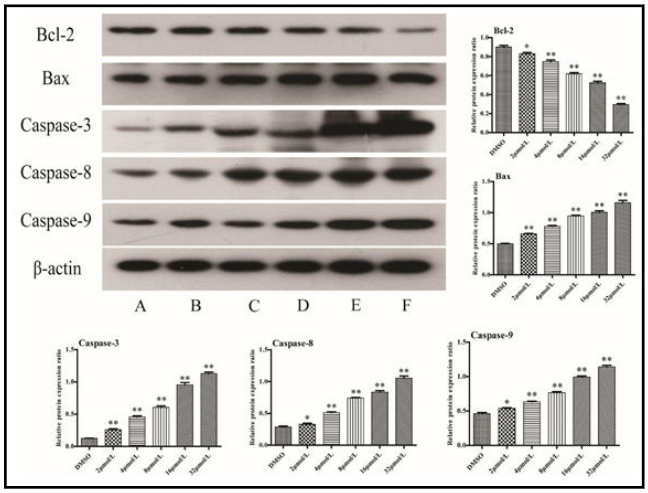

Figure 5: Effect of the Neo on the expression of Caspase-3, Caspase-8, and Caspase-9, Bax, and Bcl-2 in HepG2 cells cells *in Vitro*.

Western blotting analysis was used to detect protein expression levels of apoptotic markers on HepG2 cells, β-actin served as an internal control. A: DMSO, B: 2 μmol/L cos, C: 4 μmol/L cos, D: 8 μmol/L cos, E: 16 μmol/L cos, F: 32 μmol/L cos. Data was expressed as mean ± standard deviation from triplicate determinations. *P<0.05, ***P<0.01 *vs.* control.

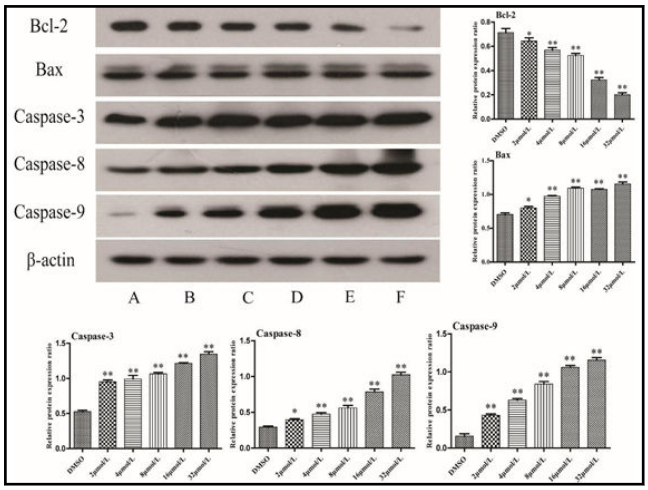

Figure 6: Effect of the Neo on the expression of Caspase-3, Caspase-8, and Caspase-9, Bax, and Bcl-2 in SMMC-7721 cells *in Vitro*.

Western blotting analysis was used to detect protein expression levels of apoptotic markers on SMMC-7721 cells, β-actin served as an internal control. A: DMSO, B: 2 μmol/L cos, C: 4 μmol/L cos, D: 8 μmol/L cos, E: 16 μmol/L cos, F: 32 μmol/L cos. Data was expressed as mean ± standard deviation from triplicate determinations.

DISCUSSION

The sesquiterpenes are widely distributed in nature. The monocyclic sesquiterpenoids are abundant in sesquiterpenes with many physiological activities including anti-inflammatory, antioxidant, anti-ulcer, cytotoxic activities and other physiological activities [12-14], among which anti-inflammatory and cytotoxic activities are the most prominent. There are many studies have shown that megastigmanes in different cancer cells have different cytotoxic activities [10] and Neo is a type of megastigmanes compound, in order to further verify the antitumor activity of Neo, the cytotoxic activities of Neo against HepG2 cells and SMMC-7721 cells *in vitro* was investigated. In this study, it was showed that Neo could inhibit the proliferation of HepG2 and SMMC-7721 cells and in a dose-dependent.

