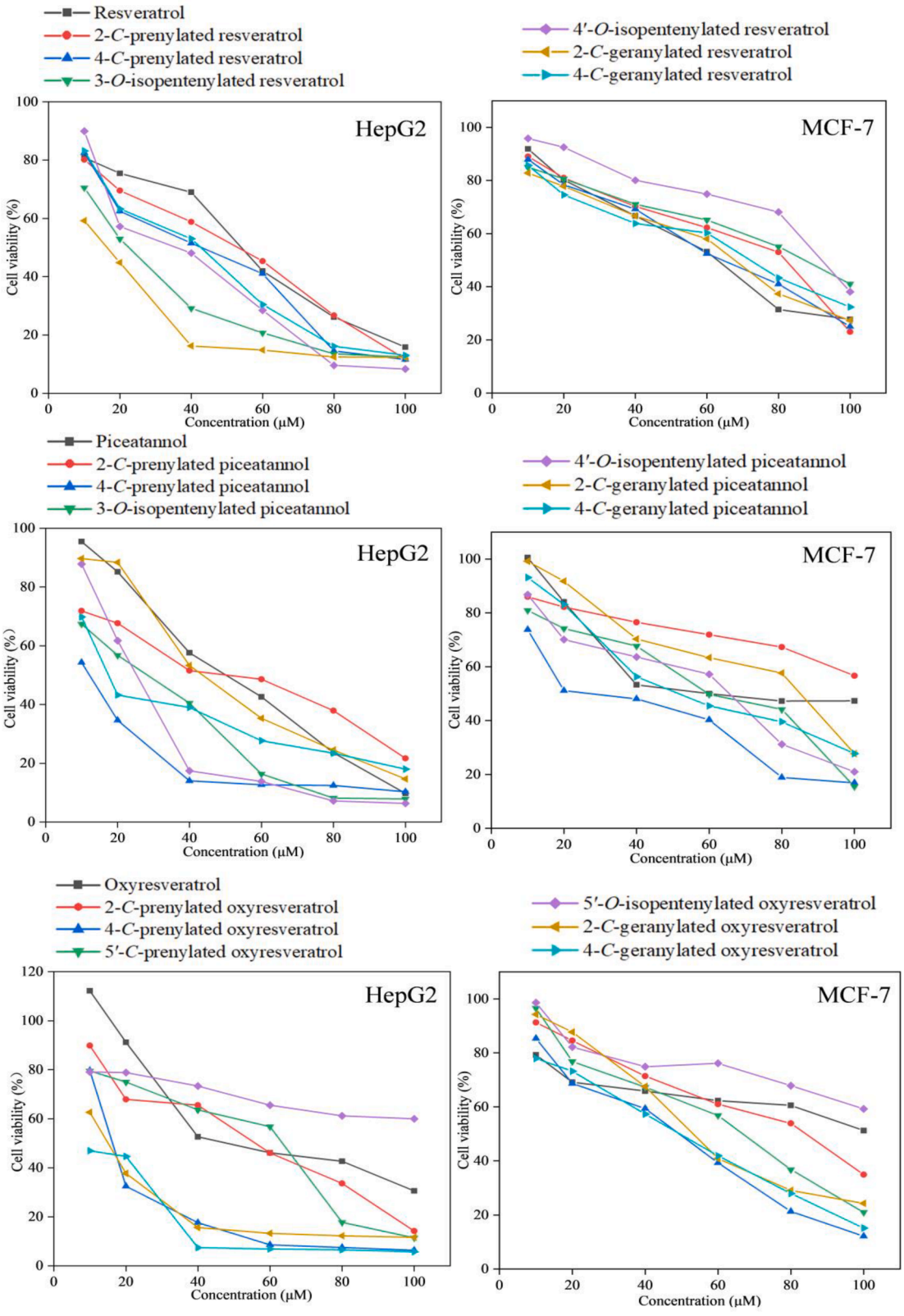


Fig. 2. (*continued*).

different regulation ability on apoptosis and cycle-related proteins of different cells, resulting in the different sensitivities of $HepG_2$ and MCF-7 cells to resveratrol.

