COREOPSIS TINCTORIA NUTT PROTECT HUMAN LIVER CELL FROM OXIDATIVE DAMAGE AND EXHIBIT ANTIOXIDANT CAPABILITY

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ABSTRACT

Background: The aim of the present study was to evaluate the antioxidant effect and the mechanism of total extract and two isolated compounds derived from *Coreopsis tinctoria Nutt.* on human hepatocyte LO2 injured by hydrogen peroxide *in vitro.*

Methods: The chemical compounds of *Coreopsis tinctoria Nutt*. were isolated and identified. The cell model with oxidative damage was set up by adding H_2O_2 in normal liver cell LO2. LO2 cells were pre-incubated with *Coreopsis tinctoria Nutt* compounds to test cell viability, nitric oxide release, caspase activity and apoptosis. The protective effect against oxidative damage were measured and the mechanism was analyzed by DPPH radical scavenging activity, ABTS+• radical-scavenging assay and reducing power (Fe3+) assay

Results: The study showed that incubation with *Coreopsis tinctoria Nutt* caused significant increase in the viability of LO2 cell, decrease of nitric oxide release, caspase activity and cell apoptosis in LO2 cell injured by hydrogen peroxide. The *Coreopsis tinctoria Nutt* and its two compounds showed antioxidant abilities.

Conclusions: *Coreopsis tinctoria Nutt* protect normal human liver cells against oxidative induced DNA damage and apoptosis by reducing nitric oxide release. One mechanism of protective effect may be radical-scavenging which is via donating hydrogen atom (H·), donating electron (e).

Keywords: *oxidative damage; Coreopsis tinctoria Nutt. antioxidant protection; liver cell LO2*

1. INTRODUCTION

Coreopsis tinctoria Nutt has been used to prevent cardiovascular disease (Sun YH, et al). Dias T, et al report it is also effect to control high glucose (Dias T, et al). But the medical function and mechanism of *Coreopsis tinctoria Nutt* have not been fully explored. This study investigates the potential antioxidant benefits of *Coreopsis tinctoria Nutt.* on human liver cells and investigate the possible molecular mechanism.

2. METHODS

2.1 Chemicals and plant compounds derived from plant extract

Flowers of *Coreopsis tinctoria Nutt.* were planted in Xinjiang (Figure 1A). Healthy flower were separated, shade dried, and pulverized mechanically (Figure 2). The crude extracts were qualitatively identified for separate chemical compounds to provide the chemical basis for further analysis (Figure 3). The isolation progress has been optimized to get the total flavonoid from *Coreopsis tinctoria Nutt* by ethanol extraction, column chromatography and crystallization by referring the methodology of other herb extraction (Dias T, et al). Trolox is a standard antioxidant purchased from Sigma, U.S.A. Total nitric oxide assay kit was purchased from Beyotime, Shanghai, China.

2.2 Cell culture

Human normal hepatocellular cell line LO2 was purchased from Chinese Academy of Science (Shanghai, China). Cells were grown in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 unit/mL), streptomycin (0.1 mg/mL), 1μg/ml glucose and 0.25U/ml insulin. The cells were incubated at 37 °C in a humidified incubator under an atmosphere of 5% CO2.

2.3 Oxidative damage induced by H_2O_2

LO2 cell was cultured in 96-well plates at a density of 2 × 105 cells/mL. The cells were treated with 100 μmol/L H_2O_2 for 24 h incubation except control group.

2.4 Measurement of cell viability

The cells were divided into control group, H_2O_2 group and high, medium and low concentrations (20, 50, and 100 μmol/L). The viability of LO2 cell was measured by a colorimetric MTT assay. After the cells were treated by H_2O_2 (100 μmol/L) and different concentrations of *Coreopsis tinctoria Nutt* and its compounds respectively for 24 h. 50 μL MTT solution (1 mg/mL) was added to each well and then incubated at 37 °C for 4 h. After medium was removed, the crystal dye was dissolved in 100 μL DMSO to read the optical density (OD) on ELISA micro-plate reader (BIO-RAD, USA) at 490 nm.

2.5 Measurement of nitric oxide release

When cells were in log phage, 100 μmol/L H_2O_2 or 20, 50, 100 μmol/L *Coreopsis tinctoria Nutt* and its compounds were added to LO2 cells. After being incubated for 24 h, cells were collected and washed twice with PBS. The cells were stained for apoptosis morphology observation. The supernatant was collected to measure nitric oxide release by using

nitric oxide assay kit.

