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In vitro study of DNA protective effect of DuZhong (E*ucommia ulmoides* Oliv) using single cell gel electrophoresis

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ABSTRACT

Introduction: DuZhong (*Eucommia ulmoides* Oliv.) is a Chinese traditional medicinal herb with antioxidant actions toward oxidative damage which is well documented in different scientific researches. Comet assay (single cell gel electrophoresis) is a well-developed technique for the analysis on DNA damage in single cell level. The purpose of this study is to compare aqueous and alcoholic crude extract of DuZhong cortex for its potential *in vitro* DNA protective effect on human lymphocytes against oxidative stress environment.

Design: Venous blood was collected from three healthy adult volunteers followed by isolation of lymphocytes. DuZhong extract was obtained by boiling in water and ultrasound-assisted alcoholic extraction. Lymphocytes were incubated to different dilutions of DuZhong extract before oxidative challenge with hydrogen peroxide solution in standard and lysed cell comet assay. The nucleus of lymphocyte was stained and manually scored under fluorescent microscope.

Results: Significant reduction in length of comet was only found in 0.1mg/L of aqueous extract undergone lysed cell comet assay while alcoholic extract did not show significant reduction with same other experimental setting. No significant reduction in comet length and no obvious difference were seen among aqueous and alcoholic extracts in standard comet assay. The result demonstrated that DuZhong shows some DNA protection action against oxidant stress for specific experimental condition.

KEYWORDS

Comet assay;
Eucommia ulmoides Oliv.;
DNA protection;
Antioxidant;
Lymphocyte.

INTRODUCTION

Reactive oxygen species (ROS) are products of normal cellular metabolism and generally representing oxygen free radicals[1]. They can also be produced from exposure to oxidizing agents such as xenobiotics, environmental pollutants or ionizing radiation[2].

From the view of oxygen supply, either too high or too low level of oxygen molecules (O_2) for tissues may promote greater generation of ROS resulting in in-

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flammation, signaling and gene activation[3]. It is believed that alternation of O_2 level in tissues affect gene expression directly.

Immunological cells like lymphocytes need ROS to carry out their normal function but excessive amount of ROS may affect lymphocytes by attacking the cellular components leading to cellular damage[4].

There are different types of ROS including free radical superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) which can be produced by intercellular or extracellular sources[1,5]. The relationship between the quantity of ROS and the extent of oxidative stress has been interested to many scientists.

Oxidative stress is a term generally describing the imbalance between cellular production of free radicals (oxidative load) and the complex network of antioxidant defense or damage repair mechanism available to detoxify and remove the damage by oxidative load[6,7]. Oxidative stress is believed to correlate with broad spectrum of diseases commonly found in human such as all inflammatory diseases, ischemic diseases, smoking-related disease and neurological diseases[8]. Susceptible target organs to oxidative stress include liver, brain, kidney and testes[9,10].

Many of the above mentioned diseases are accompanied with aging process[11]. In other word, oxidative stress is an important and unavoidable factor of aging in human being and other species of organisms. The increased production of superoxide is believed to be resulted from altered oxidative metabolism and hence disturbed mitochondrial regulation[8].

The damage of DNA can be induced by oxidants such as hydrogen peroxide with subsequent possible consequences of termination of cell cycle regression and apoptosis[12]. Alternation of base pairs and DNA structure may lead to aging, cellular senescence and possibilities of cancer and certain diseases. Although DNA damage increases the risk of cancer, the risks are thought to be lowered by DNA protecting agent[13].

DuZhong is a flowering deciduous tree and endemic species in mainland China[14,15]. DuZhong has been widely investigated for its active ingredients in terms of antioxidant, hypoglycemic and hypolipidemic actions[16,17]. Different approaches have been developed to investigate the beneficial effects of DuZhong against oxidative damage in biomolecules and ROS scavenging activity[18,19]. DNA protective effect of DuZhong against oxidant challenges in living cells has been demonstrated by the comet assay[19].

Comet assay is developed by Östling and Johanson in 1984 which is a fast, easy-to-use and sensitive technique for the analysis on DNA damage of all cells and it has been widely used for investigation on DNA repair, radiation biology and genetic toxicity[19,20]. In standard whole cell comet assay, cell are lysed to expose DNA strands after treatment with potentially genotoxic or genoprotective agent while cell lysis is performed before treatment with potential agent in lysed cell comet assay[13]. The lysed cell version allows naked DNA to be exposed to putative geno-protecting or DNA-damaging agents such that cellular bioavailability and mechanism of action can be explored[21]. By comparing the result from both types of comet assay, the possible role of cell membrane and intracellular constituents can be evaluated[13].