Compared with the other compounds used in this work, 4-*C*-prenyl piceatannol and 4-*C*-prenyl oxyresveratrol showed better inhibitory effects against $HepG_2$ and MCF-7 proliferation. Previous studies have shown that $HepG_2$ cells exhibit more sensitive behaviour than MCF-7 cells. A 50% and 100% decrease in the viability of $HepG_2$ cell have

been obtained at 0.4 and 2 M linalool, respectively. However, the same inhibitory behaviour for MCF-7 is obtained at a higher concentration (Usta et al., 2009). As mentioned above, three prenylated patterns in this study could significantly affect the cytotoxicity. Prenylation on *C*-4 of stilbenoid displayed stronger cytotoxicity on $HepG_2$ than MCF-7.

3.2. Apoptosis mechanism

Cell viability test indicated that 4-*C*-prenyl piceatannol showed the most potent antiproliferative activity. Thus, 4-*C*-prenyl piceatannol was selected to explore the anti-cancer mechanism of prenylated stilbenoids. As shown in Fig. 2, 4-*C*-prenyl piceatannol induced the apoptosis of $HepG_2$ cells. Fig. 3 shows the cytomorphology of $HepG_2$ cells after 24 h of 4-*C*-prenyl piceatannol treatment. The morphology of treated $HepG_2$ cells changed from long spindle to round gradually. The cell shrank and the adhesion to wall was weakened.

Both piceatannol and resveratrol are naturally occurring stilbenoids. They possess good antioxidant activity. Polyphenolic compounds exhibit good biological activity due to their richness in phenolic hydroxyl groups. The brain is more sensitive to reactive oxygen species than other tissues because it consumes most oxygen during respiration, in addition to the high levels of polyunsaturated fatty acids contained in neural membrane phospholipids. All these reasons contribute to the brain's vulnerability to oxidative damage. Therefore, some polyphenols with significant free radical scavenging activity, such as syringic acid, show neuroprotective activity (Ogut et al., 2022a; Ogut et al., 2019). In addition to antioxidant activity, piceatannol also possesses apoptotic activity to cancer cell (Nayyab et al., 2020). Previous study has indicated that these potent cytotoxic effects are accompanied by induction of DNA damage, increase of the proportion of cells in the sub-G(1) phase of cell cycle, and inhibition of reactive oxygen species (ROS) generation (Jin et al., 2018). In this study, ROS release from $HepG_2$ cells treated with piceatannol and 4-*C*-prenyl piceatannol were measured (Fig. 4). The treatment led to a decrease of ROS release in a dose-dependent behaviour. 4-*C*-prenyl piceatannol has a better scavenging effect on ROS. N-acetyl-L-cysteine is a strong ROS scavenger. Previous study has shown that N-acetyl-L-cysteine can significantly inhibit piceatannol-induced apoptosis (Jin et al., 2018). It suggests that piceatannol-induced apoptosis might not occur via inhibition of ROS generation.

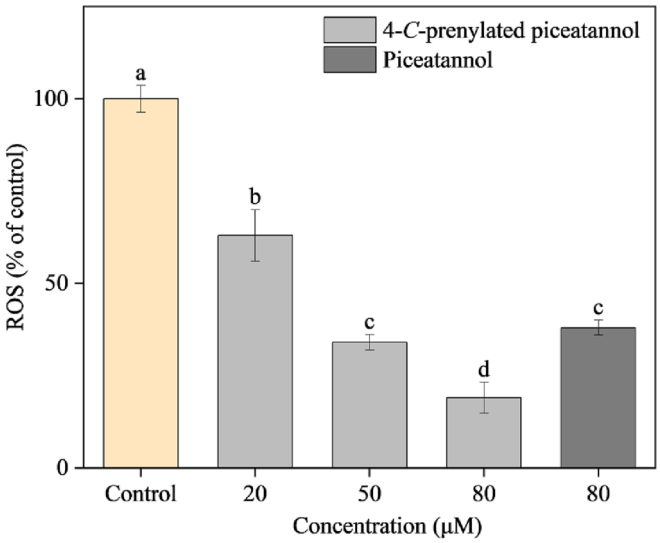


Fig. 4. Effects of 4-*C*-prenyl piceatannol and piceatannol on ROS generation in $HepG_2$ cell. Values with no letters in commom are significantly different (*p* less than 0.05).

