A Study of the *in vitro* cytotoxic activity of *Gelsemium elegans* using human ovarian and breast cancer cell lines

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Abstract The crude methanol extracts of *Gelsemium elegans* leaves were assessed for their cytotoxic activity using the microculture 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cellular viability. This study utilized two different types of human cancer cell lines, CaOV-3 (human ovarian cancer cells) and MDA-MB-231 (human estrogen receptor negative breast cancer cells), allowing for comparison of toxicity of *G. elegans* against these two cancer cells lines. Our results showed that the methanol extract of *G. elegans* exhibited high cytotoxicity against the human ovarian cancer cell line CaOV-3 with an IC_{50} value of 5µg/ml after 96 h incubation. However, *G. elegans* displayed discernibly less toxicity against the MDA-MB-231 cells with an IC_{50} value 40µg/ml after 96 h incubation and this effect was dose- and time-dependent, up to 72h and 20-30 µg/ml. In conclusion, our results demonstrated that *G. elegans* is potently cytotoxic against the human ovarian cancer cell line CaOV-3 and to a lesser extend towards the human breast carcinoma cancer MDA-MB-231 cells, suggesting that the extract is selective towards CaOV-3 cells and may have a chemotherapeutic role for ovarian cancer treatment in the future.

INTRODUCTION

Gelsemium elegans (Family: Loganiaceae), locally known as Lemuan, is an extremely poisonous plant that is indigenous to the South East Asian countries and found predominantly in Malaysia and Sarawak (Latiff *et al*., 1999; Ridley, 1925; Perry, 1980). The three popularly known species are the Asian (*G. elegans*) and the two North American related species, *G. sempervirens*, and *G. rankini* (Lin *et al*., 1989; Burkhil, 1966). *Gelsemium* is a climbing plant with dark evergreen leaves and all parts of the plant are poisonous (Ponglux *et al*., 1988; Watson & Dallwitz, 2000). Some studies on domestic animals have shown that the consumption of the leaves can cause death in goats over a 24-hour period (Thompson *et al.,* 2001). Post mortem carried out on the goats revealed the presence of neurological degeneration, cerebral cell loss, reduction of muscle mass, fat stores, atrophy of adipose tissue and reduced gastrointestinal contents.

Indigenous systems of medicine have shown that the rhizome or underground stem of *Gelsemium* has numerous therapeutic uses and has traditionally been used as a nervous system relaxant, especially to treat various types of pain including headache and pain associated with inflammatory conditions [Ponglux *et al*., 1988; Watson & Dallwitz, 2000). *Gelsemium* has also been found useful in the treatment of spasmodic disorders, such as asthma and whooping cough (Lin *et al*., 1989) and is one of homeopathy's most important remedies for influenza (Blackwell, 1990). In China, the drug is also used as a poison because its effects are very rapid (Kitajima *et al*., 1998). It disrupts the gray matter in the spinal cord affecting the respiratory centre, which ultimately slows down respiration until it is arrested, and finally causing death (Bousta *et al*., 2001).

Chemical investigations of *G. elegans* have shown that the plant contains indole and oxindole alkaloids of novel polycyclic systems (Luo *et al*., 1993; Saxton, 1965; Clardy *et al*., 1989; Sakai, 1995). Studies on laboratory animals have shown that low doses of *Gelsemium* roots had a protective effect on gastric alterations induced by experimental stress (Boustaa *et al*., 2001).

In view of this, the focus of the present study was to evaluate, for the first time, the effects of the methanol extract of *G. elegans* leaves on the growth of two types of human cancer cell lines, ovarian (CaOV-3) and breast cancer oestrogen receptor negative (MDBA-MB-231) cells. Two different types of cell lines were chosen in order to investigate if there is any selective inhibition of neoplastic cell growth by *G. elegans*.

MATERIALS AND METHODS

Cell lines

The human papillary ovarian adenocarcinoma cell line CaOV-3 and human estrogen and progesterone receptor negative breast carcinoma cell line MDA-MB-231 used in this study was kindly provided by Syamsul Ahmad, Institute of Medical Research, Kuala Lumpur. Both cell lines are adherent cells.

Cell culture

Cells were grown as monolo yer and cultured in RPMI-1640 (Flow Laboratories, Sydney Australia) medium with 2mM L-glutamine and containing 100µg/ml gentamycin sulphate, 40µg/ml Amphotericin B, supplemented with 5% (v/v) fetal bovine serum (Culture Lab, Australia). Both cell lines were maintained at 37^{o}C in a humidified atmosphere with 5% CO_2 and 95% air and were in the exponential phase of growth at the time of testing. The cells were harvested following tripsinization (0.25% trypsin-0.002% EDTA) and washed twice with phosphate buffered saline (PBS). The viability of the test cell lines exceeded 95% as determined by exclusion of the vital dye trypan blue. Cell viability assay was performed when cells reached 70% confluence and the *G. elegans* extract added to the cells after 24 h incubation.

