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An *in vitro* study of Sanchi (*Panax pseudoginseng*) for its DNA protective effect

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Panax pseudoginseng (Shachi) are well known for their antioxidant properties. The aim of this study was to explore Sanchi extracts, potential protection of human cellular DNA from oxidative challenge induced by hydrogen peroxide *in vitro*. Aqueous and alcoholic extracts of Shachi were incubated with human lymphocytes from $1 \times 10^{-2}\%$ to $1 \times 10^{-7}\%$ (w/v) for 30 mins. Pre-treated cells were then stressed with 50 μ M hydrogen peroxide. Washed cells were subjected to the standard and the lysed cell comet assays. Results showed that water extracts of Sanchi at specific concentrations were able to diminish human DNA damage induced by hydrogen peroxide in both standard and lysed cell comet assays. No effect was observed in ethanolic extract. More pronounced protective effect in lysed cell comet assay model implied cell membrane limited the availability of active ingredients of Sanchi to nucleus.

Keywords: acellular comet assay; DNA; hydrogen peroxide; lysed cell; oxidative challenge

Introduction

The integrity of human DNA and the maintenance of genomic stability are crucial to our health. Replication, transcription and protein synthesis will be affected by damaged DNA, which in turn lead to impairment of cellular growth, development and metabolism (de Boer et al., 2002). However, DNA of the human body is constantly and unavoidably exposed to oxidative attacks. Normally, in a healthy subject, the oxidative status is balanced by the antioxidant defence mechanisms. When the oxidative status becomes imbalanced due to over-production of reactive oxygen species (ROS), depletion of antioxidants, or both, the body is said to be sustaining oxidative stress (Hekimi, Lapointe, & Wen, 2011). Many studies have pointed out that the most effective method to reduce DNA damage is antioxidant supplement. Some natural antioxidants, including traditional Chinese herbal medicine (TCM), have attracted interest because their long history of consumption and relatively less concern on toxicity than synthetic chemicals (Yen & Hsieh, 2000).

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Supplementation of some medicinal herbs has been used successfully in some cases either to protect against age related diseases or to increase the average life span of some species. Therefore, much attention has been focused on the antioxidative effects of these herbs because of their great market values for promoting health.

Sanchi, also known as notoginseng, Sanchi root or Sanqi, is an important Chinese medicinal herb from the root of plant *Panax notoginseng* (Chan & Tomlinson, 2000). It was first used by ethnic groups in Southwest China and became more popular and widely accepted since Ming Dynasty (Zhang & Fang, 2003). Although *P. notoginseng* belongs to the same genus species as *Panax ginseng*, which is the king of traditional herbal medicines that helps to improve overall health and restore the body balance, the potency of *P. notoginseng* is quite different from *P. ginseng* and is less expensive (Sun, 2004). In TCM, Sanchi is used for pain relief and hemostasis (Chinese Pharmacopoeia Commission, 2005). This herb has also been used to treat patients in China with circulatory disorders for hundreds of year (Chan & Tomlinson, 2000). Some of the active constituents isolated from Sanchi are similar to those found in ginseng (Ng, 2006). Experimental and supplementation studies of the antioxidant effects of Sanchi have been performed and encouraging results are found. For example, trilinolein isolated from Sanchi has been shown to have an antioxidant effect which supports its long history of use in treating circulatory disorders (Chan, Thomas, & Tomlinson, 2002). Ginsenoside Rg1 and Re isolated from Sanchi have also shown a cardioprotective effect against oxidant-mediated injury by its radical scavenging properties (Xie et al., 2006; Zhu et al., 2009). However, the scientific findings of Sanchi on geno-protective effect are still inadequate. Further scientific studies are required to confirm the reputed health benefits of Sanchi, particularly in relation to oxidative stress and DNA protection.

The comet assay or single cell gel electrophoresis, which can be applied to in vitro and in vivo systems, is a relatively simple but sensitive technique for measuring and analysing DNA damage in individual cell (Szeto, 2007; Wong, Szeto, Collins, & Benzie, 2005). Two versions of comet assay are commonly employed. When they are used in conjunction, the mechanism of antioxidants action can be partly elucidated (Szeto, Collins, & Benzie, 2002). The aim of the current study was to investigate the geno-protective effect of aqueous and alcoholic extracts of Sanchi on human lymphocytic DNA using two versions of the comet assay (standard comet assay and lysed cell/acellular comet assay) in parallel in order to elucidate the cellular bioavailability and mechanism for genoprotection.