2.6 Protective effect against DNA damage

Coreopsis tinctoria Nutt extracts were dissolved in methanol (5 mg/mL). Different concentration (0-100 μL) of mixture were then put into mini tubes. After evaporating, 400 μL of phosphate buffer (0.2 mol/L, pH 7.4) was added to the tube. 50 μL DNA sodium (10.0 mg/mL). 50 μL H2O2 (50 mmol/L), 50 μL FeCl3 (3.2 mmol/L) and 50 μL Na_2EDTA (1 mmol/L) were added. Then 50 μL of ascorbic acid (18 mmol/L) were added to initiate reaction in a water bath at 55 °C for 20 min, 250 μL of trichloroacetic acid (10g/100mL water) was then added to terminate reaction. The color was then developed by addition of 150 μL of TBA (2-thiobarbituric acid) (0.4 mol/L, in 1.25% NaOH aqueous solution) at 105 °C for 15 min. The tubes were cooled and absorbance was measured at 530 nm against the blank buffer as control. The percent of protection against DNA damage is expressed as follows: Protective effect % = (1 - A/A0) ×100

2.7 DPPH radical scavenging activity

Free radical scavenging activity of the extracts was carried based on the scavenging activity of stable DPPH. *Coreopsis tinctoria Nutt* extracts (5 mg/mL) were added to each tube and then were incubated at room temperature in dark room for 30 min. The scavenging activity on the DPPH radical was determined by measuring the absorbance at 519 nm with a spectrophotometer (Unico 2100, Shanghai, China). Radical scavenging activity was calculated using the formula: The DPPH• inhibition percentage was calculated as: Inhibition % = (1 - A/A0) ×100%, where A is the absorbance with samples, while A0 is the absorbance without samples.

2.8 ABTS+• radical-scavenging assay

The ABTS+• -scavenging activity was measured as described. The ABTS+• was generated by 0.35 mL ABTS diammonium salt (7.4 mmol/L) mixed with potassium 0.35 mL persuylfate (2.6 mmol/L). The solution was kept in the dark room for 12 h for radical generation, and then diluted with 1:50 ethanol. To determine the radical-scavenging activity, 1.2 mL ABTS+• reagent was diluted by 0.3 mL of *Coreopsis tinctoria Nutt* extracts (0.08-0.4 mg/mL). After incubation for 5 min, the absorbance at 734 nm was read on a spectrophotometer (Unico 2100, Shanghai, China). The percentage inhibition was calculated as: Inhibition % = (1 - A/A0) ×100% in which, A0 is the absorbance of the mixture without sample, A is the absorbance of the mixture with sample

2.9 Reducing power (Fe^{3+}) assay

Ferric (Fe^{3+}) reducing power was determined by mixing C*oreopsis tinctoria Nutt* extracts with $Na_2HPO4/KH2PO4$ buffer (0.2 mol/L, pH 6. 6) and 250 μL $K_3Fe(CN)6$ aqueous solution (1 g/100 mL) at 50 °C for 20 min. 250 μL of trichloroacetic acid (10 g/100 mL) t was added to the mixture and centrifuged at 3500 r/min for 10 min. The 400 μL supernatant was then aliquoted into 400 μL $FeCl_3$ (0.1 g/100 mL in distilled water) and incubated for 90 s. The absorbance was measure at 700 nm (Unico 2100, Shanghai, China). The relative reducing ability of the sample was calculated by using the formula： Relative reducing effect % = (A-Amin)/ (Amax-Amin) ×100% in which,

Amax is the maximum absorbance and Amin is the minimum absorbance in the test. A is the absorbance of sample.

2.10 Statistical analysis

Data are given as the mean ± SD of three measurements. The IC50 values were calculated by linear regression analysis. Significant differences were performed using one-way ANOVA ($p < 0.05$) with SPSS software (v.12, SPSS, USA)