Plant extract

The methanol extract of *G. elegans* leaves was kindly provided by Prof. Dato' Laily Din from Department of Chemistry, Faculty of Science and Technology, UKM, Bangi. The leaves of *G. elegans* were collected from the UKM campus in Bangi. Voucher specimens are registered in the herbarium of UKM in Bangi (KMS 5281). For the preparation of the methanol extract, leaves were washed and dried in a ventilating

drier at 35^{o}C for 72 h. The dried samples were pulverized (3 kg) and extracted twice with methanol (12 L) for 72 h. The solvent was then evaporated to dryness under reduced pressure resulting in the crude extract (130 g; 4.3%).

MTT Assay

The cytotoxic potential of the plant extract was determined following incubation of model cells using the MTT assay (Mosmann, 1983). The assay uses a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), to assess cellular metabolism and hence viability. In metabolically active cells, MTT is reduced by the mitochondrial enzymes succinate dehydrogenase with the formation of insoluble purple formazan crystals. These are then solubilised, and the absorbance measured spectrophotometrically at 570nm (Carmichael *et al*., 1987).

A concentrated stock solution of each test compound was prepared in dimethyl sulphoxide (DMSO) and stored at –20^{o}C until required for use. Prior to the assay, the stock solution was rediluted to the required dilutions using blank RPMI-1640 media. The maximum percentage of DMSO present in the wells was 0.5% (v/v) and this was incorporated as a negative control in all experiments. Cultures containing vinblastine (0-100µg/ml) were used as a positive control for CaOV-3 cells and 5-fluorouracil (0-70µg/ml) for MDA-MB-231 cells.

Cells were seeded at a density of 4x10^{3} cells/well into a sterile 96-well plate and allowed to adhere overnight. Then 20µl of the appropriate extract solution in the concentration range of 0-100µg/ml was added. Cells were incubated with the extract for a period of 24, 48, 72, or 96 h. Following the required incubation period, 50µl of MTT was added to each well and the plates were incubated at 37^{o}C in a humid atmosphere with 5% CO_2 and

95% air for 4 h. The media was then gently aspirated, and 150µl DMSO was added to dissolve the formazan crystals.

The amount of formazan product was measured spectrophotometrically at 570 nm using a Tuneable VERSA max microplate reader (Molecular Devices, Sunnyvale, CA). Each extract concentration had three replicates per assay, and each experiment was carried out on three separate occasions. The IC_{50} value, defined as the drug concentration causing a 50% reduction in cellular viability, was calculated for the extract at 96 h incubation. This value was used as a means for comparing the cytotoxicity of the extract for each of the two cell lines used in this experiment.

Statistical Analysis

The results represent the mean ± SEM and are the average of three values per assay and each assay was repeated three times. Statistical evaluation of the untreated control cells along with the extract and solvent-treated cells was calculated using Student's t-test. A probability of 0.05 or less was statistically significant.

RESULTS AND DISCUSSION

The crude methanol extract derived from the leaves of *G. elegans* leaves was evaluated as a cytotoxic agent, using the MTT assay, against the human papillary ovarian adenocarcinoma cell line CaOV-3 and human estrogen and progesterone receptor negative breast carcinoma cell line MDA-MB-231. The cytotoxic effect of the crude extract was also compared to that of commercial drugs (vincristine for CaOV-3 cells and 5-florouracil for MDA-MB-231 cells). Two different types of human cancer cell lines were evaluated in order to establish if the cytotoxic effect of *G. elegans* was exclusive to any one neoplastic cell line.

The IC_{50} values for the continuous exposure of CaOV-3 cells over a 96 h period to *G. elegans* extract are shown in Figure 1. The results indicate that the extract is potently cytotoxic to the ovarian cell line with an IC_{50} value of 5µg/ml after 96 h incubation. An additional series of experiments were carried out in order to establish if the *G. elegans* extract was cytotoxic in a dose- and time-dependent manner to the CaOV-3 cells. Results from these studies are presented in Figure 1, and clearly indicate that significant cytotoxic activity was evident after 24 h exposure at a concentration as low as 10µg/ml ($P<0.05$) and this effect increased in a dose-dependent manner up to 30µg/ml. Significant cytototoxic activity was also observed after 48 and 96 h incubation and this was dose-dependent up to 30µg/ml, respectively ($P<0.05$). However, the cytotoxic effect of *G. elegans* in these cells abated after 72 h of incubation. Treatment with 20µg/ml *G. elegans* extract at 24 h resulted in approximately 60% viable CaOV-3 cells ($P<0.05$) and maximum cytotoxic effect was obtained after 96 h incubation whereby almost no viable

cells were present ($P<0.005$). The cytotoxic effect of the crude extract was found to be more potent than that of vincristine (IC_{50} = 58µg/ml) indicating that the positive control is not suitable for CaOV-3 cells.

