



ANTI-CANCER EFFECT OF *PLANTAGO DEPRESSA* ETHANOLIC EXTRACT IN B16F10 SKIN CANCER CELLS

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ABSTRACT

Plantago depressa Willd. is a distributed herb which traditionally used for treating wound, fever, cancer and inflammation in some temperate asian countries. This present study aimed to investigate the anti-cancer activity of ethanol extract of *Plantago depressa* Willd. plant on B16F10 skin cancer cells. Cells were treated with different concentration of the PDE (25, 50, 100, 200 µg/mL) with or without lipopolysaccharide (LPS) stimulation to evaluate its effect on cell viability. Furthermore, anti-cancer activity was evaluated by checking viability of B16F10 skin cancer cells in various concentration of the PDE extract (0.6, 1.2, 2.5 and 5 µg/mL), using CCK-8 assay. The results indicated that (i) the PDE treatment did not influence on cell viability of HaCaT skin normal cells dose-dependently; and (ii) PDE exhibits anti-cancer activity through inhibition of proliferation of B16F10 skin cancer cells.

KEYWORDS: *Plantago depressa* Willd., *Plantago depressa* ethanolic extract, anti-cancer effect, B16F10 skin cancer cells, *in vitro* testing.

ABBREVIATIONS

LPS: Lipopolysaccharide

PDE: *Plantago depressa* ethanolic extract

DMSO: Dimethylsulfoxide

CCK-8: Cell Counting Kit – 8

N.C: Negative control

INTRODUCTION

Plantago depressa Willdenow belonging to Plantaginaceae, is an medicinal plant which distributed in some temperate countries as Mongolia, Pakistan, E Russia, Bhutan, India, Kashmir, Kazakhstan, Korea, China [6, 9, 22]. Plantaginaceae

genus has been reported to have anti-leukemia, anti-carcinoma, antiviral and immune modulation activities. The upper parts of plant are used not only as crude drug but also as commercial tea for anti-phlogistic purpose [2]. It has been reported

containing various bioactive compounds which cure many diseases, such as anti-inflammatory, anti-oxidant, anti-aging properties; cough and wound healing activity [7]. Moreover, *Plantago* sp. also contains mineral elements, vitamin C, β -carotene and anti-nutritional factors to evaluate their nutritive value in human diet [20]. Besides being recorded as a traditional medicine, nowadays, plantain plants are

appreciated in many more aspects. In addition, *P. depressa* has high content of aucubin, iridoid glycoside which has anti-inflammatory and anti-cancer reaction [16] or plant stress resistance [21]. Indeed, the present study was built up to verify the anti-cancer activity of *Plantago depressa* ethanolic extract on B16F10 skin cancer cells.

MATERIALS AND METHODS

Extraction of plant material

Samples of *Plantago depressa* were collected from plant geographical region of Middle Khalkh dry steppe of Mongolia (47.88°N, 106.90°E in latitude and longitude) in July 2015. The plant species for sampling was identified according to morphological characters by Grubov, 1982. [23].

The whole plant of various *Plantago depressa* plant (200 g) was extracted with ethanol (1 Litter) at room temperature. The *Plantagodepressa* ethanolic extract (PDE) was evaporated to obtain powdered sample. The extract was dissolved in dimethylsulfoxide (DMSO) to give 0.1 v/v concentration and used at appropriate concentrations ($\mu\text{g}/\text{mL}$).

Cell line, culture media, cell treatment

B16F10 skin cancer cells were obtained from the American Type Culture Collection (Cryosite, Lane Cove, NSW, Australia). B16F10 skin cancer cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin. The cells were cultured at 37°C in a humidified incubator with an atmosphere of 5% carbon dioxide (CO₂).

Murine B16F10 skin melanoma cells were used to assess anti-cancer effect of PDE. Cytotoxicity was evaluated using HaCaT skin normal cells. Cells were treated with increasing concentrations of the PDE

extract (0.6, 1.2, 2.5 and 5 $\mu\text{g}/\text{mL}$) to determine its effect on cell viability.

Cytotoxicity was accessed using HaCaT skin normal cells. Cells were treated with increasing concentrations of the PEE extract (50, 100, 200, 400 $\mu\text{g}/\text{mL}$) to determine its effect on proliferation.

