

Evaluating the Anticancer Activity of *Hedyotis diffusa* Water Extract Against Human Breast Cancer MCF7 Cells

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Abstract: *Hedyotis diffusa* has been used to treat cancers in traditional Chinese medicine for many years. Recently, several anthraquinone compounds were extracted from *Hedyotis diffusa* and identified as its anticancer ingredients. These compounds are able to induce apoptosis in several types of cancer cells. In this study, we showed that the anticancer activity of *Hedyotis diffusa* was highly selective, possessing high cytotoxicity towards human breast cancer MCF7 cells but almost no cytotoxicity against the mammary epithelial MCF12A cells. The *Hedyotis diffusa* water extract also elevated the energy metabolism in both MCF12A and MCF7 cells; but the ATP/ADP ratio was still maintained at about 1.0 in the MCF12A cells to avoid either apoptosis or uncontrolled proliferation. In addition, Sheshecaojing, a health product of *Hedyotis diffusa* marketed in China, gave a dose-dependent cytotoxic response against the MCF7 cells.

Keywords: cytotoxicity, energy metabolism, apoptosis, ATP/ADP ratio, herbal extraction.

INTRODUCTION

Traditional herbal medicine has existed for thousands of years, and herbs are still commonly used as therapeutic agents worldwide. A variety of herbal products have been proposed to possess anticancer or cancer inhibition properties [1-4]. Recently, herbal remedy has been emerging as a promising supplement to modern cancer therapies [5]. According to a study conducted by the Northern California Cancer Center, almost 13% women with breast cancer use herbal remedies [6]. However, the clinical application of herbal medicines is significantly impeded due to insufficient information on their active ingredients as well as the underlying functional mechanisms in spite of recent developments in nutraceutical research.

Hedyotis diffusa is an herbal medicine widely used as an anti-inflammatory agent in traditional Chinese medicine [7-9]. Because inflammation is a crucial component of cancer progression [10], *Hedyotis diffusa* has been included in therapies against various types of cancer for a long time without even knowing its active ingredients [11-13]. The

ethanol extract from *Hedyotis diffusa* was shown to induce cell apoptosis in the HT-29 human colon cancers [14]. Recently, several anthraquinone compounds were extracted from *Hedyotis diffusa* and claimed to be the active anticancer ingredients [15, 16]. Both 2-hydroxy-3-methylantraquinone (HMAQ) and 2-hydroxy-1-methoxyanthraquinone induced cell apoptosis in human lung adenocarcinoma SPC-1-A, breast cancer Bcap-37 and hepatoma HepG2 cells via mitochondria-dependent caspase-3 activation [15]; and HMAQ promoted cell apoptosis in human breast cancer MCF7 cells through the endoplasmic reticulum mediated Ca²⁺/calpain/caspase-4 pathway [16].

Cancer cells predominantly rely on aerobic glycolysis instead of mitochondrial oxidative phosphorylation for their ATP generation. Targeting the energy metabolism in cancer cells has recently obtained more research attentions as a novel therapeutic method in treating breast cancers. It is still unclear whether the *Hedyotis diffusa* water and ethanol extracts, as well as the extracted anthraquinone compounds, possess any effects towards the energy metabolism in either normal or cancer cells. Thus, in the current study, we showed that the *Hedyotis diffusa* water extract increased the energy metabolism in both normal mammary epithelial MCF12A and the breast cancer MCF7 cells. However, the ATP/ADP ratio was still maintained at about 1.0 in the MCF12A cells, suggesting that the *Hedyotis diffusa* treatment was well regulated to avoid uncontrolled proliferation

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in MCF12A. In addition, the combination with other anticancer herbal medicines such as *Scutellaria barbata* might decrease the anticancer activity of *Hedyotis diffusa*.

MATERIALS AND METHODS

Materials

Dried *Hedyotis diffusa* was purchased from a traditional Chinese medicine store in Calgary, Alberta, Canada. SheshECAOJING (containing 5 g *Hedyotis diffusa*, 1 g *Scutellaria barbata*, 1 g *American ginseng* and 3 g sucrose in each 10 g package) was manufactured by Guangzhou Medicines and Health Products I/E Corporation (Guangzhou, Guangdong Province, China) and purchased from a Chinese herbal store in Saskatoon, Saskatchewan, Canada. Human mammary epithelial cell line MCF12A and breast cancer cell line MCF7 were purchased from Cedarlane Canada (Burlington, Ontario, Canada). The CytoTox 96[®] Non-Radioactive Cytotoxicity Assay kit was purchased from Promega Corporation (Madison, Wisconsin, USA).