In order to extract the contents from medicinal herbs like DuZhong in effective and controlled manner, different approaches of extraction have been attempted. Extraction methods include boiling water extraction with or without enzyme-assisted, microwave irradiation and ultrasonic technique[19,22-24].

The aims of the present study were to investigate aqueous and alcoholic crude extract of DuZhong cortex for its potential *in vitro* DNA protective effect against oxidative stress and compare the antioxidant activity among two extraction methods with standard comet assay and lysed cell comet assay.

EXPERIMENTAL

Materials

DuZhong was purchased from a traditional Chinese medicine store (Tung Fong Hung Medicine Co. Ltd.) in Hong Kong. The chemicals with highest purity grade available were used. Histopaque 1077, Type VII low melting point agarose, phosphate buffered saline (PBS) powder, ethidium bromide, hydrogen peroxide (H_2O_2) solution, Triton X-100, disodium ethylenediaminetetraacetic acid (EDTA) dihyrate and Tris were from Sigma (St. Louis, MO, USA). Agarose

3:1 was from Amersco (Solon, OH, USA). Sodium chloride was from BDH (Poole, UK). Sodium hydroxide was from RdH (Sigma-Aldrich, Germany). Hydrochloric acid was from Merck (Darmstadt, Germany) and absolute methanol from International Laboratory, USA.

Experiment apparatus are listed as follow: fluorescence microscope (Microphot-Fx, Nikon, excitation filter ranged 510-560nm, Tokyo, Japan), refrigerator and freezer (4°C and -20°C, Sanyo, Japan).

Sample collection

Fresh blood were obtained by venous blood collection from three healthy volunteers (2 males and 1 female aged 29-33 years, mean = 31) for each time of experiment. These samples were ready for further procedures harvesting lymphocytes described in part of method of comet assay.

Preparation of herb water extract

One gram of finely cut dried cortex of DuZhong was soaked in 100 mL deionized water and then boiled for both 30 minutes. After cooling, the mixture was filtered through Whatman No. 1 filter paper and made up to 100 mL mixture with deionized water as 1% w/v stock solution of DuZhong extract. The stock solution was kept at -20ºC as aliquot until use.

Preparation of herb alcoholic extract

DuZhong powder of 0.5 gram and 10 mL of absolute methanol were added into 50mL centrifuge tube. The tube was loosely capped and ultrasonicated for 30 minutes. The tube was centrifuged for 15 minutes for 3000 rpm at 15ºC for supernatant retaining with two changes of fresh solvent. The extract was concentrated by evaporation in 37ºC and then aliquoted into plastic tube for 1 mL each and kept at -20ºC until use.

The dry mass of DuZhong extract was determined by defrosting and drying one set of alcoholic extract at 55 ºC oven overnight for making up 1% w/v stock solution.

Comet assay

Comet assay is used to assess DNA damage in lymphocytes under oxidative challenge in this research setting, following the procedure developed and further modified in various research topics[13,21].

One side of microscope slides was pre-coated with 1% standard agarose solution. For each comet assay, eighty five µL of warm 1% standard agarose was added to the pre-coated slides followed by covering with 18 x18 mm cover slip and refrigerated for solidification. One hundred µL of venous fresh blood was mixed with 1mL of PBS in a microtube. After 4ºC refrigeration for 30 minutes, Histopaque 1077 was underlaid under the mixture inside the microtube followed by 1500 rpm spinning for 5 minutes at 4ºC centrifuge. One hundred µL of lymphocytes was retrieved into a new microtube containing 1 mL of PBS followed by centrifugation with same condition applied. Lymphocytes obtained were ready for following steps of both standard and lysed cell comet assay. Comet assays were carried out in triplicate as follow:

For standard comet assay, different dilutions of DuZhong water or alcoholic extract from 10^{-2} to 10^{-6} of original 1% w/v stock solution (i.e. 0.1 g/L to 10 µg/L of DuZhong extract) were prepared with PBS. These dilutions were added into each microtube containing 50 µL of lymphocytes for incubations. After 30 minutes, the microtubes were cold centrifuged followed by supernatant removal and PBS washing.