In order to further explore the mechanism of 4-*C*-prenyl piceatannol induced apoptosis of $HepG_2$ cells, we determined the expression of apoptosis-related genes in $HepG_2$ cells. The mRNA expression levels of *Caspase-3*, *Caspase-9*, *CDK2* and *Cyclin A2* were measured. The results are shown in Fig. 5. 4-*C*-prenyl piceatannol could upregulate the expression of *Caspase-3*, *Caspase-9* and downregulate the expression of *CDK2* and *Cyclin A2* in $HepG_2$ cells. This might explain the apoptotic mechanism of 4-*C*-prenyl piceatannol.

Caspase is closely related to the apoptosis of eukaryotic cells. The apoptotic executioner *Caspase-3* can mediate the death receptor pathway, mitochondrial pathway and activate the other Caspase

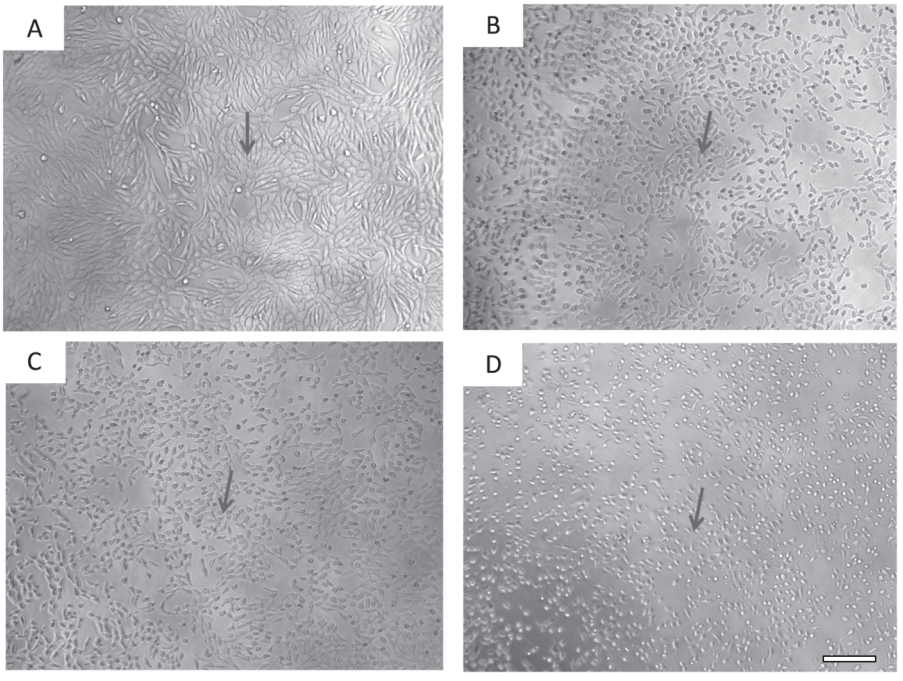


Fig. 3. Morphology of $HepG_2$ cells treated by 4-*C*-prenyl piceatannol for 24 h. Concentration: 0 μM (A), 20 μM (B), 50 μM (C), 80 μM (D).

