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Full Length Article

Effective Composition Extraction and Antioxidant Activity of *Dioscorea nipponica*

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Abstract

Dioscorea nipponica Makino is well known and applied as folk medicine for bronchitis and rheumatoid. However, minimal research is available regarding its antioxidant capacity. To better understand antioxidant activity of *D. nipponica*, petroleum ether, ethyl acetate and n-butyl alcohol were used as solvent extraction and antioxidant activity was evaluated by DPPH, super-oxide anion and hydroxyl radical scavenging assays combined with online HPLC-ABTS(+) system. The mechanism of antioxidant was performed by analyzing some genes expression of zebrafish embryos after treated with ethyl acetate extraction. Our results indicated that the extraction of ethyl acetate and n-butyl alcohol showed better antioxidant activity and many antioxidant compounds of ethyl acetate were obtained. Extraction from ethyl acetate decreased the expression of p53, p21 genes and increased that of TERT gene while mdm2 gene expression exhibited a little change. These results proved that *D. nipponica* is effective in prevention and treatment of cardiovascular disease. © 2018 Friends Science Publishers

Keywords: Antioxidant; *Dioscorea nipponica*; Effective composition; Free radicals scavenging; Gene expression

Introduction

Now-a-days, the humans are being encountered by a number of unprecedented diseases, which probably will continue in the future. From the later stage of 20th century, significant changes have taken place in human disease spectrum. Main diseases affecting human health are transformed from infectious diseases into cardiovascular and cerebrovascular diseases, malignancies and diabetes and other non-communicable diseases (Nakatani, 2016). As we all know, the main reason for the aging of the human body is the decreasing function of remove free radicals in the body with age. Reactive oxygen species (ROS) such as superoxide anion, hydroxyl radicals and hydrogen peroxide are continuously produced as a by-product of mitochondrial metabolism in metabolic pathways (Panth *et al.*, 2016; Jetly *et al.*, 2017; Wiens *et al.*, 2017). The ROS damage proteins, DNA and other biological macromolecules through the redox reaction leading to protein denaturation, cross-linking, loss of enzyme activity, gene mutation and decreased immune function, which bring about approximately 85% of human diseases such as a cerebrovascular disease, liver injury and cancer (Galant *et al.*, 2017; Wang *et al.*, 2017).

Compared with currently available therapeutic options, natural products are becoming popular over the world and widely accepted as conservative clinical therapy. Much public awareness and scientific interest has encourage to explore towards the effect of natural products in the field of health prevention and disease treatment due to their effectiveness, convenience, less side-effects and relatively low cost (Tundis *et al.*, 2010; Brown *et al.*, 2016). So far, more and more studies have shown that natural antioxidants can effectively remove free radicals to inhibit lipoprotein oxidative and prevent lipid peroxidation and atherogenic dyslipidemia which imply that natural antioxidants have a wide range of development and utilization prospects (Orekhov *et al.*, 2013; Zhang *et al.*, 2017). The extra intake of antioxidants is an inevitable way to remove harmful free radicals. There is an urgent need for finding out the clinical medicines especially for those natural anti-oxidants which are valuable in the treatment of diseases.

Discorea nipponica Makino belongs to Dioscoreaceae growing in mountainous areas of the Chinese Changbai Mountain or Korean peninsula and applied in analgesia, anti-inflammation, relaxing muscles and tendons, relieving cough, preventing asthma and others revealed by modern pharmacological research. It is regarded as a source for the sterodial sapogein diosagenin with a variety of bioactivities which make it having considerable application prospect. Due to these therapeutic properties, some wild *D. nipponica* resources have become endangered. And some wild species have been tamed to cultivated ones to meet the medical demands for medicine. Previous studies indicated that in order to get high yield and content of saponin, the best

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growth years for cultivated species of *D. nipponica* is three-year old and optimum harvest stages is in the late of September (Zhou *et al.*, 2016). Currently, limited data was elucidated that extrac of *D. nipponica* has the antioxidant property *in vitro*. In this study, HPLC-ABTS(·+) screening system was used to identified antioxidant components of different organic solvent from *D. nipponica* on basis of traditional extraction and isolation of natural antioxidant compounds after exploring anti-oxidative ability of different solvent extracts *in vitro*. Then the antioxidant activity mechanism of ethyl acetate extractions were analyzed in zebrafish embryonic cells. The results will contribute to further study on composition purification of antioxidant from *D. nipponica*.