Cell viability assay

The cytotoxicity of PDE was determined by a Cell Counting Kit-8 assay (CCK-8) according to the manufacturer's Dojindo Laboratories (Tokyo, Japan). Values for cytotoxicity measured by CCK-8 assay were expressed as the concentration that causes 50% decrease in B16F10 cell viability (IC₅₀, $\mu\text{g}/\text{mL}$). Briefly, B16F10 cells were plated at a density of 1×10^4 cells per well in a 96-well plate, and were incubated at 37°C for 24 hours. The cells were treated with different concentrations of PDE or vehicle alone, and incubated at 37°C for every additional 24, 48 and 72 hours. After incubation, 10 μL of CCK-8 solution was added to each well and incubated under the same conditions for another 3 hours and the resulting color was assayed at 450 nm using a microplate reader (Tecan, Switzerland). Each assay was carried out in 3 replicates. For control studies, 0.05 % DMSO was used. All quantitative data are representative of at least three independent experiments.

RESULTS AND DISCUSSION

Effect of *Plantago depressa* extract on HaCaT skin normal cell viability

Despite a period in which pharmaceutical companies cut back on their use of natural products in drug discovery, there are many promising drug candidates in the current development pipeline that are of herbal origin. After all, traditional cytotoxic chemotherapy although kills cancer cells by indirectly inducing apoptosis unfortunately, side effects are brutal, and most tumors become resistant [11]. The present study demonstrated the

cytotoxicity indices as a measure of percentage cell mortality recorded by CCK assay in HaCaT skin normal cells, in a dose dependent manner at the end of 24 hours incubation with extract.

To evaluate the effects of PDE on cell proliferation and identify its therapeutic potential we indicated, for the first time, the potent cytotoxic activity of different concentrations of PDE did not significantly influence on normal cell line viability (Fig. 1). A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damages to

normal cells, meaning minimum side effects. This ideal situation is reachable by inducing apoptosis in cancer cells. Cell cycle modulation by various

natural and synthetic agents is gaining widespread attention in recent years [1].

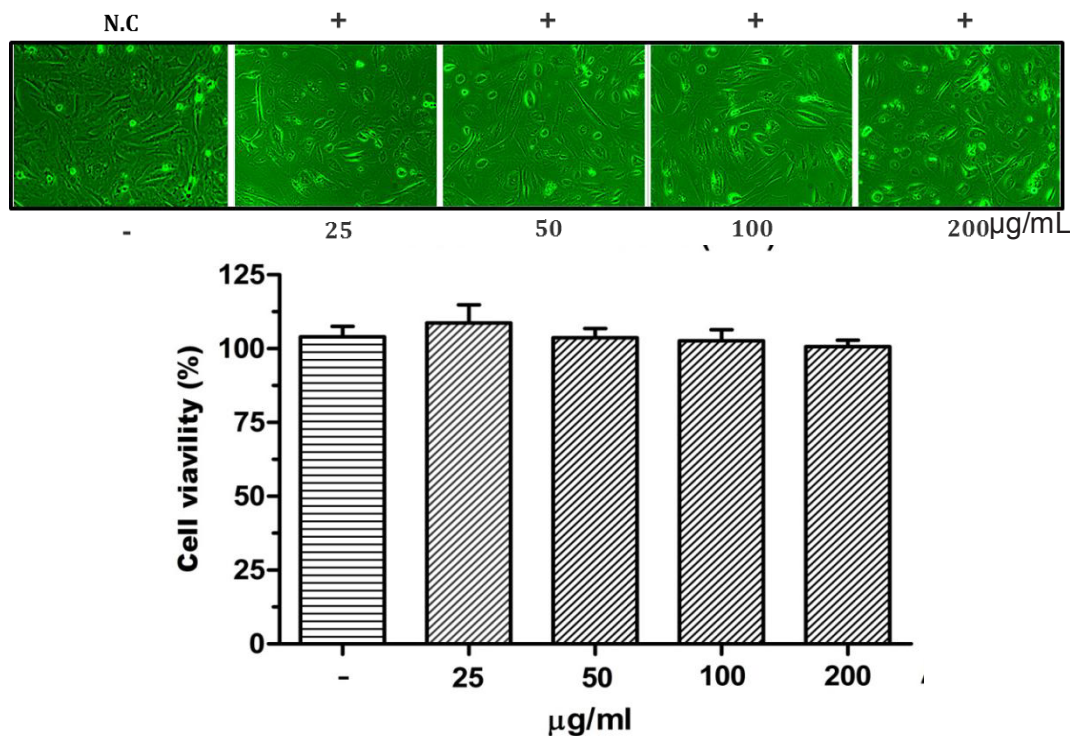


Figure 1. Effect of *P. depressa* ethanolic extract on HaCaT skin normal cell viability after 24 hour incubation.