Extract Preparation

The *Hedyotis diffusa* water extract was prepared by boiling 1 g of chopped *Hedyotis diffusa* in 100 mL deionized water for 1 hr. The solution was allowed to cool down at room temperature for at least 2 hr before the top supernatant was used for cell treatments. For convenience, the extract solution was defined as concentration of 10 g/L (i.e. 10 g of *Hedyotis diffusa* boiled in 1 L of water). The extract solution of 5 g/L was prepared by diluting the above preparation with deionized water. The SheshECAOJING solution (40 g/L) was prepared by boiling 1 package (10 g) of SheshECAOJING in 250 mL water for 1 hr. The other SheshECAOJING solutions (concentrations from 0.31 g/L to 20 g/L) were prepared by serial water dilution of the above preparation.

Cell Culture and Cytotoxicity Assay

Both MCF12A and MCF7 cells were cultured in T-75 cell culture flasks under the recommended culture conditions by Cedarlane Canada. The cells were incubated under a humidified, 5% CO₂ atmosphere at 37°C with the culture media changed every 2-3 days. The cells were then collected from the T-75 cell culture flasks, re-suspended in appropriate culture media, and plated in 96-well culture plates with each well containing about 5 × 10⁴ cells. The cells were allowed to grow for another day before being treated with either the *Hedyotis diffusa* water extract or SheshECAOJING solution for 24 hr. The cytotoxicity was measured using the CytoTox 96[®] Non-radioactive Cytotoxicity Assay kit from Promega Corporation. Cells treated with the culture media were used as the negative controls. The cytotoxicity was calculated using the following equation:

$$\text{Cytotoxicity (\%)} = \frac{\text{Experimental} - \text{Control}}{\text{Maximum (Lysis)} - \text{Control}}$$

ATP and ADP Measurements

The concentrations of ATP and ADP in *Hedyotis diffusa* treated MCF12A and MCF7 cells (maintained at least 80% confluence after treatment) were measured with HPLC-UV

method as described by Olkowski *et al.* [17]. Briefly, the cells were quickly washed with 1 mL phosphate buffered saline (PBS) twice after 24 hr treatment. Ice cold perchloric acid (0.35 mL of 0.7 M) was added into each well. The cells were detached from the plate using a cell scrubber. The cell mixtures were then homogenized and centrifuged at 12000 rpm for 5 min. The supernatants were collected and neutralized with 2 M potassium hydroxide to bring pH close to 7.0. The supernatants were subsequently filtered through 0.45 μm filter, and 10 μL of each sample was injected into a C-18 column using gradient. Two mobile phase components used included 20 mM potassium phosphate buffer (pH 7.0) and 100% methanol. The gradient was 100% phosphate buffer from 0 – 6.5 min, 100% methanol from 6.5 – 12.5 min, and 100% phosphate buffer from 12.5 – 25 min for column re-equilibration, which was sufficient to achieve stable baseline conditions. The ATP and ADP levels in the samples were monitored at 210 nm.

RESULTS AND DISCUSSION

Selective Anticancer Activity Against Human Breast cancer MCF7 Cells

The cytotoxicity of the *Hedyotis diffusa* water extract was measured towards human mammary epithelial MCF12A and breast cancer MCF7 cell lines under two different concentrations (Fig. 1). The water extract did not exhibit any cytotoxicity against the mammary epithelial MCF12A cells under either concentration. However, it showed 13% cytotoxicity at concentration of 5 g/L and 45% cytotoxicity at concentration of 10 g/L, respectively, against the breast cancer MCF7 cells. This indicated that the anticancer activity of *Hedyotis diffusa* is highly selective. Since no cytotoxicity was observed even at concentration of 10 g/L in the MCF12A cells, the *Hedyotis diffusa* water extract may be pushed to much higher concentrations in order to achieve better cytotoxic effect against the breast cancer MCF7 cells. Although direct administration of pure HMAQ (> 20 μM) induced apoptosis in the MCF7 cells [16], further studies are definitely required to answer the following two questions. The first question is whether HMAQ can induce apoptosis in the MCF7 cells under its naturally occurring concentrations in *Hedyotis diffusa*; and the second question is whether any