One mL of 50 µM H_2O_2 was added to each microtube and then kept in fridge followed by cold spinning both for 5 minutes. The lymphocytes remained in microtubes were washed by PBS with supernatant removal and then tapping for re-suspending. Cover slips were removed from standard agarose pre-coated slides just before carrying out cell embedding. Another 85 µL of 1% low melting point (LMP) agarose in PBS was added into the microtube. The microtube was tapped and then 85µL of mixture was transferred to the centre position of second agarose layer on the slide. Cover slip was placed again followed by 5 minutes of refrigeration.

Working lysis solution was then prepared by adding 0.4 mL of Triton X-100 to 40 mL stock lysis solution in a staining jar. Slides with cover slip removed were placed into the staining jar keeping at 4ºC for one hour. The slides were placed into another jar containing 40 mL of electrophoresis solution and left for 20 minutes with two changes for DNA unwinding and break-

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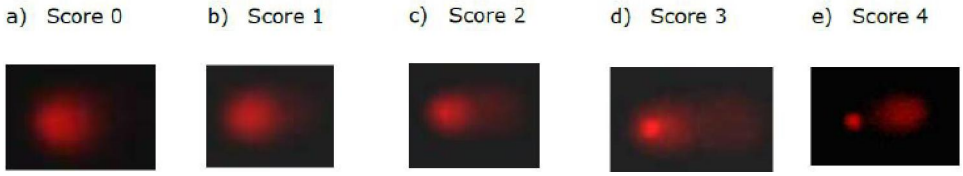

Figure 1 : Microscopic images of lymphocyte stained with 2mg/L ethidium bromide showing different length of "comet" tail in accordance its extent of DNA damage from Score 0 (not damaged) to Score 4 (severely damaged) using fluorescence microscope

ing. The slides were then placed gently on the platform of the electrophoresis tank in a complete row with blank slides. Electrophoresis was run at 25V of voltage and 0.3A of current for 30 minutes in cold environment. The slides were placed into staining jar containing tap water at 4ºC for 5 minutes with two changes. The slides were removed from the staining jar for drying in 37ºC oven for 30 minutes.

For lysed cell comet assay, the cell embedding step which is same as that in standard comet assay was carried out first in lysed cell comet assay. After that, the slides without cover slips were dipped into working lysis solution for one hour. Different dilutions of DuZhong water or alcoholic extract from 10^{-3} to 10^{-7} of original 1% w/v stock solution (i.e. 10 mg/L to 1 µg/L of DuZhong extract) were made up to 20mL mixture with phosphate buffer.

Slides were transferred to a staining jar containing 0.4 M phosphate buffer for 10 minutes at room temperature (RT) and different dilutions of DuZhong extract for 37 ºC incubation for 30 minutes. After incubation, the slides were transferred to staining jar containing 0.4 M phosphate buffer at RT for 10 minutes, 40mL of 50 µM H_2O_2 at 4 ºC for 5 minutes and then 0.4 M phosphate buffer at RT again for 10 minutes. The slides were then placed into another staining jar with electrophoresis solution and left for 20 minutes with two changes for DNA unwinding and breaking.

The electrophoresis step was carried out with same protocols as standard comet assay.

Result analysis

The slides were stained with 40 µL of 20 mg/L ethidium bromide for instant viewing and scoring using fluorescence microscope. The score related to the length of lymphocyte comet was recorded for statistical analysis using Prism 5.0 for Windows software (GraphPad Software, CA, USA). One way ANOVA test followed by Dunnett's Multiple Comparison Test were adopted in current study.

RESULTS

The length of comet tail was manually scored from score 0 to 4 as illustrated in Figure 1. The comet scores of lysed cells of water extraction of DuZhong with the concentration of 0.1 mg/L (10^{-5} dilution) showed significant differences ($p<0.05$) to positive control (H_2O_2) as shown in Figure 2A. The mean comet scores of this concentration of DuZhong water extraction was 107 while that of positive control was 380. The significant differences in comet scores in this set of comet assay indicate that water extract of DuZhong had an obvious protective effect of on DNA of lysed lymphocytes against oxidative challenge.

Differences between various concentrations of alcoholic extract of DuZhong and positive control in lysed cell comet assay was not significant. The comet score of alcoholic extract ranged from 372 to 387 while the positive control was 389. The result of comet scores in this setting was shown in Figure 2B.

No statistical significant difference was found on both hot water and alcoholic extracts of DuZhong in standard comet assay. The comet scores of water extract lay between 359 and 384 while the positive control was 383. The scores of alcoholic extract were 337 to 374 while the corresponding positive control was 342. The plotting of comet scores using water extraction and alcoholic extraction in standard version of comet assay was shown in Figure 2C and 2D respectively.

