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Antiproliferative and Apoptotic Activity of Crude Methanolic Extract of *Pandanus odoratissimus* Linn. on Human Oral Cancer Cells

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Abstract

To evaluate the anticancer activity of crude methanol extract of *Pandanus odoratissimus* Linn. on a human oral cancer cell line (KB). Oral cancer is one among the various cancers that affect majority of the population in India. In order to overcome toxicity of chemotherapy and disfiguration through surgical procedures, the researchers are targeting phytochemicals because of their anticancer properties. Cell growth was observed when the methanol extracts was 12.5 µg/mL (32%, 36% and 39%), 25 µg/mL (42%, 45% and 46%), 50 µg/mL (45%, 50% and 55%) and 100 µg/mL (57%, 61% and 62%) after 24 h, 48 h and 72 h treatments respectively, at extract concentrations. The IC50 values of 100 µg/mL, 50 µg/mL and 25 µg/mL were determined for 24 h, 48 h and 72 h treatment, which indicated that the extract is only highly cytotoxic after 72 h treatment. Apoptotic cell death is a highly organized physiological process to eliminate damaged or abnormal cells.

Keywords: *Pandanus odoratissimus*; KB cell lines; Apoptotic activity

Introduction

Cancer is characterized by a rapid and uncontrolled formation of abnormal cells, which may mass together to form a growth or tumour, or proliferate throughout the body, initiating abnormal growth at other sites. If the process is not arrested, it may progress until it causes the death of the organism [1]. Oral cancer can result from poor life style choices such as smoking tobacco and consumption of alcohol that are considered as major risk factors in the development of this type of cancer [2,3]. Tobacco contains known carcinogens such as N'-nitrosonornicotine and aromatic hydrocarbon benzo-pyrene, N-nitrosomine (4-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK). These compounds have been linked to oncogenesis capable of inducing tumors of the oral and nasal cavities, lungs, esophagus and pancreas [4]. Biological agents can also cause oral cancer. Genetic predisposition is another important risk factor in the development of oral cancer. Evidence shows that certain individuals inherit genetic dispositions that result in the inability to metabolize carcinogens as well as repair DNA damage thus causing cancer. The *human papillomavirus* was able to cause oral carcinoma [5]. Treatment of oral cancer has been achieved through the use of either surgery or radiation or both depending on the severity of the disease. These treatment options result in side effects such as vomiting, nausea, hair loss, fatigue, mouth sores and complications like mucositis [6].

Apoptosis or programmed cell death is a highly organized physiological process to eliminate damaged or abnormal cells. It also plays a major role in embryogenesis where apparently normal cells undergo apoptosis. It is involved in maintaining homeostasis in multicellular organisms [7].

Traditional medicine refers to the application, approach, knowledge and belief in incorporating plant and animal based properties in remedies for the purpose of treating or preventing disease as well as to maintain the well-being of an individual. Herbal remedies have been used to cure a variety of disorders like diabetes, sexual malfunction, and urinary tract infections in females, cardiovascular diseases, and weight control and used to cure many other ailments. Plants have a long history of use in the treatment of cancer. Plants have played an important role as a source of effective anti-cancer agents and it is reported that over 60% of currently used anti-cancer agents are derived from natural sources, including plants, marine organisms and micro-organisms [8].

Pandanus odoratissimus L. is said to be a restorative, deodorant, indolent and phylactic, promoting a feeling of wellbeing and acting as a counter to tropical lassitude. It may be chewed

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as a breath sweetener or used as a preservative on foods. It is also said to possess healthful properties, including antiviral, anti-allergy, antiplatelet, anti-inflammatory, antioxidant and antitumor activity [9].

Materials and Methods

Collection of plant material

Aerial leaf parts of *Pandanus odoratissimus* Linn. sps. were collected from Gurmitkal, near Gulbarga, north Karnataka, India. The botanical identification was made by Dr. Shiddamallya N, Scientist, National Ayurveda Dietetics Research Institute (NADRI), Bangalore. A voucher specimen was deposited in department (RRCBI- 12749).

Preparation of extracts and stock solutions

The extract was obtained by infusion and maceration from 200 g of plant material. The material was weighed chopped and extracted with solvent. The infusion was prepared with 50 gm of dried leaves in 2 ml × 200 ml of methanol respective to its temperature and solid matters were removed by filtration. After this preliminary step, the same plant material was extracted in boiling distilled water at the same condition and the maceration was done following the aforementioned process at room temperature 28°C overnight. The solvent was removed by rotary evaporation. The yield (w/w) of the infusion and maceration of methanol was 3.78 and 1.78 respectively in terms of newly collected plant material.

Dimethyl sulfoxide (DMSO) was used to dissolve each extracts and sterilized using 0.22 µm syringe filters (Axiva, Scichem Biotech) for further use.

Antiproliferative assay

A KB (oral cancer) cell line was procured from National Centre for Cell Science (NCCS) Pune, used to study antiproliferative activity. Cells were grown in DMEM-F12 (Sigma, Germany), having 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin solution. Cells were incubated in CO_2 incubator containing 5% CO_2 at 37° temperature.