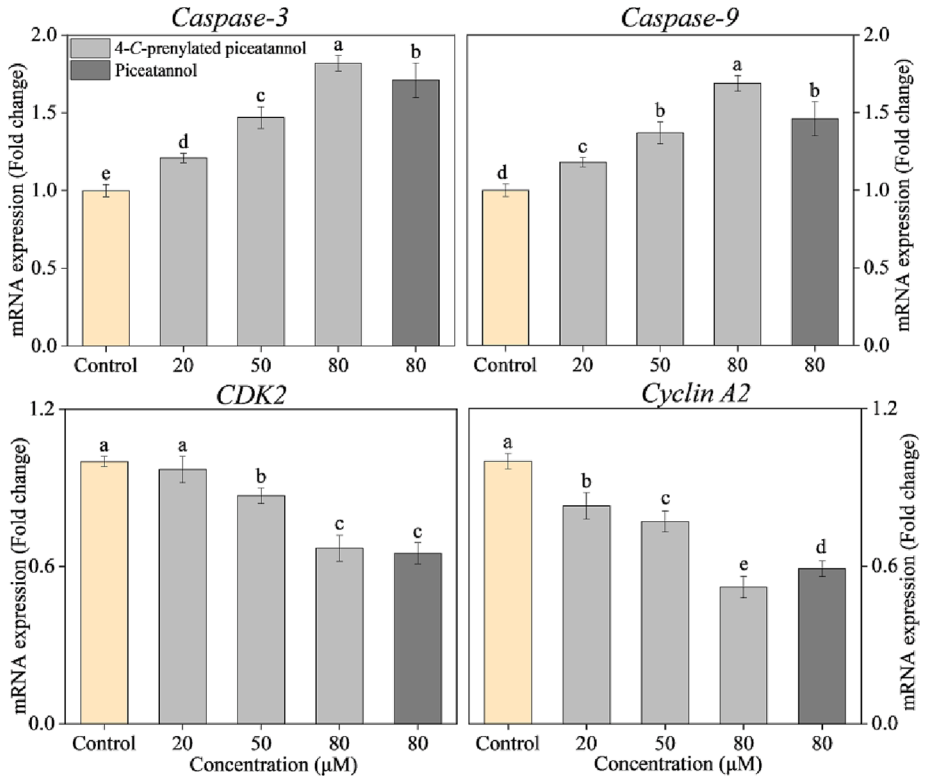


Fig. 5. Effects of 4-*C*-prenyl piceatannol and piceatannol on mRNA expression of genes related to apoptosis. The expression levels of mRNA were normalized to the vehicle control by using GAPDH as the house keeping gene. Values with no letters in commom are significantly different (*p* less than 0.05).

members, further inducing apoptosis (Ji et al., 2010). It is also an important component of cytotoxic T lymphocyte (CTL) killing mechanism (Adrain et al., 2005). Thus, prenylated piceatannol inhibits proliferation and induces apoptosis of $HepG_2$ cell. Caspase signaling pathway is highly involved in the apoptotic mechanism. *Caspase-9* is an important protein of the mitochondrial pathway that controls intracellular apoptosis. In the mitochondrial apoptotic pathway, cytochrome *C* is released from mitochondria to activate *Caspase-9*, then the active *Caspase-9* promote the enzymolysis of *Caspase-3* precursor to activate *Caspase-3*. *Caspase-3* is the major executioner Caspase. Both the mitochondrial pathway involved by *Caspase-9* and other non-mitochondrial pathways ultimately induce apoptosis through executioner Caspase (Li et al., 1997). Therefore, the mechanism of $HepG_2$ apoptosis induced by 4-*C*-prenyl piceatannol include upregulation of apoptosis-related factors such as *Caspase-3* and *Caspase-9*. However, apoptosis is regulated by many factors and is a signal transduction cascade.

Compounds that can regulate the cell cycle will affect the proliferation of cancer cells. Cyclin-dependent kinases (*CDK*s) that promote transition through the cell cycle are considered to be a key therapeutic target. *CDK*2 is one member of *CDK*s, which are relevant for DNA replication in higher eukaryotes. SNS-032 is a potent and selective inhibitor of *CDK*2. It can effectively kill chronic lymphocytic leukemia cells *in vitro* and induce apoptosis of tumor cells (Meng et al., 2013). Mitosis is thought to be triggered by the activation of *CDK*-cyclin complexes. *Cyclin A2* is a major regulator of cell cycle progression and its synthesis is necessary for progression to S phase. *Cyclin A2* is accumulated in somatic cells from the end of G1 phase and persists until metaphase of mitosis. It binds and activates *CDK2* in G1 and S phases (Gong et al., 2007). 4-*C*-prenyl piceatannol may affect cell proliferation and promote apoptosis by down-regulating *CDK*-cyclin complexes expression. Since apoptosis is a complex process, whether the other apoptotic factors are involved in 4-*C*-prenyl piceatannol-induced $HepG_2$ apoptosis needs further investigation.

3.3. *Affinity to apoptotis-related enzymes*

Apoptosis and the associated signaling proteins are linked to drug discovery in cancer. And sometimes this phenomenon induced by probiotic bacteria (Khodaii et al., 2022). Caspase-3 and Caspase-9 are apoptotic signaling proteins, which associate with cancer (Chen et al., 2008; Zhang et al., 1998). It is of interest to study the interaction of these two proteins with the drug candidate. These signaling proteins are linked in several cancers and drug resistance. Previous studies have shown that piceatannol shows anti-cancer activity which can inhibit tumor cell proliferation, induce apoptosis, and inhibit tumor cell invasion and migration. In this study, prenylated piceatannol (4-*C*-prenyl piceatannol) showed better anticancer acticity than piceatannol. Thus, the optimal binding features of apoptosis-associated signaling proteins *Caspase-3* and *Caspase-9* with 4-*C*-prenyl piceatannol were investigated. 4-*C*-prenyl piceatannol and receptor structure features is of great significance for the design of a more effective inhibitor.