Materials and methods

Chemicals and reagents

For preparation of test agent, methanol was purchased from International Laboratory, USA. For isolation of lymphocytes, Histopaque 1077 was from Sigma (St. Louis MO, USA). For comet assay, type VII low melting point (LMP) agarose, phosphate buffered saline (PBS) tablets, disodium ethylenediaminetetracetic acid (EDTA) dehydrate, Tris[hydroxymethyl]aminomethane (Tris), hydrogen peroxide solution, ethidium bromide and Triton X-100 were from Sigma (St. Louis MO, USA); standard agarose was from Amresco (Solon, OH, USA); sodium chloride was from

BDH Chemicals Ltd (Poole, England); disodium hydrogen phosphate, sodium dihydrogen phosphate and sodium hydroxide were from Riedel-de Haen (Germany).

Preparation of test agent

Sanchi was purchased from Tung Fong Hung Medicine Co. Ltd, Hong Kong. For water extraction, the herb was cut into small pieces, and 1 g of herb was soaked in 100 ml deionised water for 30 min at room temperature. This procedure enhanced the constituents of the dry medicinal herb to be dissolved in water. The mixture was then boiled for 30 min using a hotplate and allowed to cool for another 30 min. The mixture was then filtered through Whatman no. 1 filter paper. The filtrate was made up to 100 ml with deionised water and this was regarded as 1% (w/v) stock solution of extract (initial extract). The stock solution was aliquoted and kept at -20°C until used.

For alcoholic extraction, the herb was ground to fine powder using a mortar and pestle, and 0.5 g powdered herb was weighed into a 50 ml centrifuge tube. Ten ml of 70% methanol was added into the tube and kept in an ultrasonic bath for 30 min for dissolution. The mixture was then centrifuged at 3000 rpm for 15 min at room temperature. The extraction procedure was repeated twice with fresh solvent. Three methanolic extracts were pooled and reduced to approximately 3 ml in a 37°C water bath overnight. Finally, the extract was made up to 10 ml with deionised water and kept at -20°C until used. The known volume of this herb extract was dried in petri dishes at 55°C for 48 h to determine the dry mass of herb. One per cent (w/v) stock solution of extract (initial extract) was used in the experiment.

Isolation of lymphocytes

Fresh blood was taken for each experimental run. Lymphocytes from four healthy volunteers (three males and one female, 33–37 years of age, mean = 33) were harvested from venous blood. Brief medical history of all donors was taken, and they were not taking any herbal medicine nor nutritional supplement a month prior to blood taking. To achieve this, 100 μl of fresh heparinised blood was added to 1 ml cold PBS immediately in microtube after collection. This was mixed gently, and 100 μl of Histopaque 1077 was added at the bottom of the tube for separation of lymphocytes. The tubes were spun at 1500 rpm for 5 min at room temperature. Lymphocytes were retrieved in 100 μl from just above the boundary between the PBS and Histopaque layer using pipette, and added to 1 ml PBS in another microtube. The centrifugation step was repeated and as much supernatant as possible was removed from the pelleted lymphocytes using pipette.

Standard comet assay

One millilitre of Sanchi extracts or PBS (as control) was added to the microtube containing the washed lymphocytes and the cell suspensions were mixed gently. Water and alcoholic extracts of Sanchi were tested at between $1 \times 10^{-2}\%$ to $1 \times 10^{-6}\%$ (w/v) and $1 \times 10^{-3}\%$ to $1 \times 10^{-7}\%$ (w/v), respectively. Cell suspensions were incubated at 37°C for 30 min. The tubes were then spun at 1500 rpm for 5 min at room temperature, the supernatant was discarded, cells were washed once with 1 ml