Wang and co-workers (Wang *et al*., 2001) evaluated the *in vitro* effects of *gelsemium* alkaloids from *Gelsemium sempervirens L*, using the liver carcinoma cell line, HepG2. They found that the extracts of *gelsemium* alkaloids inhibited the growth of HepG2 cells in a dose-dependent manner. This inhibition of growth was via apoptosis and this is a preferred mode of cell death in cancer treatment. In the present study with *G. elegans* leaves, we observed a similar dose-dependent effect of the extract against the human ovarian cancer (CaOV-3) cell line. Additional experiments demonstrated that the *C. elegans* crude extract was cytotoxic in a dose- and time-dependent manner towards the CaOV-3 cells up to 20-30µg at all incubation times, with the exception of 72 h.

The effects of methanol extract of *G. elegans* against the human receptor negative breast carcinoma cell line MDA-MB-231 cells are shown in Figure 2. The results show that *G. elegans* exhibited less cytotoxic activity in the MDA-MB-231 cells compared to the CaOV-3 cells (Fig. 1) with IC_{50} of 40µg/ml after 96 hours of incubation. Additional experiments were also performed every 24 h for four days in order to determine if the extract was cytotoxic in a dose- and time-dependent manner against the MDA-MB-231 cells. The results in Figure 2 show that there was cytotoxic activity as early as after 24 h incubation ($P<0.005$) and this increased in a dose-dependent manner up to 70µg/ml. A further reduction in cell proliferation was observed after 48 and 72 h at a concentration of

10µg/ml. The 24 h treatment of 20µg/ml *G. elegans* extract inhibited cell proliferation of MDA-MB-231 cells by approximately 50% and maximum cytotoxic effect was obtained after 72 h incubation whereby only approximately 20% viable cells were present ($P<0.05$). This study shows that the crude extract of *G. elegans* is more effective in inhibiting growth of CaOV-3 cells than the MDA-MB-231 cells.

The lower cytotoxic effect of *C. elegans* against the MDA-MB-231 cells compared to the CaOV-3 cells might be due to the selectivity of the extract towards the ovarian CaOV-3 cancer cells than the MDA-MB-231 breast cancer cells. The strong cytotoxic effect of *G. elegans* extract against the CaOV-3 cells implies that the methanol extract may have a potent anti-tumour effect on proliferation of ovarian cancer cell lines.

Further studies are currently underway in our laboratory with the aim of extracting *gelsemium* alkaloids from *G. elegans* and determining its mode of molecular action and cytotoxic effect on a variety of other human cancer cell lines.

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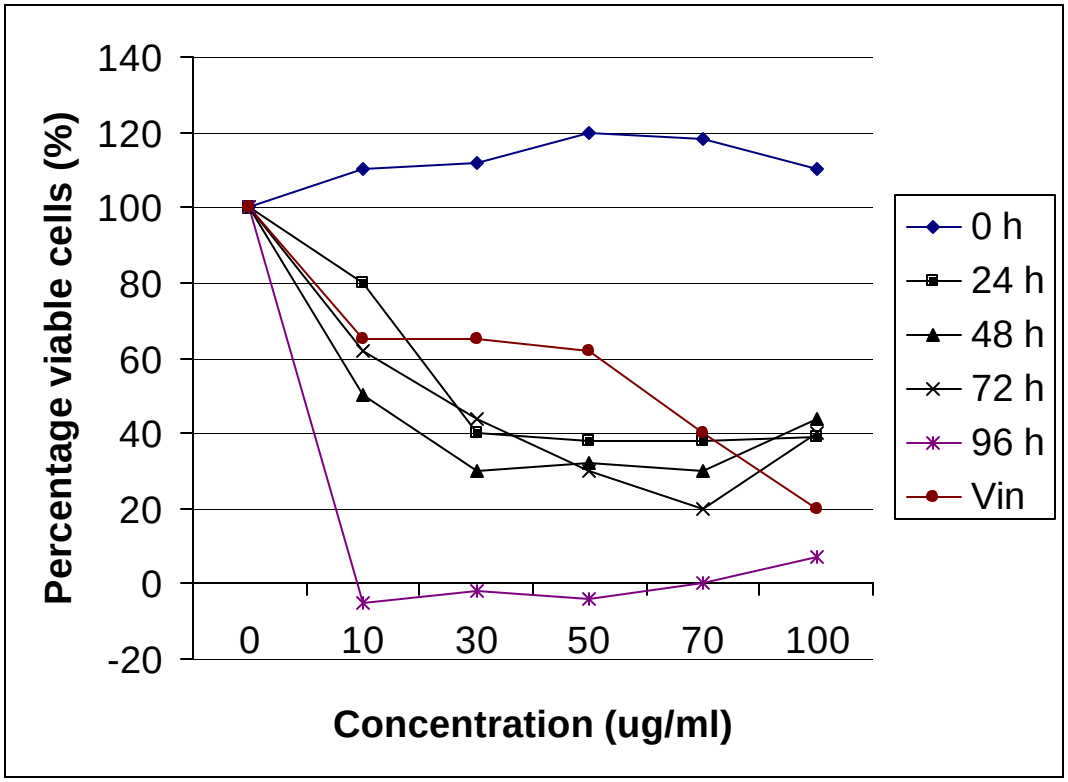