3. RESULTS

3.1 Plant compounds derived from plant extract

Flowers of *Coreopsis tinctoria Nutt*. were planted in Dabancheng, Xinjiang (Figure 1A). Healthy flower were separated, shade dried, and pulverized mechanically. The crude extracts were qualitatively examined for chemical compounds to provide the chemical basis for development and utilization of this plant. The extraction progress has been optimized to get the total flavonoid from *Coreopsis tinctoria Nutt* by ethanol extraction, column chromatography and crystallization. The separate compounds from *Coreopsis tinctoria Nutt*. have been isolated and identified. The compounds include the total flavonoid (XJE) and two major compounds（XJ01 ,XJ02 with the highest production were applied in the antioxidative study (Figure 1B). *Coreopsis tinctoria Nutt*. were extracted with water under reflux. The extracted liquids were pooled and concentrated under vacuum. Then ethanol was added to the concentrate and filtered. The supernatant was concentrated under vacuum to get the crude extract (Figure 2). Quantitative analysis was carried out by HPLC on Shimadzu Prominence LC-20A liquid chromatographic system (Shimadzu instruments company, Japan) comprising binary pumps, a PDA detector and LC solution software (Figure3) with the operation condition reported (Eugene NO et al)

3.2 Protection Effect on H_2O_2 injured cell viability

H_2O_2 remarkably decreased the viability of cell, while XJE, XJ01 and XJ02 (20, 50, and 100 μM) relived the cell damage induced by H_2O_2 in a dose-dependent manner after 24 h treatment (Figure 4A). The result suggests that XJE, XJ01 and XJ02 protects LO2 cell from oxidative damage.

3.3 Effects of Coreopsis tinctoria Nutt on inhibiting nitric oxide release in H2O2 injured LO2 cell

Incubation of LO2 cell with H2O2 for 24 h caused a significant increase in nitric oxide release in the medium, while incubation of the cell with Coreopsis tinctoria Nutt (20, 50, and 100uM) significantly attenuated the increased nitric oxide level (Figure 4B).

3.4 Measurement of Antioxidant Activity

The antioxidant activity was analyzed using in vitro methods such as DPPH radical scavenging, ferric reducing antioxidant power activity, ABTS radical cation scavenging activity (Figure5).

Hydroxyl radical (•OH) is extreme reactive and easily damage DNA to produce malondialdehyde (MDA) and various oxidative lesions. MDA combines 2-thiobarbituric acid (TBA) to yield thiobarbituric acid reactive substances

(TBARS) which present a maximum absorbance at 530 nm. The absorbance of A530nm can reflect the amount of MDA and DNA damage. So in the study the decrease of A530nm value indicates a protective effect against DNA damage. As seen in Figure 5, *Coreopsis tinctoria Nutt* extracts showed a protective effect against DNA damage in a dose dependent manner and the IC50 values were significantly higher than positive control-trolox, a well known antioxidant agent.

The DPPH method is used for examining the general antioxidant activity because DPPH itself is a stable nitrogen-centered free radical. The color changes was caused by the formation of diphenyl picryl hydrazine, a stable diamagnetic molecule. Our results show that the DPPH radical scavenging activity of XJE, XJ01 and XJ02 was increased in a dose-dependent manner (Figure 5).

The Fe3+reducing assay provides fast reproducible results, measures the ability of an antioxidant to reduce Fe3+ to Fe2+), which is blue in color and can be detected. Fe3+reducing assay showed a dose-dependent manner.

Figure 5 also shows ABTS radical scavenging activity of XJE, XJ01 and XJ02 was increased in a dose-dependent manner. Figure 5 suggest that XJE, XJ01 and XJ02 exhibited appreciable antioxidant ability at all three test concentrations. The most substantial antioxidant capacity was observed at the highest concentration (100 Um).

4. DISCUSSION

People live under many degradative stresses, such as ROS and free radicals. These reactive species extensively cause the oxidative damage to the bimolecular nucleic acids, proteins, lipid damage and as a result oxidative stress related diseases such as cancer, ageing, heart failure, ulcer occur (Sanchez-Moreno C, et al).Many natural herbs are good antioxidants and possible protective agents against oxidative damage of the human body. In this study, the antioxidant activity of Coreopsis tinctoria Nutt extracts and its two compounds were investigated.

Photochemical studies suggested that total phenolics and total flavonoid can be responsible for the antioxidant ability in plants (Demo A); We then determined the total phenolics and total flavonoid contents in Coreopsis tinctoria Nutt extracts. The data showed that Coreopsis tinctoria Nutt extracts contained high amounts of total phenolics and flavonoid.

There are two pathways for natural phenolic antioxidant to protect DNA oxidative damage: one is to scavenge the •OH radicals so that its attack is reduced; the other is to repair the deoxynucleotide radical cations damaged by •OH radicals. We then tested the repaire function by measuring the DPPH· and ABTS+· radical-scavenging abilities.