The docking data of *Caspase-3* and *Caspase-9* with the 4-*C*-prenyl piceatannol are given in Table 2. Their MM-GBSA scores were −58.644 and −61.324 kcal/mol, respectively. The binding free energy of receptor to substrate is an important thermodynamic factor. The calculation of the free energy of the complex system, especially the absolute binding free energy, is of great significance for evaluating the binding ability of the enzyme to the substrate, predicting the binding of small molecules to the target and studying the enzyme reaction. Accurate free energy prediction can better understand the structure and function of biomolecules and contribute to rational drug design. A variety of calculation methods are proposed, among which MM-GBSA method is widely used for free energy calculation based on empirical equation. This method mainly uses kinetics sampling to decompose energy into van der Waals action energy under vacuum, electrostatic action energy, solvation energy and conformational change induced entropy change. This method has been widely applied to interaction between macromolecules and small molecules (Kollman et al., 2000).

Table 2
The molecular docking output of Caspase-3 and Caspase-9 with 4-*C*-prenyl piceatannol.

Protein name	MM-GBSA (kJ/mol)	Hydrogen bonds residues	Distance (Å)	π – Stacking residues	Distance (Å)	Hydrophobic interaction residues	Distance (Å)
Caspase-3	−58.644	Asp 2	3.16	Phe 256	5.30	Val 4	3.61
		Asp 2	2.80				
		Thr 62	3.20				
		Thr 62	2.87				
		Ser 251	3.14				
Caspase-9	−61.324	Arg 355	3.34	–	–	Arg 355	3.42
		Arg 355	2.86				
		Arg 355	3.33				
		Asp 356	2.64				

As mentioned above, prenylated piceatannol was found to exhibit anticancer activity by inducing apoptosis. The molecular docking assay of 4-*C*-prenyl piceatannol with apoptosis proteins indicated the presence of high affinity between them. The steric structure between proteins and 4-*C*-prenyl piceatannol are illustrated in Fig. 6. Table 2 provides data for hydrogen bonding between interacting residues with 4-*C*-prenyl piceatannol. The predicted active site residues in *Caspase-3* were Asp 2, Val 4, Thr 59, Gly 60, Met 61, Thr 62, His 121, Phe 128, Val 134, Trp 206, Ser 251 and Phe 256. Val 4 interacted with 4-*C*-prenyl piceatannol by hydrophobic interactions. Phe 256 interacted with 4-*C*-prenyl piceatannol by π-π stacking. Asp 2, Thr 62 and Ser 251 interacted with 4-*C*-prenyl piceatannol by hydrogen bonds. Asp 2 and Thr 62 formed a hydrogen bond with two hydroxyl groups on 4-*C*-prenyl piceatannol. Val 4, Met 61, Phe 128, Val 134, Trp 206, Phe 256 formed hydrophobic zones.

The predicted active site residues in *Caspase-9* were Arg180, Thr 181, His 237, Gln 285, Ser 353, Trp 354, Arg 355, Asp 356, Pro 357, Lys 358