DISCUSSION

DuZhong is a common traditional Chinese medicinal herb, which is used widely for lowering blood pressure and improving the tone of kidney and liver[17,18,23].

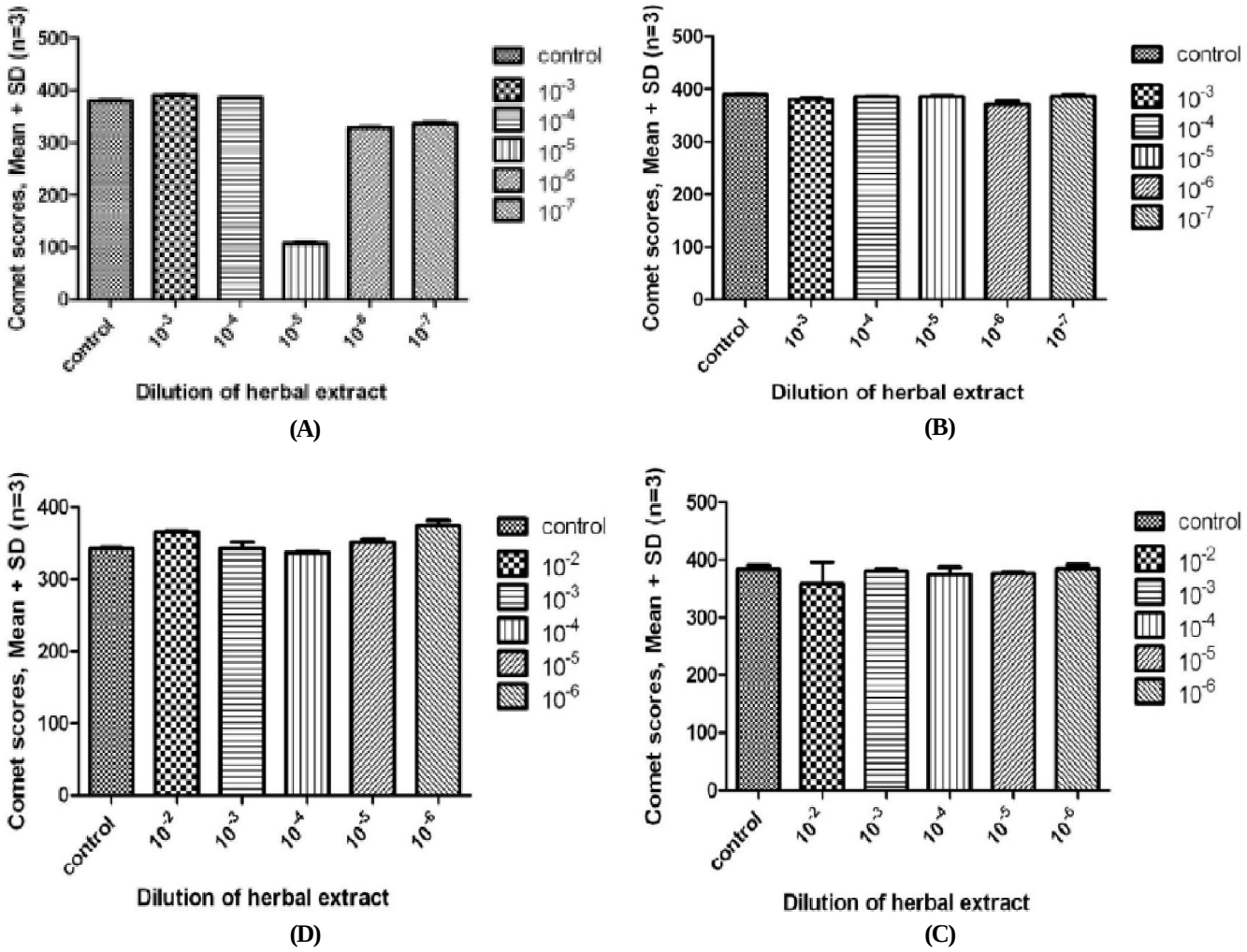

Figure 2 : Mean total comet score with standard derivation (n=3) in the presence of DuZhong extract from 10^{-2} to 10^{-6} of original 1% w/v stock solution (0.1g/L to 10µg/L) in standard comet assay and 10^{-3} to 10^{-7} of original 1% w/v stock solution (10mg/L to 1µg/L) in lysed cell comet assay. Control used in each experimental setting was 50 µM H_2O_2. A: DuZhong water extract in lysed cell comet assay. B: DuZhong alcoholic extract in lysed cell comet assay. C: DuZhong water extract in standard comet assay. D: DuZhong alcoholic extract in standard comet assay

It has the ability to minimize oxidative status and lipid peroxidation in clinical areas as well as food preservation[18,25,26]. Plants rich in natural antioxidant such as phenolics was found to prevent oxidative damage.[18,27] The use of DuZhong on oxidative stress related diseases deserves attention[18].