The DPPH and ABTS+· radical-scavenging assays showed that Coreopsis tinctoria Nutt extracts can effectively eliminate DPPH• radical (Figure 5) and ABTS+·radical (Figure 5). Because a reductant is not necessarily an antioxidant while an antioxidant is commonly a reductant. We further tested the reducing power of Coreopsis tinctoria Nutt extracts as a significant indicator of its potential antioxidant activity. Figure 5 showed that Coreopsis tinctoria Nutt extracts exhibited reducing power on Fe3+. It indicate that the protective effect of Coreopsis tinctoria Nutt extracts against DNA oxidative damage was induced by their radical-scavenging

ability of donating hydrogen atom (H·) and electron (e).

5. CONCLUSION

Snow mountain herbal medicine Coreopsis tinctoria Nutt can effectively protect against hydroxyl-induced DNA damage. One mechanism of protective effect may be radical-scavenging which is via donating hydrogen atom (H·), donating electron (e). Its antioxidant ability can be mainly attributed to the chemical structure of flavonoid or total phenolics.

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Figure. 1. *Coreopsis tinctoria Nutt*

The flower of *Coreopsis tinctoria Nutt* grown in South Xinjiang China (Figure 1A) and the basic chemical structure of its major compounds (Figure 1B).

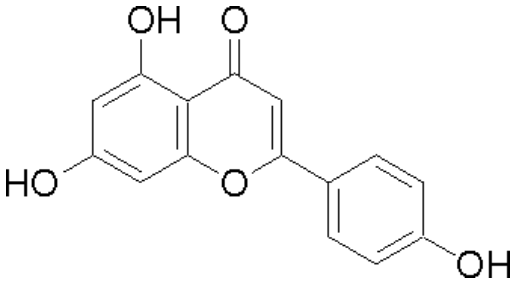

Figure. 2. *Coreopsis tinctoria Nutt* extraction

The flower of *Coreopsis tinctoria Nutt* were separated, shade dried, and pulverized mechanically and then processed by ethanol extraction, column chromatography and crystallization. *Coreopsis tinctoria Nutt*. were extracted with water under reflux. The extracted liquids were pooled and concentrated under vacuum. Then ethanol was added to the concentrate and filtered. The supernatant was concentrated under vacuum to get the crude extract.

Figure. 3. *Coreopsis tinctoria Nutt* separation and identification

The flower of Coreopsis tinctoria Nutt extract was separated and identified by quantitative high performance liquid chromatographic analysis. Quantitative analysis was carried out by HPLC on Shimadzu Prominence LC-20A liquid chromatographic system (Shimadzu instruments company, Japan) comprising binary pumps, a PDA detector and LC solution software.

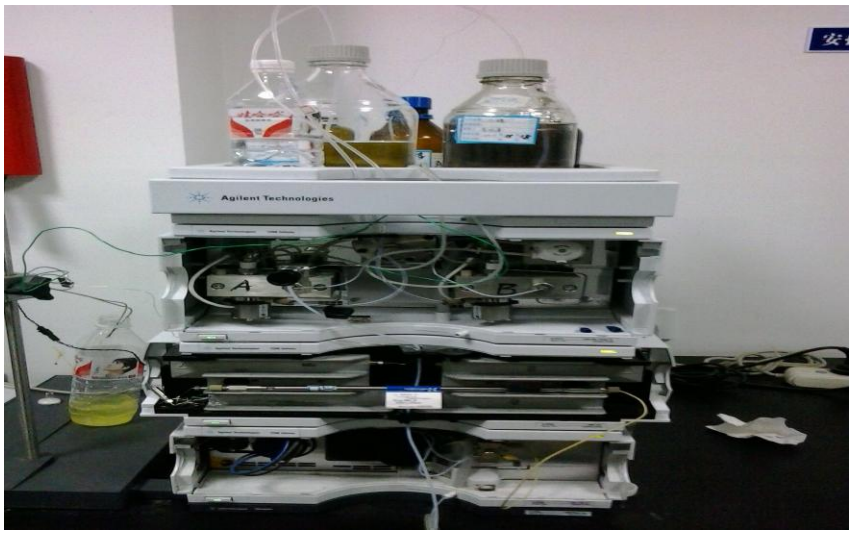

Figure. 4. Effect of *Coreopsis tinctoria Nutt.* on increasing cell viability in H_2O_2 injured LO2 cell by inhibiting NO release in the cells damaged by H_2O_2.