cold PBS, and the centrifugation step was repeated. Pre-treated lymphocytes were stressed by addition of 1 ml of 50 μ M cold H₂O₂ in PBS to each tube and all tubes were kept refrigerated. After 5 min, the tubes were spun again and the pellet was washed with 1 ml cold PBS, after which the cells were again pelleted by centrifugation as before. The pelleted lymphocytes were gently mixed with 85 μ l of 1% pre-warmed (40°C) 1% (w/v) LMP agarose in PBS and quickly pipetted onto a microscopic slide which had been pre-coated with two layers of 1% (w/v) standard agarose in PBS. The microscopic slides were pre-coated with 1% (w/v) standard agarose in PBS, and 85 μ l of 1% (w/v) standard agarose in PBS was added onto pre-coated slides on the day of experiment. Cover slips (18 \times 18 mm) were applied and the slides were placed at 4°C for at least 5 min until the gel layer solidified. For lysis of cells, the slides were immersed in lysis solution (0.1M Na₂EDTA, 2.5M NaCl, 10 mM Tris and 1% Triton X-100, pH 10) after gently removing the cover slips at 4°C for 1 h in the dark in order to lyse the cell and nuclear membranes and remove histones from around the DNA. Afterwards, the slides were immersed in 40 ml of cold electrophoresis solution (0.3M NaOH and 1M Na₂EDTA, pH >13) in a clean Coplin jar at 4°C for 20 min. This step was repeated for DNA unwinding and breaking before electrophoresis, which was run at 25 V (constant voltage setting) for 30 min in a 0.3 A (by adjusting the buffer level in the tank) with ice packs neighbouring the electrophoresis tank to keep the tank cool during electrophoresis. After electrophoresis, the slides were placed in a staining jar and washed two times (5 min each) with tap water to remove the alkaline buffer. Finally, the slides were oven dried at 37°C for about 30 min before the slides were stained with 40 μ l aqueous ethidium bromide (2 μ g/ml) and covered with cover slips for scoring.

Lysed celllacellular comet assay

Eighty-five microlitre of 1% pre-warmed (40°C) 1% (w/v) LMP agarose in PBS was added to the microtube containing the washed lymphocytes and quickly pipetted onto a microscopic slide which had been pre-coated with two layer of 1% (w/v) standard agarose in PBS. Cover slips (18 \times 18 mm) were applied, and the slides were placed in at 4°C for at least 5 min until the gel layer solidified. Lysis of cells was then performed as described previously in standard comet assay. After 1 hour of lysis, slides were transferred to a Coplin jar containing 0.4 M phosphate buffer (pH 7.4) for 10 min at room temperature. Each slide was then transferred to a petri dish containing 20 ml of Sanchi extracts or phosphate buffer (as control), and incubated at 37°C for 30 min. Water and alcoholic extracts of Sanchi were tested at between 1 \times 10⁻²% to 1 \times 10⁻⁶% (w/v) and 1 \times 10⁻³% to 1 \times 10⁻⁷% (w/v), respectively. Slides were then transferred to phosphate buffer for a further 10 min at room temperature, after which slides were transferred to a Coplin jar containing 50 μ M cold H₂O₂ in phosphate buffer and kept refrigerated. After 5 min the slides were then immersed in fresh phosphate buffer for 10 min at room temperature for removing H₂O₂, and transferred to a clean Coplin jar containing 40 ml of cold electrophoresis solution (0.3M NaOH and 1M Na₂EDTA, pH >13) at 4°C for 20 min. This step was repeated, and the remaining steps were then performed according to the procedures described previously in standard comet assay.

Scoring

Slides were coded and 100 cells per slide were scored in each of the three independent experiments using a fluorescence microscope (Microphot-Fx with excitation filter G: Ex 510-560, Nikon, Tokyo, Japan) by manual scoring by another team member. Different levels of DNA damage were graded from 0 to 4, where four was the most damaged for each comet image according to the comet intensity and tail length visualized (Szeto et al., 2011). Results are presented as the mean \pm SD of comet score of 100 cells.

Statistical analysis

ANOVA with *post hoc* Dunnett's *t*-test was used to investigate differences in DNA damage scores between cells with or without pre-treatment with various concentrations of different types of Sanchi extract. Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used. Results were considered as significantly different at *p* value < 0.05.

Results

Results obtained using standard comet assay and lysed cell comet assay are shown in Figures 1 and 2. Different levels of DNA damage were associated with the comet tail intensity and length visualised. Water extract of Sanchi at 1×10^{-3} dilution showed statistically significant DNA protective effects against H_2O_2 challenge as compared with the control in standard comet assay (Figure 1a), and it also gave highest levels of DNA protection followed by water extract of Sanchi at 1×10^{-4} dilution in lysed cell comet assay (Figure 2a). However, no significant DNA protective effect was found in cells treated with alcoholic extract of Sanchi at all dilution in standard comet assay (Figure 1b) as well as lysed cell comet assay (Figure 2b).