Different studies on DuZhong have been carried out before. Many of them focused on the therapeutic effect of the herb using animal model while related study on inhibitory effect on oxidative stress has been carried out using standard comet assay alone[2,16,27]. The current study further investigated whether roasted cortex of DuZhong protects DNA against oxidative stress with comparison of antioxidant activity among two extraction methods with two versions of comet assay.

Results showed that significant reduction of comet score of lymphocytes pretreated with specific concentration of hot water roasted cortex DuZhong extract (0.1mg/L) and oxidative challenge comparing with control (oxidative challenge of 50 µM H_2O_2 only) ($p<0.05$).

Only hot water extract of DuZhong exhibited DNA protective effect in lysed lymphocytes against oxidative challenge. No protection was seen in standard comet assay and alcoholic extract demonstrated ineffective in protecting DNA at all concentrations tested. The results of DNA damage prevention are consistent with previous study showing the inhibitory effect on damage of purified DNA by DuZhong extract[18].

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Absent of protection in standard comet assay but narrow range protection in lysed cell comet assay suggested the possibility of cell membrane effect[28]. Molecules with DNA protecting effect were not able to pass through plasma membrane. Although limited access to cellular DNA was observed, this does not rule out the antioxidant to be exerted in extra-cellular environment. The protective action may be mediated via iron binding or scavenging of ROS while possible interaction with intracellular components can be considered also[27].

Comet assay results in the present study indicated that DNA protective effect was from narrow range of concentration of DuZhong aqueous extract. The limitation of protective effect may be caused by two major factors: insufficient protective power in low concentration of active ingredients and possible counteracting pro-oxidative action at high concentration. Roasted cortex extract of DuZhong may cause oxidative DNA damage due to the presence of Maillard reaction oxidative by-product during roasting process[19]. However, such suggestions may provide idea to further investigate possible DNA damage effect in the herbs affecting the accuracy of the similar study.

In the current study, only simple extraction methods and solvents were adopted. Only crude extracts were obtained because traditional Chinese medicines are generally prepared in simple boiling procedure. Herbal medicine is also commonly prepared in the form of 'wine' particularly when it is taken as supplement. It has also been shown that ultrasound-assisted extraction benefits of higher yield and shorter extraction time comparing with traditional extraction methods[23,30]. Furthermore, higher concentration of phenolic contents obtained and higher inhibiting ability on lipid peroxidation using alcoholic extraction other than aqueous extraction have been demonstrated in various studies on different medicinal plants[31,32]. However, only aqueous extraction providing significant result indicated that active protective ingredient of DuZhong may be soluble in water only but not alcoholic agents. Use of other combinations of extractions other than hot water seems to have little effect for this species of herbal plant based on the result of current study.

However, the current study provided little information about the type of DNA protective agents and way of protection given which is remained for further investigation. Besides, the possibility of DNA protection and repairing ability, beneficial effects on other cell types and other systems of human bodies such as immune system and actions to the extent higher than cellular level by DuZhong should not been ruled out such that it can be considered in follow-up studies.

There are some limitations on this study. Small number of subjects (n=3) in current study may cause statistical error despite low standard deviation (around 10) among test samples was recorded. Larger sample size in further studies may produce more representative result.

The current study focused on beneficial effect of DuZhong to oxidative damage. However, more work can be included in further studies like effects on other body systems like immune system while various vital parameters can be also taken into account in order to construct more comprehensive and systematic overview of the herb concerned.

In conclusion, aqueous DuZhong cortex extract exhibited DNA protective effect on naked DNA while alcoholic extracts showed ineffective protection against hydrogen peroxide mediated damage. Further study on individual compounds in DuZhong is needed to explore the additional potential beneficial effect in health maintenance.

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CONFLICT OF INTEREST

No conflict to disclose.

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