Materials and Methods

Plant Material and Animals

All the cultivated *D. nipponica* samples were collected from the experimental field in Tonghua Horticultural Institute and identified by Professor Qin Jiamei, School of Life Science, TongHua Normal University. 3-year-old roots were collected and dried on natural shade, then smashed by Chinese medicine grinder (Q-500B2). Separated the samples in bag after over 100 mesh. Zebrafish were raising in standard fish facility conditions including a 14:10 h light/dark cycle, retaining the temperature of water at 28°C, feeding with living brine shrimp twice every day. Embryos at 8 h post-fertilization (hpf) were placed into a 24-well microplate (Millipore Co., Bedford, MA, USA).

Analytical Methods

Extraction and isolation: The dried plant root powder of *D. nipponica* (100 g) was extracted with 60% ethanol after smash (1 g powder/30 mL solvent) for three times (2, 1.5 and 1 h, respectively). Through cooling and filteration, solvent in the filtrates were recovered to obtain the ethanol dry extract; then extracted three times with petroleum ether, ethyl acetate and n-butyl alcohol. The extracts of each solvent were respectively combined and filtered to obtain petroleum ether, ethyl acetate and n-butyl alcohol extract. The extracts from each solvent obtained were dried in a fume hood and stored under room temperature until further use.

***In vitro* antioxidant assay:** The extracts of petroleum ether, ethyl acetate and *n*-butanol were prepared in five different concentrations for test, respectively, 2.5, 5, 7.5, 10 and 12.5 mg/mL. Using the clearance rate as the evaluation index, DPPH, superoxide anion and hydroxyl radical scavenging assays were performed to compare the antioxidant properties of the obtained fractions. The DPPH radical scavenging activity was evaluated using method previously described with modification (Kapewangolo *et al.*, 2013; Cheng *et al.*, 2016). Various concentrations of different solvent extracts were mixed with 90 μM DPPH. Since DPPH is light sensitive, incubation was done in the dark at room temperature for 30 min. The absorbance of the resulting solution was measured using a plate reader at 520 nm. Vitamin C was used as a positive control.

The scavenging rate of superoxide anion was determined by pyrogallol autoxidation method (Semsei and Zs-Nagy, 1985; Li *et al.*, 2012; Wang *et al.*, 2016). The reaction mixture consisted of Tris-HCl buffer solution, pyrogallol autoxidation, HCl and various concentrations of extracts. The reaction mixture was incubated at 37°C for 10 min in a water bath and then added with 37°C preheated pyrogallol solution except tube for regulating zero without extracts. After pyrogallol complex for 4 min reaction was terminated in HCl (0.01mol/L), the reaction mixture was measured spectrophotometrically at 352 nm.

The scavenging activity of sample extracts on hydroxyl radicals scavenging was measured by $FeSO_4$-salicylic acid method according to the method (Smirnoff and Cumbes, 1989; Li *et al.*, 2012). The reaction mixture consisted of $FeSO_4$, hydrogen peroxide, sodium salicylate and various concentrations of extracts. The reaction mixture was incubated at 37°C for 1h in a water bath. After incubation the absence of the hydroxylated salicylate complex was measured spectrophotometrically at 562 nm. The percentage of free radical scavenging activity was calculated by the following formula:

$$\text{Scavenging activity (\%)} = [1 - (A_1 - A_2)/A_0] \times 100$$

Where A_0 was absorbance of the control without extracts, A_1 was the absorbance in the presence of the extract, and A_2 was the absorbance without developer.

HPLC-ABTS(·+) analysis of the extracts: The antioxidant activity of individual compounds was monitored by the Agilent 1,200 HPLC-DAD equipment. The extract was separated with a ZorbaxSBC 18 column (250 mm×4.6 mm internal diameter 5 μm, Agilent, America), which was kept at 40°C. The mobile phase consisted of formic acid and acetonitrile. The elution gradient is shown in Table 1. The flow rate was maintained at 1 mL/min and then the elution was linked to a reaction coil (15 m×0.25 mm), which was dominated by a temperature controller (Waters Corporation, USA). At the same time, ABTS ·+ solution described previously was transported via a pump (Waters Corporation, USA) to the reaction coil at 0.5 mL/min. In the reaction coil (40°C), each fraction in sequence was reacted with the working solution of ABTS·+. Finally, the reaction mixture was delivered into the DAD recording at 280 nm for positive peaks and 734 nm for negative peaks. Agilent Chemistation Software was used for analyzing data (Lee *et al.*, 2014; Le Grandois *et al.*, 2017; Wang *et al.*, 2017).