Discussion

The comet assay is a relatively simple, speedy and inexpensive technique for measuring *in vitro* and *in vivo* DNA damage. By modifying the electrophoresis conditions, or using lesion-specific enzymes, different types and levels of DNA damage can be detected. Furthermore, by using appropriate versions of comet assay, the effect of the antioxidants on cellular DNA as well as the possible mechanisms of their action on DNA can be explored (Szeto, 2007). For example, as seen in this study, parallel testing using both standard comet assay and lysed cell comet assay may help in understanding the cellular bioavailability and the mechanism of action of Sanchi. Because of the numerous advantages of the comet assay, it has been widely applied in nutraceutical research, DNA repair studies, supplementation trials and environmental biomonitoring (Wong et al., 2005).

In this study, results showed that water extract of Sanchi demonstrated DNA protective effect against oxidative stress only at high concentration tested. It is possible that Sanchi has a concentration-dependent antioxidant effect. It has been demonstrated that some antioxidants possess pro-oxidant or genotoxic effect under certain circumstances, particularly at high concentration (Panayiotidis & Collins,

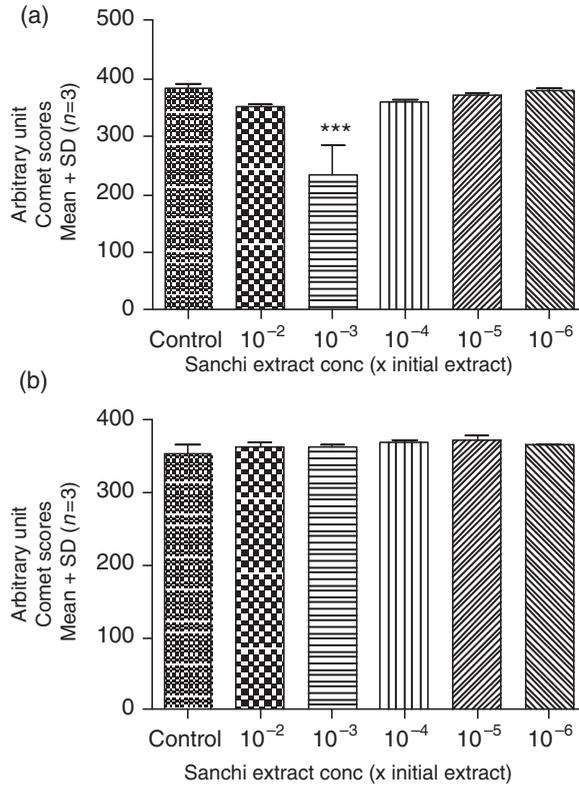


Figure 1. Effect of (a) water extract (b) alcoholic extract on DNA of human lymphocytes in standard comet assay. Data are expressed as the mean \pm standard deviation of comet scores relative to that in control (PBS-treated) cells obtained in three separate experiments. Cells were pre-incubated in different dilutions of Sanchi extract followed by H_2O_2 challenge ($50 \mu\text{M}$); *** $P < 0.0001$ compared to the positive control, Dunnett's test.

1997). However, there was no indication that Sanchi induced DNA damage at any concentrations tested in the present study. Our results showed also that the water extract of Sanchi at 1×10^{-4} dilution demonstrated significant DNA protection in lysed cell comet assay but not in standard comet assay, which implies that the cellular bioavailability or response to antioxidative components of Sanchi was low in whole cells. Because the pretreatment of cells with the Sanchi extract comes before the lysis step in the standard version of comet assay, the cell membrane may prevent entry of certain types of molecule into cells due to the large molecular size or high polarity, thus limiting the DNA protective effect of the Sanchi extract (Wong et al., 2005). This could help in explaining the absence of protective effect of Sanchi extract at 1×10^{-4} dilution seen in whole cells. The lysed cell comet assay allows direct contact of naked DNA or nucleus with the Sanchi extract by removing cell membrane prior to exposure to the Sanchi extract. It is particularly useful for assessing the effect of the cell membrane and intracellular systems on oxidative damage (Szeto et al., 2002).