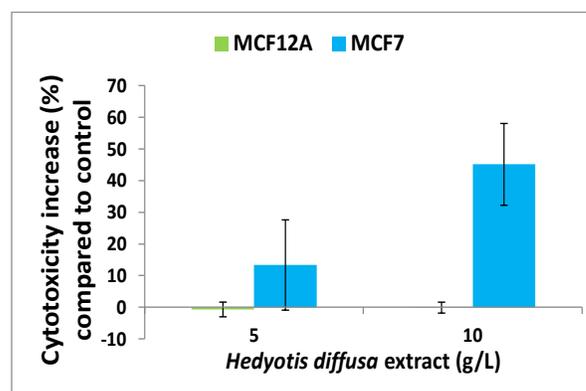


Fig. (1). Cytotoxicity (%) of the *Hedyotis diffusa* water extract in human mammary epithelial MCF12A cells (shown in green) and breast cancer MCF7 cells (shown in blue).

other ingredients such as flavonoids are also involved in the apoptotic effect of *Hedyotis diffusa*.

Effects on Energy Metabolism

Cell growth and function are closely associated with the cellular energy production. Thus, the effect of the *Hedyotis diffusa* water extract on energy metabolism was investigated in both MCF12A and MCF7 cells. As shown in Fig. (2a), the ATP level was increased by about 2.8 folds in the MCF12A cells. Since normal differentiated cells rely on mitochondrial oxidative phosphorylation (producing 36 ATPs per glucose molecule) for their energy production [18, 19], *Hedyotis diffusa* is likely increasing the mitochondrial oxidative phosphorylation in the MCF12A cells. However, the ATP/ADP ratio was still maintained at about 1.0 (Fig. 2b), indicating that the *Hedyotis diffusa*-treated MCF12A cells were well regulated by cellular signaling pathways to avoid uncontrolled proliferation. In the MCF7 cells, the ATP level was elevated by 3.6 folds (Fig. 2a). Because cancer cells predominantly rely on aerobic glycolysis for their ATP generation (producing 2 ATPs and 1 lactate per glucose molecule), the higher ATP level in the MCF7 cells might be due to increased share of the mitochondrial oxidative phosphorylation (as observed in the MCF12A cells) in the total energy metabolism rather than elevated aerobic glycolysis. One of the benefits of aerobic glycolysis to the cancer cells is to increase their biomass in preparation for proliferation [19].

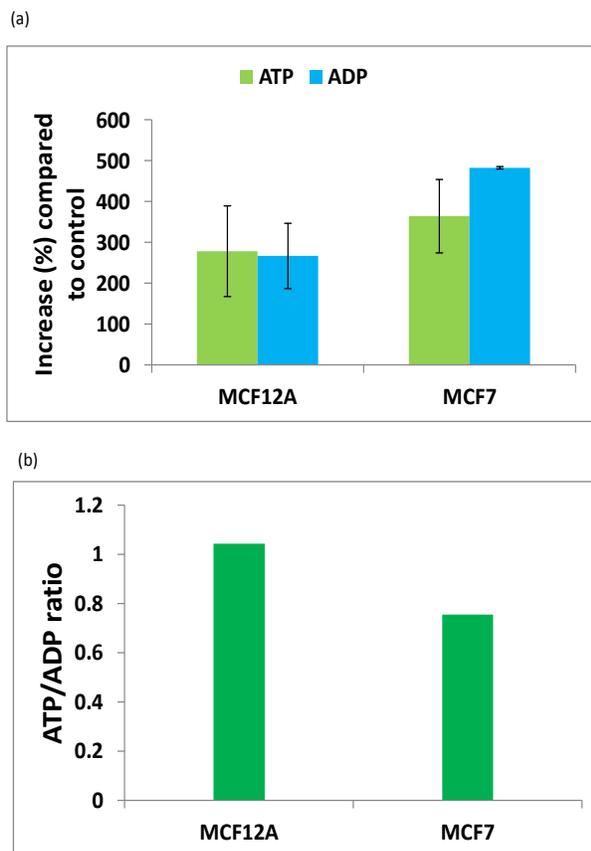


Fig. 2. (a) ATP and ADP levels in the MCF12A and MCF7 cells after *Hedyotis diffusa* treatment for 24 hrs. (b) ATP/ADP ratio in the treated MCF12A and MCF7 cells.