Data are means \pm SD (n=3) and not significantly different ($p < 0.05$).

Anti-cancer effect of *Plantago depressa* ethanolic extract on Murine B16F10 skin melanoma cells

The current study investigated anti-cancer activity of extract of *P. depressa* in B16F10 skin melanoma cells. HaCaT skin normal cells were used to evaluate cytotoxic effect of PDE. CCK-8 assay was used to measure PDE effects on cell proliferation. The cells were incubated for 24 hours in the presence or absence of PDE concentration (0.6, 1.2, 2.5 and 5 µg/mL) (Fig. 2). Dose-dependent studies revealed IC50 of 1.32µg/mL for *P. depressa*. The anti-cancer effect of the ethanolic extract from *Plantago depressa* Willd.on B16F10 skin cancer cells is summarized in Figure 2 and 3. The results showed that the extract possessed anti-cancer activity with different levels. A dose-dependent inhibition of cell proliferation was observed for most of the extract

tested in this study. It is clear that the more concentration of PDE increased the more cell viability of tumor cell growth went down, especially the concentration at 5 µg/ml (31.4; 34.0; 20.5% at 24, 48 and 72 hours after incubation, respectively), followed by other ethanolic extract concentrations of *P.depressa* plant.

The investigation for anti-cancer properties from natural sources has been successful worldwide. Bioactive compounds have been isolated and nowadays are used to treat human tumors. The ethnopharmacological knowledge is helpful to lead the search for plants with potential cytotoxic activity. Different species of *Plantagogenus* have been reported to be utilised as remedies against cancer, however, there is not any scientific validation especially *P. depressa* Willd.

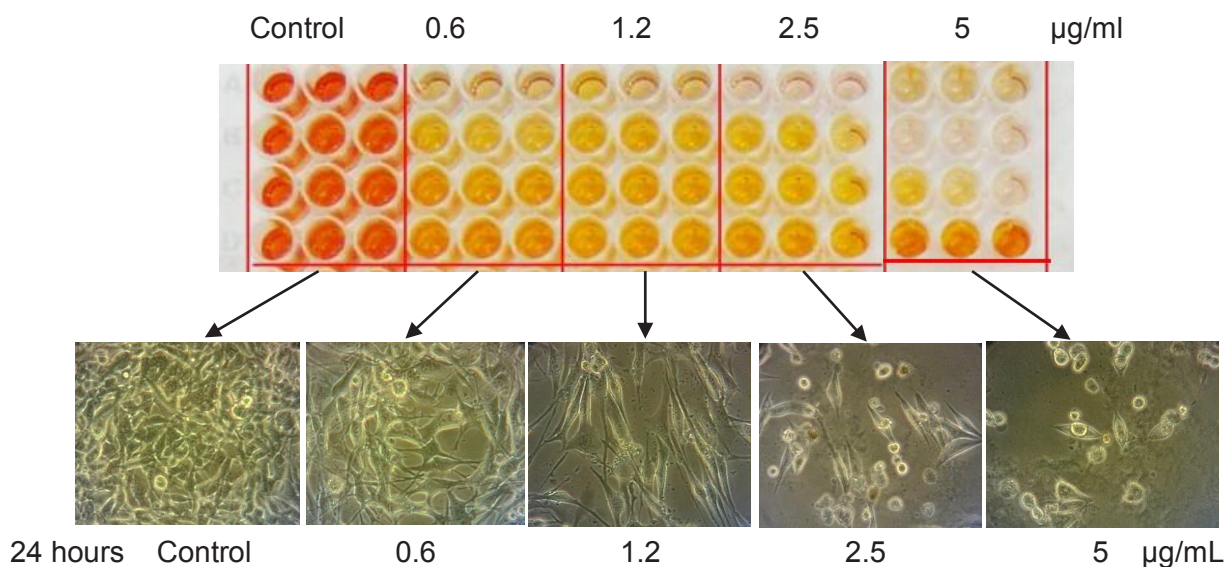


Figure 2. Cell morphology and population of murine B16F10 skin melanoma cells responded in various doses of *Plantago depressa* ethanolic extract after 24 hours incubation