No DNA protection was conferred by alcoholic extracts of Sanchi at any concentrations tested in both versions of comet assay in the present study. The difference in potency between the organic extracts and water extracts in protecting

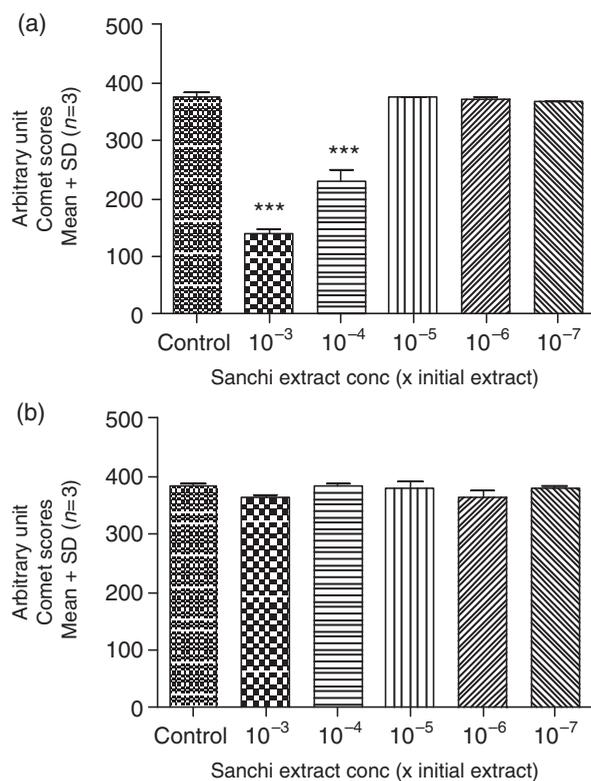


Figure 2. Effect of (a) water extract and (b) alcoholic extract on DNA of human lymphocytes in lysed cell comet assay. Data are expressed as the mean \pm standard deviation of comet scores relative to that in control (phosphate buffer-treated) cells obtained in three separate experiments. Cells were pre-incubated in different dilutions of Sanchi extract followed by H₂O₂ challenge (50 μ M); *** $P < 0.0001$ compared to the positive control, Dunnett's test.

lymphocytic DNA suggested that the majority of active compounds of Sanchi might be more water soluble. This finding comes in agreement with earlier report that the amounts of four active constituents of Sanchi by water extraction were higher than those of alcoholic extraction (Dong, Zhao, Huang, Leung, & Tsim, 2005). However, it must be noted that negative results do not indicate the absence of genoprotective effect of Sanchi. In general, Chinese herbs are composed of a wide variety of chemical components to which their benefit is attributed. The chemical constituents of Sanchi have been investigated for many years, and several classes of compounds have been isolated, including triterpene saponins, polysaccharides, flavonoids, peptidoglycans, nitrogen-containing compounds, and various ubiquitous compounds such as fatty acids, carbohydrates and phenolic compounds (Liu et al., 2009; Sun, 2004). It is possible that not all the constituents of Sanchi are responsible for genoprotective activities as Sanchi has been shown to have a variety of beneficial effects in various pharmacological studies, including haemostatic, anti-thrombotic, improving micro-circulation, preventing cardiac and cerebral ischemia, anti-atherosclerotic, protecting the liver from ischemia-reperfusion injury, preventing kidney interstitial fibrosis, hepatoprotective, anti-tumour, immunity modulatory, and anti-aging effects

(Gu, Huang, & Ge, 2005; Ng, 2006; White, Fan, Song, Tsikouris, & Chow, 2001; Yao & Li, 2002; Yoshikawa, Morikawa, Yashiro, Murakami, & Matsuda, 2001). Also, some of these constituents may be more soluble in water and some in organic solvent. In addition, the active constituents of Sanchi may be present in insufficient quantities in crude extract to show the geno-protective effect with the concentrations tested in present study.

In summary, water extracts of Sanchi at specific concentrations were able to diminish human DNA damage induced by hydrogen peroxide. The possible active constituents of Sanchi responsible for the genoprotective activities and their structures await elucidation. The use Sanchi for treatment of patients with ROS-related diseases deserves further study.

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