In Vivo Antioxidant Assay

According to the *in vitro* antioxidant and HPLC-ABTS(·+) screening results of different organic solvent extracts of *D. nipponica*, embryos cell developed for 8 h were incubated in

different concentrations of ethyl acetate extracts solution, which was respectively 0, 7.5 and 12.5 mg/mL and kept in the dark for 24 h at 28°C. Then, embryos cells were rinsed thoroughly 8 times with egg water. Stained embryos were anesthetized with MESAB (0.5 m*M* 3-aminobenzoic acid ethyl ester, 2 m*M* Na2HPO4) and mounted in a depression slide for observation using methylcellulose. By using a fluorescence microscope, they were visualized and images were captured for <60 sec. In addition, expression profiling of genes that related to the *p53* signaling pathway was detected by semi-quantitative RT-PCR and agarose gel electrophoresis (Geng *et al.*, 2011).

Total RNA was extracted from embryos using the TRIzol reagent. Semi-quantitative PCR was performed with an initial incubation for 5 min at 94°C, followed by 22 cycles of incubation at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. The primers for amplification of *p53* and *p21* were the same including CTCTCCCACCAACATCCACT/ACGTCCACCACCATT TGAAC for p53 forward/reverse and CGGAATAAA CGGTGTCGTCT/CGCAAACAGACCAACATCAC for p21 forward/reverse. The primers forward/reverse for amplification of *mdm2* was GACTACTGGAAGTGTCCCAAAT/GTCCACTCCAT CATCTGTTTCT and *TERT* forward/reverse was. GTGTGTGTGTCCTGGGTAAA/CAGCCTGAGGTCT AA GAAGATG Subsequently, PCR products were loaded into gels for agarose gel electrophoresis (Robu *et al.*, 2007).

Statistical Analysis

Statistical analyses in figures expressed as mean significant difference (SD) and differences between groups were assessed by analysis of variance (ANOVA). Differences were considered to be statistically significant if $p < 0.05$. All statistical analyses were carried out using SPSS for Windows, Version 11.5 (SPSS, Chicago, IL).

Results

Effects on DPPH Scavenging

DPPH is 1,1-Diphenyl-2-picrylhydrazyl as a monitoring reaction material in the chemical reaction of antioxidant properties of material for the position and intensity of the electron paramagnetic resonance signal. It is a stable free radical with purple color. Results showed that *n*-butanol and ethyl acetate extract have significant scavenging effect on DPPH. With an increase in *n*-butanol concentration, DPPH clearance rate was enhanced and reached the highest. Differences were considered significant between 2.5 to 5.0 mg/mL for DPPH clearance rate ($p<0.05$). DPPH clearance rate of ethyl acetate extract decreased with the increase of concentration. The removal rate of DPPH was 93.38% at 2.5 mg/mL, but there was no significant difference (Fig. 1).

Table 1: The elution gradient of HPLC

Time (min)	0.05% FA in water (%)	ACN (%)	Flow (mL/min)
0	95	5	1
30	50	50	1
35	0	100	1
40	0	100	1
40.1	95	5	1
45	95	5	1

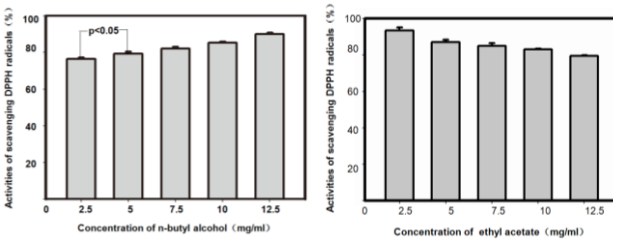

Fig. 1: Antioxidant activities of *n*-butanol and ethyl acetate extractions were determined by the DPPH assay. DPPH scavenging was measured at different concentration (2.5, 5.0, 7.5, 10.0 and 12.5 mg/mL) of *n*-butanol and ethyl acetate extractions. Value are mean (n=3) ± SD