Carcinogenesis is a long and multistep process affected by many factors. The cell cycle is a primary factor which is a very sophisticated process and different compounds have different effects on the cell cycle, the G0/G1 and G2/M phases are critical points in this process. It was found that the volatile oil significantly inhibited the proliferation of hepatocellular carcinoma HepG2 cells and SMMC-7721 cells cultured *in vitro* may be due to the increase of G2/M phase and S phase, accompanied by the decrease of G_0/G_1 phase [15]. The effect of Rosiglitazone may be prevent the retention of hepatocellular carcinoma HepG2 cells in G_0/G_1 phase and inhibit the cell migration to G2/M phase to a certain extent [16]. The Drug-containing serum of *Clerodendrum Bunge* blocks hepatocellular carcinoma MHCC97-H cells from G2/M phase to G_0/G_1 phase and further reducing S phase, thereby interfering with DNA replication to inhibit cell proliferation [17]. Therefore, it is important to analyze the effect of substances on cell proliferation by detection for the cell cycle, our study found that the Neo could block the cell cycle arrest in G_0/G_1 phase, it indicated that Neo can block the cell cycle of both types of cells to some extent and it was a great significance for the study of anticancer mechanism.

Apoptosis is a physiological process of cells. In recent years, many studies have shown that the occurrence and development of tumors were related and may be caused by abnormalities in the process of cell apoptosis [18,19]. Bcl-2 proteins and Bax proteins belongs to Bcl-2 family and then were key factors in the process of cell apoptosis which were expressed abnormally in various tumors, and then have become one of the targets of anticancer drugs [20]. Cell apoptosis may be induced by inhibition of Bcl-2 or activation of Bax [21], the lower the ratio of Bcl-2/Bax and the higher mortality of the cell, therefore, the up-regulation of Bax protein expression or the down-regulation of Bcl-2 protein expression might be related to the apoptosis of various tumor cells including liver cancer [22]. In addition, the study indicated that there are two apoptotic pathways including the "intrinsic" cytochrome C/Caspase-9 pathway and the "extrinsic" Caspase-8 pathway [23], caspase family proteins were also play a key role of cell apoptosis. Caspase-9 as a promoter of caspase which were necessary for apoptosis signal through mitochondrial pathways and activated on apoptotic complex, and thus activate caspase-3 [24]. Caspase-8 was also as a promoter of caspase and thus activate caspase-3 eventually leads to cell apoptosis [25]. When Caspase-3 was activated by upstream pathway and as a key "killer" of cell apoptosis [26] and induces cascade reactions leading to cells apoptosis [27]. Research suggests that costunolide can activate caspase-3 to mediate apoptosis of esophageal cancer cells by up-regulation of Bax protein expression and down-regulation of Bcl-2 protein expression [28], breast cancer McF-7 cells apoptosis induced by curcumol through regulating the expression of Bcl-2 protein and Bax protein and down-regulating the ratio of Bcl-2/Bax [29], and many other studies have shown that inducing apoptosis was important for inhibiting the development of liver cancer [30]. Therefore, the study investigated the effect of Neo on the expression levels of the key regulators of apoptotic pathways in HepG2 cells and SMMC-7721 cells including Bax, Bcl-2, Caspase-3, Caspase-8 and Caspase-9. In this study, western blotting analysis showed that Neo induced apoptosis of HepG2 cells and SMMC-7721 cells by up-regulating the expression of Bax, Caspase-3, Caspase-8 and Caspase-9 proteins, and down-regulating the expression of Bcl-2 protein. The results of the study were consistent with those in the literature, and it was indicating that Neo promoted apoptosis in liver cancer cells and has significance for the study of anti-cancer mechanisms.

CONCLUSION

In conclusion, Neo showed inhibition effect on proliferation of HepG2 cells and SMMC-7721 cells *in vitro*. The survival rate of the two cells decreased and cell apoptosis rate increased in a dose-dependent and time-dependent way. The mechanism of antitumor effect may be related to cell cycle arresting in the G_0/G_1 phase. The up-regulating expression of Bax, Caspase-3, Caspase-8 and Caspase-9 proteins and down-regulating the expression of Bcl-2 proteins in both cells may also show the mechanism of cell apoptosis when the dose of Neo is increasing. This study suggested that Neo may be a promising candidate or major compound for the

development of liver cancer, providing data support for further animal and clinical trials.

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DATA AVAILABILITY STATEMENTS

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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