Therefore, the increase in mitochondrial oxidative phosphorylation may not be favorable for the MCF7 cells as it will decrease the biomass accumulation as well as the proliferation in the MCF7 cells. Furthermore, the ATP/ADP was reduced to 0.75 in the MCF7 cells after the *Hedyotis diffusa* treatment (Fig. 2b), suggesting that the cells were undergoing apoptosis. Although the identity of the compounds that may increase mitochondrial oxidative phosphorylation in the cells is unclear, one potential candidate is the iridoid glycosides, which have been identified as another family of active ingredients in *Hedyotis diffusa* [20, 21] and shown to ameliorate mitochondrial energy metabolism in rat cortical neurons experiencing oxygen-glucose deprivation [22].

Anticancer Activity of SheshECAojing

Single herbal remedy is quite rare in traditional Chinese medicine. *Hedyotis diffusa* is usually used in combination with *Scutellaria barbata*. SheshECAojing, consisting of 50% *Hedyotis diffusa*, 10% *Scutellaria barbata*, 10% American ginseng and 30% sucrose, is marketed as an anti-inflammatory health product in China. Since both *Scutellaria barbata* and American ginseng possess anticancer activities [23-26], SheshECAojing was expected to exhibit better cytotoxic effect against the cancer cells than *Hedyotis diffusa*. In Fig. (3), SheshECAojing was shown to impose little cytotoxicity to the MCF12A cells; however, it gave an almost dose-dependent cytotoxic response in the MCF7 cells. The cytotoxicity was 32% at concentration of 20 g/L for SheshECAojing (equivalent to 10 g/L of *Hedyotis diffusa*), which was much less than that of 45% for *Hedyotis diffusa* alone. This decrease in cytotoxicity might be due to the addition of sucrose since sucrose is generally believed to diminish or deplete herbal functions in traditional Chinese medicine. However, the cytotoxicity for SheshECAojing was 26% at concentration of 10 g/L (equivalent to 5 g/L of *Hedyotis diffusa*), which was 13% higher than that for *Hedyotis diffusa* alone. Therefore, further studies are needed to clarify the influences of *Scutellaria barbata*, *American ginseng* and sucrose on the anticancer activity of *Hedyotis diffusa*.

CONCLUSIONS

Hedyotis diffusa is an herbal medicine that has been used to treat cancers in traditional Chinese medicine for a very

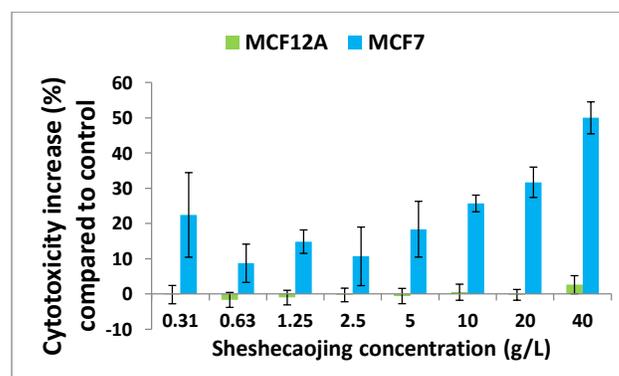


Fig. 3. Cytotoxicity (%) of SheshECAojing against human mammary epithelial MCF12A cells (shown in green) and breast cancer MCF7 cells (shown in blue).

long period of time. In this study, we showed that *Hedyotis diffusa*, as well as the commercial health product SheshECAojing, exhibited highly selective cytotoxicity against human breast cancer MCF7 cells. The *Hedyotis diffusa* water extract stimulated the ATP generation in both normal mammary epithelial MCF12A and breast cancer MCF7 cells possibly via increasing the mitochondrial oxidative phosphorylation. However, the increase in mitochondrial oxidative phosphorylation may not be beneficial to the MCF7 cells.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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