Effects on Superoxide Anion Radical Scavenging

The $O_2^{\cdot-}$ as the main cause of oxygen poisoning, $O_2^{\cdot-}$makes polysaccharide depolymerization, nucleic acid chain destruction and unsaturated fatty acid peroxide changes such as enzyme failure, membrane damage, genetic mutation and mitochondrial oxidation. In a certain range of concentration, the $O_2^{\cdot-}$ clearance rate went up with the increase of the concentration. At 12.5 mg/mL, the influence of *n*-butanol and ethyl acetate extracts on clearance rate reached the highest level, respectively. As shown in Fig. 2, *n*-butanol extracts under the concentration from 2.5 to 10 mg/mL had a significant level of $O_2^{\cdot-}$ clearance ($p<0.01$). Due to very low scavenging activity of petroleum ether extract on the superoxide anion, there is no data statistics.

Effects on Hydroxyl Radical Scavenging

Hydroxyl radicals (-OH) are recognized as the most active species in biological systems and can lead to oxidative damage to DNA, proteins and fats *in vivo*. For the organism, hydroxyl radicals possess the traits including the most active, toxic and harmful, which can react with any molecule in living cells at extreme speed leading to free radical chain reaction in the body and damage to the organism in a large scale. From Fig. 3, it is indicated that *n*-butanol and ethyl acetate extracts have significant scavenging effect on hydroxyl radicals and show a certain dose effect that the ability to remove hydroxyl radicals increases with increasing concentration of the extract between 2.5 and 12.5 mg/mL. The petroleum ether extract had low scavenging activity of hydroxyl radicals, and the data were not listed because there was almost no difference dispose.

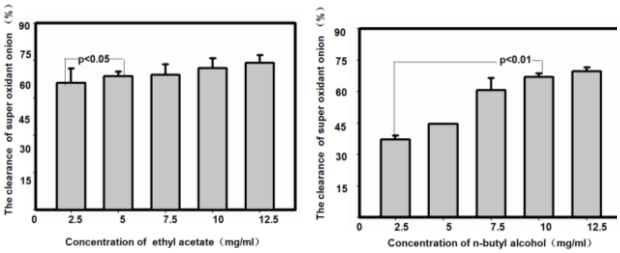

Fig. 2: influence of different organic solvent extracts of *D. nipponica* on the clearance of superoxide anion radical. Superoxide anion radical scavenging was measured at different concentration (2.5,5,7.5,10 and 12.5 mg/mL) of *n*-butanol and ethyl acetate extractions. Value are mean (n=3) ± SD

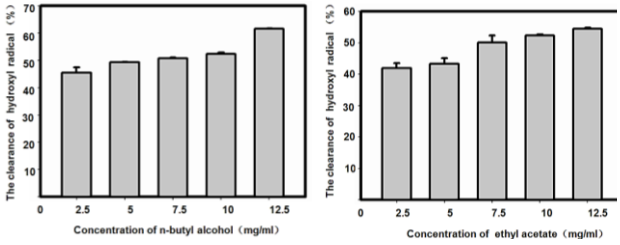

Fig. 3: Free radical scavenging activity of *n*-butanol and ethyl acetate extracts were assayed by hydroxyl radicals. Hydroxyl radical scavenging was measured at different concentration (2.5,5,7.5,10 and 12.5 mg/mL) of *n*-butanol and ethyl acetate extractions. Value are mean (n=3) ± SD

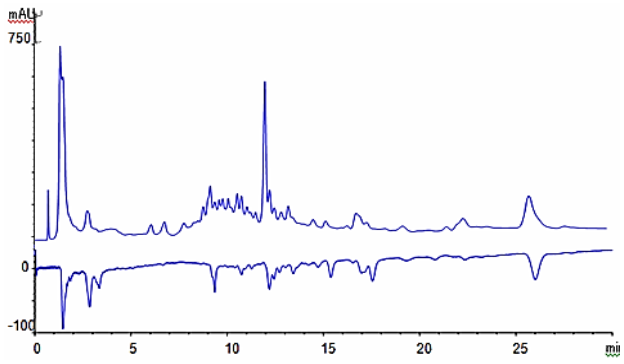

Fig. 4: Online HPLC-ABTS (·+) assay of ethyl acetate extraction from *D. nipponica*