Incubation of LO2 cell with H_2O_2 for 24 h caused a significant increase in NO release in the medium, while the pre-incubation with *Coreopsis tinctoria Nutt* (20, 50, and 100uM) significantly attenuated the increased NO level (Figure 2B) and recover the cell viability (Figure 2A). Results were shown as mean ± SD (n = 8). *P < 0.05, compared with control.

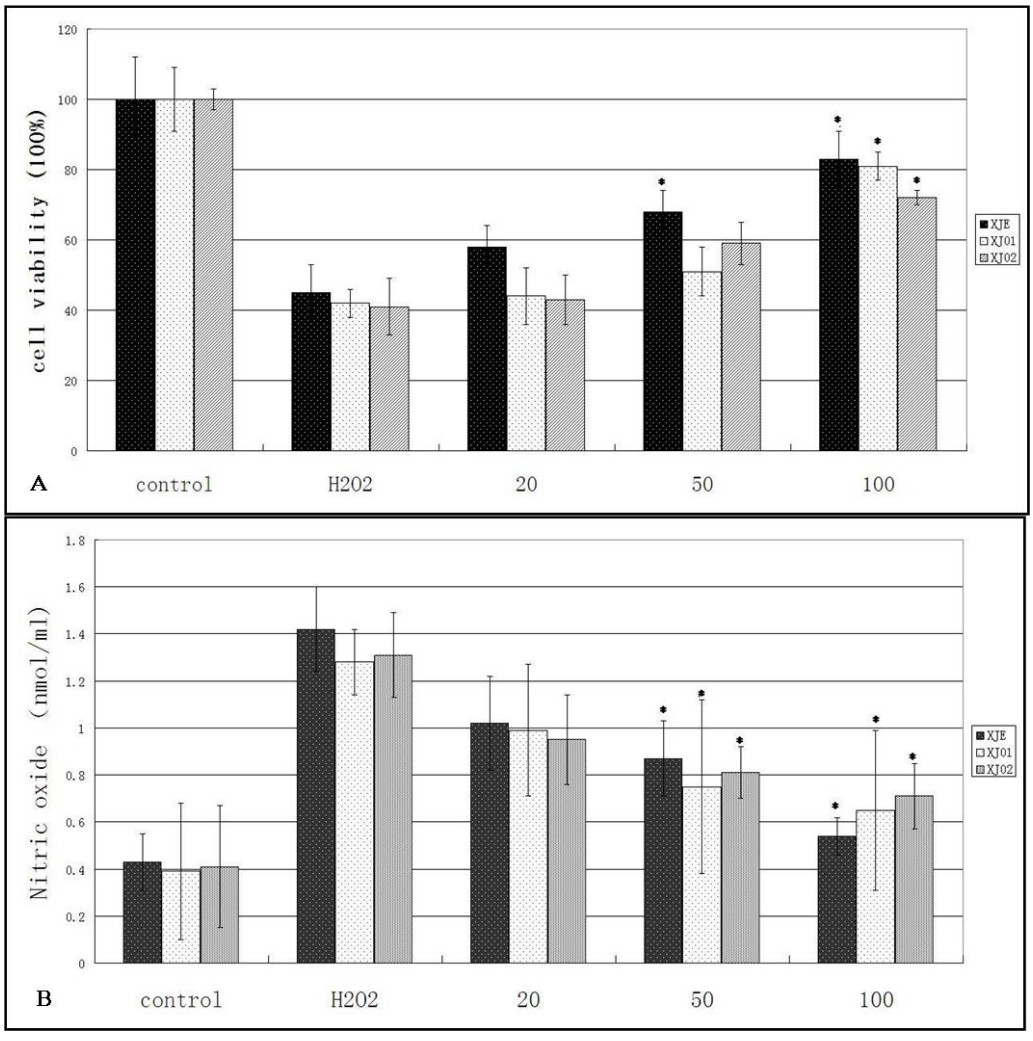

Figure 5 Antioxidant activity of *Coreopsis tinctoria Nutt* in H_2O_2 injured LO2 cell

The test of antioxidant assays include (A) protective effect on DNA damage; (B) DPPH· scavenging; (C) ABTS+· scavenging (D) Fe^{3+}-reducing; Trolox was used as the positive controls. Each value is expressed as Mean±SD (n=3). IC50 value was defined as the concentration of 50% effect percentage and expressed as Mean±SD (n=3).