In one study, [8] indicated that methanolic extracts from 7 *Plantago* species used in traditional medicine among them *P. major*, were evaluated for cytotoxic activity against three human cancer cell lines: the human renal adenocarcinoma (TK-10), the human breast adenocarcinoma (MCF-7) and the human melanoma (UACC62) cell lines) using the sulphorhodamine B (SRB) assay *in vitro*. *P. major* and the other 6 *Plantago* species showed cytotoxic activity on the breast adenocarcinoma (MCF-7) and melanoma (UACC-62) tumoral cell lines in a concentration-dependent manner at the recommended NCI (USA) doses. None of the extracts, with the exception of *Plantago bellardii*, showed cytotoxic activity against renal adenocarcinoma (TK-10) cells. It is thought that the cytotoxic activity depends basically on flavonoids, flavone and luteolin present in the extract. Luteolin exhibited inhibitory effect on various human cancer cell lines, such as renal A-549, ovary SK-OV-3,

melanoma SK-MEL-2, XF-498, HCT15, gastric HGC-27, breast MCF-7 and human leukemia cells [12, 18, 19] showed that hot water extracts of *P. major* L. and *P. asiatica* L. possessed effects of immunomodulatory activity on human mononuclear cells proliferation. In addition, Okazaki et al. (2002) reported that fibers had a remarkable effect in preventing colorectal cancer and other cancer risks. Our results revealed that *Plantago depressa* extract has growth inhibitory and cytotoxic effects on B16F10 skin cancer cells. These preliminary results could be justified by the cytotoxic activity of the flavone, luteolin-7-O- β -glucoside, the major flavonoid in all species of *Plantago*. Luteolin-7-O- β -glucoside is known to be the responsible agent for the anti-cancer activity of the plant. Similar results have been established by [17], who isolated luteolin as an active component of *Terminalia arjuna* in cancer cell lines, which justifies the underlying use of these species in traditional cancer treatment.

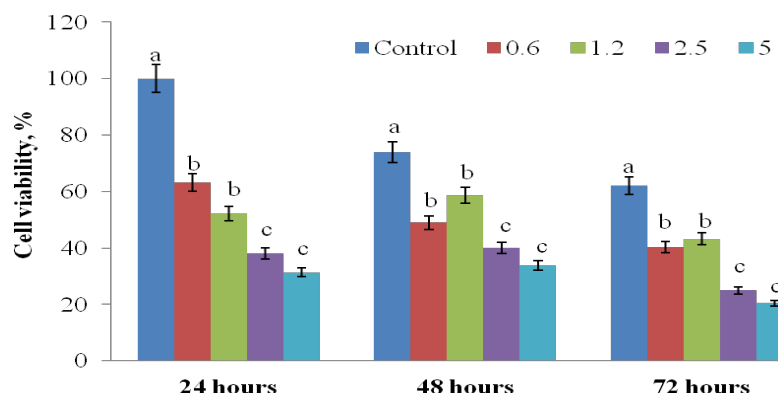


Figure 3. The effect of concentration of *P. depressa* ethanolic extract on skin cancer cell viability after incubation

Means of 4 replicates with 1 well each. Means with same letters indicate no significant difference by Duncan's multiple-range test; ns: not significant; **: significant at 0.01 level.

Besides containing flavonoids, *Plantago* sp. also has high content of aucubin which have anti-cancer effect [10, 16]. Luteolin has been shown to inhibit a series of human cancer cell lines (renal A-549, ovary SK-OV-3, melanoma SK-MEL-2, XF-498, HCT15, gastric HGC-27) [15, 19] breast MCF-7 [12] and human leukaemia cells [18]. The precise mechanism responsible for the cytotoxic activity of Luteolin-7-O- β -glucoside, the major flavonoid found in all species of *Plantago* genus, is not thoroughly

understood. The topoisomerase-mediated DNA damage seems to be a candidate mechanism, by which some flavonoids may exert their cytotoxic potential [13, 14]. According to [5], Luteolin-7-O- β -glucoside acted as a potent DNA topoisomerase I poisons as well as its aglycon luteolin. [8] mentioned that Luteolin-7-O- β -glucoside acting as a topoisomerase I poison. Thus, there is now preliminary scientific validation for the use of some of these medicinal plants for anti-cancer.

CONCLUSION

Under *in vitro* condition, *Plantago depressa* ethanolic extract doses ranging from 0.6 - 5 μ g/mL significantly inhibited the B16F10 skin cancer cells, with low IC50 values defining their promising anticancer property according to NCI; however,

further analysis are needed to confirm their total compound content. Hence, *Plantago depressa willd.* is a potential candidate for the development of pharmacological agents useful in the treatment of cancer diseases.

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