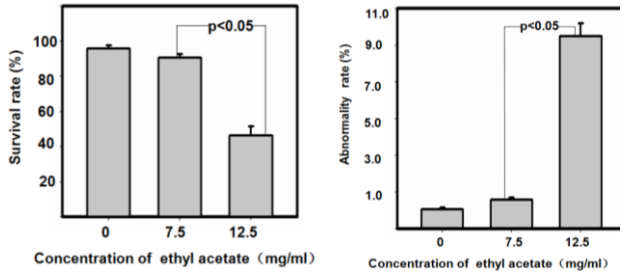

Fig. 5: The percentage of survival and abnormality rate of zebrafish embryos after treated with different concentration of ethyl acetate extracts at 24 h post-fertilization (hpf). Data are presented as mean ± SD (n=30)

Phytochemical Analysis of Antioxidant Activity

The antioxidant activity of petroleum ether, *n*-butanol and ethyl acetate extracts was studied by HPLC-ABTS + antioxidant activity screening system. The system was characterized by combination of HPLC and detector of free radical scavenging system ABTS +, which detect the positive peak value of various separated components from samples by HPLC system. After HPLC separation, the components enter the reaction cell and then trigger ABTS+• reduction followed by negative peak. As shown in the chromatogram (Fig. 4), a large amount of antioxidant active components were found in the ethyl acetate extract and the negative peaks were observed in the ABTS + detection corresponding to the positive peak of separated components from the extracts. However, for petroleum ether and *n*-butanol extracts, no negative peaks were observed.

Ethyl Acetate Extract Effect on Zebrafish Embryonic Cells Growth and Development

In view of the results of antioxidant experiment *in vitro* and HPLC-ABTS (·+) analysis, the dose of ethyl acetate extract that impact on embryonic cells was determined by incubating embryos in different concentrations of ethyl acetate extract, and then analyzed the effect of each treatment by survival rate and abnormality rate of zebrafish cell after 24 h administration (Fig. 5 and 6). The results indicated that lower concentration had little effect on growth and development of zebrafish, however, the survival and the mortality of zebrafish was significantly affected by the higher concentration group at the concentration of 12.5 mg/mL. In addition, the zebrafish cell abnormality rate also significantly increased which may lead to the main cause of zebrafish lethal.

Ethyl Acetate Extract Effects on Aging Related Genes Expression of Zebrafish Embryos

The *p53-p21* is an important senescence-related signaling pathway, and the transcriptional level of *p21* is activated by *p53*, which mainly mediated by telomere-dependent and various stress conditions such as DNA damage caused by aging. Some studies found that natural effective ingredients can activate *ERK* via the *p53* signaling pathway in MCF-7 breast cancer cells (Xia *et al.*, 2014). There is also the hypothesis that different types of intrinsic and extrinsic stress signals likely converge on the activation of the *p53* protein, the Rb protein, or both during senescence (Herrera and Jagadeeswaran, 2004). In this context, we examined whether zebrafish share similar mechanisms for the response to ethyl acetate extract of *D. nipponica*.

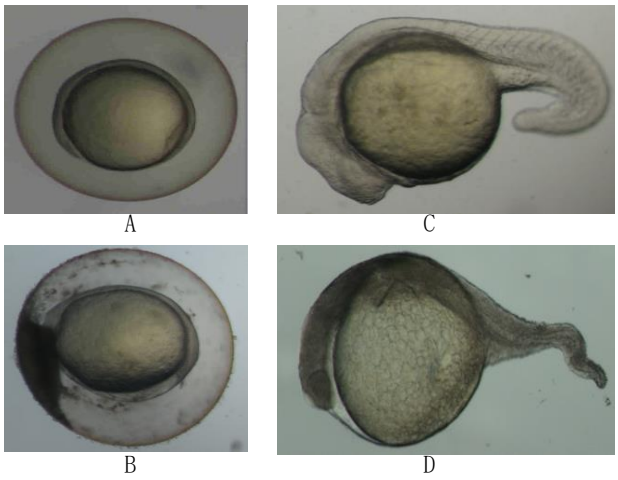

Fig. 6: Phenotypes detected after 12 and 24 (hpf). A and C non-treated embryos, B and D treated with 12.5 mg/ml of ethyl acetate extraction from *D. nipponica*