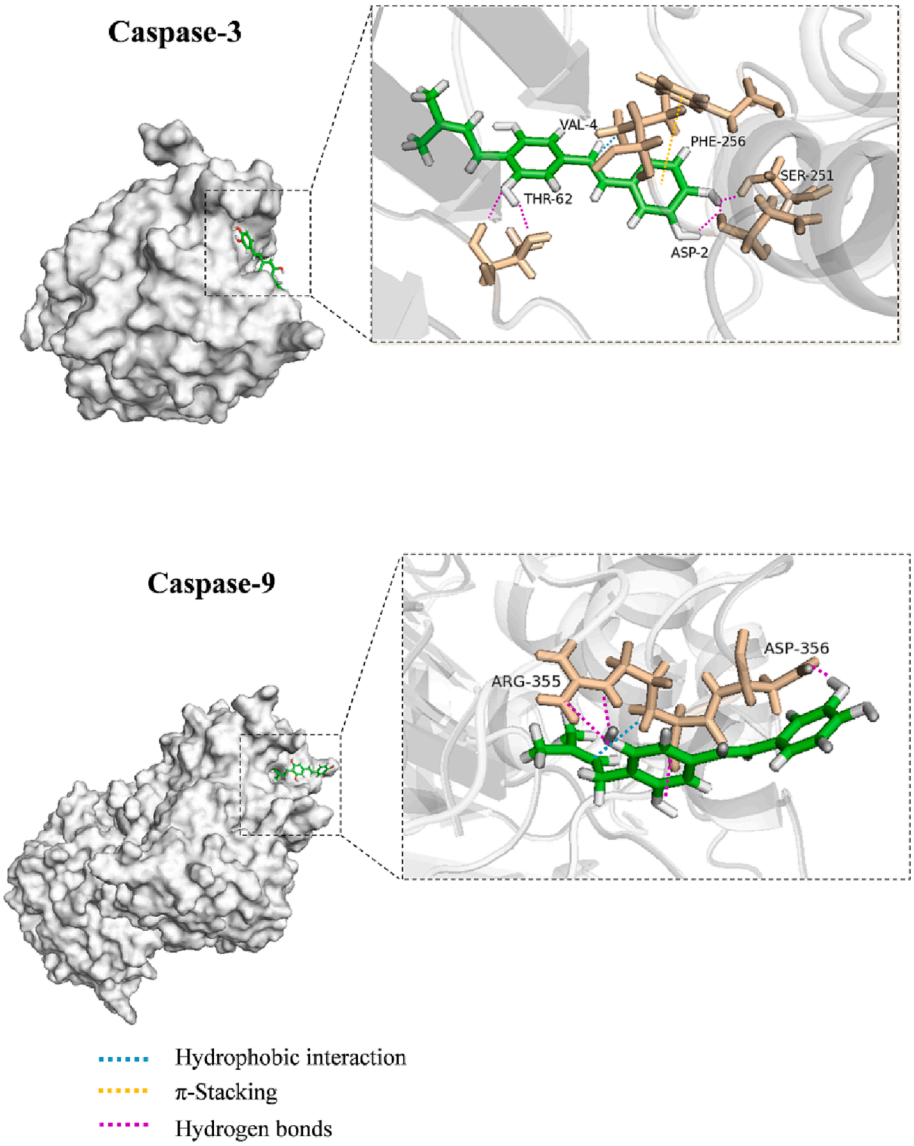


Fig. 6. The steric structure of Caspase-3 (A) and Caspase-9 (B) interacting with 4-*C*-prenyl piceatannol.

and Ser 361. Arg 355 interacted with 4-*C*-prenyl piceatannol by hydrophobic interactions and hydrogen bonds. As shown in the picture, Arg355 formed three hydrogen bonds with 4-*C*-prenyl piceatannol. Asp356 interacted with 4-*C*-prenyl piceatannol by hydrogen bonds.

Understanding the mode of action between compounds and proteins at the atomic level is important for the design of more effective drugs. The hydroxyl substitution on aromatic ring is important to anticancer activity. 4-*C*-prenyl piceatannol forms hydrogen bonds with proteins mainly with the hydroxyl and all the hydrogen bonds in the 4-angstrom range. This might be the reason why the anticancer activity of piceatannol is superior to resveratrol. The introduction of prenyl side chain improves the lipophilicity. The binding features provide evidences to regulate the activities of caspase-like proteins by 4-*C*-prenyl piceatannol. This could be another possible apoptotic mechanism for 4-*C*-prenyl piceatannol.

4. Conclusions

As mentioned above, the cancer cell proliferation inhibition activities of eighteen prenylated stilbenoids were investigated. Most of stilbenoids and their prenylated derivatives showed good anti-proliferation activities against $HepG_2$ and MCF-7 cells. A dose-dependent manner was observed. $HepG_2$ cell was more sensitive than MCF-7 cell to these prenylated stilbenoids. 4-*C*-prenyl piceatannol showed the highest cancer cell proliferation inhibition activity. Substitution site of the prenyl group has a greater effect on the proliferation inhibition activity than prenyl length. The 4-*C*-prenyl substitution had an important effect on the improved proliferation inhibition activity of stilbenoid. Upregulation of apoptosis-related proteins *Caspase-3* and *Caspase-9* was the possible apoptotic mechanism of 4-*C*-prenyl piceatannol.

CRediT authorship contribution statement

Ting Zhou: Formal analysis, Investigation, Writing – original draft. **Yueming Jiang:** Formal analysis. **Bin Zeng:** Formal analysis. **Bao Yang:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

No data was used for the research described in the article.

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