Figure 1. Cytotoxic effect of *Gelsemium elegans* leaf extract in CaOV-3 ovarian carcinoma cell line. Cells were treated at a concentration range of 0-100 µg/ml for 96 h (with 24 hourly observations). Results are expressed as % viability of the solvent treated control cells. The data shown represent mean ± SEM of three independent experiments. (vin = vincristine effect on CaOV-3 was done for 96 h incubation).

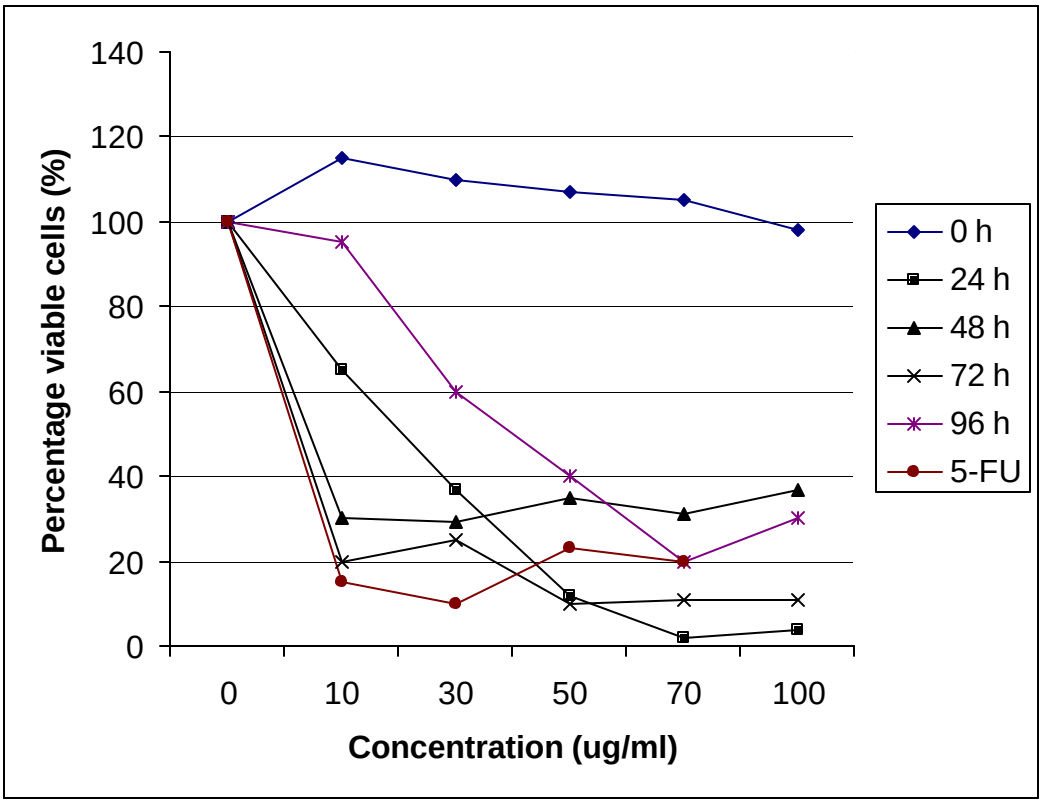

Figure 2. Cytotoxic effect of *Gelsemium elegans* leaf extract in MDA-MB-231 breast carcinoma cell line. Cells were treated at a concentration range of 0-100 µg/ml for 96 h (with 24 hourly observations). Results are expressed as % viability of the solvent treated control cells. The data shown represent mean ± SEM of three independent experiments. (5-FU= 5-fluorouracil was done for 96 h incubation)