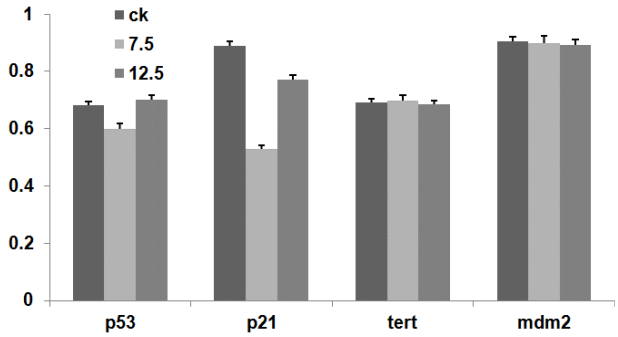

Fig. 7: The influence of different concentration of ethyl acetate extract on aging related genes expression of zebrafish embryos. Data are presented as mean ± SD (n=30)

To further investigate *p53*-dependent transcriptional responses in the treated embryos, we examined the expression of *p53*, along with that of response genes such as *p21*, *mdm2*, using semi-quantitative RT-PCR and the *β*-actin gene as a control of baseline expression. Compared to 0 mg/mL groups, the results showed that the expression level of *p53*, *p21* were decreased 7.83 and 34.08%, respectively compared to control (Fig. 7), while the expression levels of *mdm2* gene was no significant change and the expression of telomerase reverse transcriptase gene that allows to maintain the telomere ends was increased (Ben-Porath and Weinberg, 2004; Jaskelioff *et al.*, 2011; Concetti *et al.*, 2013).

Discussion

Antioxidant determination *in vitro* is an important method to evaluate antioxidant in food and pharmaceutical fields due to its simple anti-oxidation detection system, rapid operation and low cost, which is increasingly widespread. The antioxidant activity of natural plant is usually selected by the following steps including the extraction, separation and identification of various components, and then the antioxidant activity of each component were determined. HPLC-ABTS (+) system is mainly formed by high performance liquid chromatography with efficient separation ability and ABTS + • reagents. In separation process, the separated components can be detected whether it has antioxidant activity to ensure the selected components for purification and structural identification on purpose, which can improve the screening efficiency of the antioxidant activity. In view of the high efficiency and accuracy of online screening for antioxidant active substances, a large number of researchers have recently began to use the principle of HPLC-ABTS(·+) system to screen natural antioxidant active ingredients (Xu *et al.*, 2015; Obata and Nakashima, 2016).

Most of higher organisms are developed from single cell zygote and life in the embryonic period is sensitive to exogenous studied by many scholars for its good monitoring significance (Sun and Liu, 2017). Not only does zebrafish grow under *in vitro* condition after external fertilization, but embryo body show transparent, which can be directly observed for its growth and development under the microscope. As a tumor suppressor gene, *p53* gene is characterized by a variety of significant functions, which play a role in transcriptional activation and regulate a series of target gene transcription expression when organism face internal and external stimulation such as DNA damage, hypoxia and radiation stress causing DNA damage repair, cell cycle arrest, cell apoptosis and other stress responses (Lagares *et al.*, 2017; Liu and Zhou, 2017). The transcriptional level of *p21*, which is mainly mediated by telomere-dependent and aging resulting from various adversaries, such as DNA damage, is dependent on *p53* activation (Aix *et al.*, 2016; He *et al.*, 2017). Currently, as a most critical *p53* negative regulatory factor in cell, *Mdm2* combines with *p53* to form a complex for inhibiting *p53* transcription activity. *Mdm2* expression is too strong to block *p53*-mediated trans-activation leading to loss of *p53* function associated with gene instability and cell proliferation. DNA damage gives rise to *Mdm2* inactivation and elevated *p53* levels (Lessel *et al.*, 2017).

Conclusion

The antioxidant activity of *n*-butanol extract and ethyl acetate extract for DPPH, $O_2^{\cdot -}$, -OH and *n*-butanol extract and ethyl acetate extract clearance was better than that of the other compounds, which present positive correlation. Although there was no significant difference of gene expression among different concentration of ethyl acetate extraction, 7.5 mg/mL ethyl acetate extraction lead to p53, p21 gene decreased 7.83 and 34.08%, respectively

compared to control. The results provoke further studies of the ethyl acetate extract for obtaining compounds and identifying the chemical structure in order to provide experimental basis for natural antioxidant drugs development.

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