Maintenance of KB cell lines

Cell culture flasks were selected by the method of confluency. They were observed under inverted microscope. In order to maintain cell line, the technique of subculturing was performed. This is to facilitate cell growth by extraction of cells from the existing medium and placing them into a fresh new medium. For cell maintenance, enzymatic methods using TPVG were prevalently used. Growth medium is then extracted completely from the flasks and the cells were further subjected to incubation at 37°C after the addition of the enzyme. This, initially, detach the cells from the surface.

Cytotoxic activity

In MTT assay, cytotoxicity of methanol extracts of all selected plant extracts were determined on KB (oral cancer) cells depended on the reduction of MTT dye (Sigma, Germany) by calculating the quantity of insoluble formazan produced in live cells. Briefly, in 96-well plate the cells were inoculated in 100 µl at number varying from 10,000 to 20,000 cells/well based on cell growth characteristics. Then the 96-well plates were kept at 37°C and 5% CO_2 humidified incubator for 24 h before addition of plant extracts. Furthermore, methanol extracts dissolved in 2% DMSO were added into a 96-well plate at different concentration (25, 50, 100, 200 µg/ml) by diluting it in medium. After 24 h, in each well 20 µl of MTT reagent (5 mg/ml) was added. After 4 h of incubation 100 µl DMSO was added, this was followed by 1 h of incubation at 37° to dissolve formazan crystals. Using an ELISA plate reader (Promega, USA) absorbance was taken at 570 nm and background wavelength at 750 nm [10].

Following equation was used to calculate the percentage of cell viability: Cell viability (%) = (OD sample (mean)/(OD control (mean) × 100, where, OD is the optical density; OD of sample: Mean absorbance of treated cells and OD of control: Mean absorbance of control cells.

Treatment of KB cells with *Pandanus odoratissimus* extract: Aliquot of the extract (25 micro grams and 50 micro grams) was added and incubated with KB cell lines.

Isolation of DNA

1 × 10 to the power of 6 cells were incubated with 100 µl of cell lysis buffer at room temperature for one hour. This was centrifuged for 15 min at 3000 rpm at 4°C to sediment the cell debris. To the supernatant equal volume of phenol: chloroform: isoamyl alcohol mixture was added to the supernatant and mixed well. This was centrifuged at 5000 rpm for 15 min. The supernatant was transferred to new tube. The 3rd step was repeated once. To the final aqueous phase 40 µl of 3.5 M ammonium acetate was added, to this ice cold isopropanol was added to precipitate the DNA. This was incubated at -20°C for 1hour, followed by the centrifugation at 10000 rpm for 15 min. The pellet was retained and washed with 70% ethanol and stored in 20 µl to 50 µl of TE buffer. The samples were analyzed in 2% agarose gel stained with Ethidium bromide.

DNA fragmentation by agarose gel electrophoresis

The extracted DNA is loaded to Agarose gel with the loading dye, DNA fragments was visualized under UV transilluminator. DNA fragmentation was observed with all the two concentrations of *Pandanus odoratissmus* extract on oral cancer cell lines by agarose gel electrophoresis method. Apoptosis has been characterized biochemically by the activation of a nuclear endonuclease that cleaves the DNA into multimers of 180 to 200 base pairs and can be visualized as an 'oligosomal ladder' by standard agarose gel electrophoresis. This proves that *Pandanus odoratissmus* extract shows apoptotic activity on the oral cancer cells by degrading its DNA [11].

Results

Cytotoxicity study was performed using *Pandanus odoratissimus* methanol extracts of on KB cell line. In some cases, dose-dependent inhibition of KB cell growth occurred, especially when the cells were treated at higher concentrations. Cell growth was observed when the methanol extracts was 12.5 µg/mL (32%, 36% and 39%), 25 µg/mL (42%, 45% and 46%), 50 µg/ mL (45%, 50% and 55%) and 100 µg/mL (57%, 61% and 62%) after 24, 48 and 72 h treatments at concentrations. The IC50 values of 100 µg/mL, 50 µg/mL and 25 µg/mL were determined for 24, 48 and 72 h treatment, which indicates that the extract is only highly cytotoxic to KB cells after 72 h treatment.

DNA Fragmentation Analysis

Induction of apoptosis on KB cells by Pandanus odoratissmus Linn Sps. extracts was validated by DNA fragmentation analysis using gel electrophoresis technique. The DNA bands obtained from both extract-treated KB produced ladder pattern as observed from Lane 1 to 4 (Figure 1). A ladder formation was used to indicate that the DNA has undergone fragmentation, and each fragment corresponded to a band in the ladder.

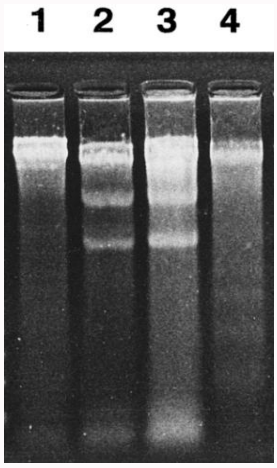

Figure 1: The DNA fragmentation by agarose gel electrophoresis of methanolic extract of *Pandanus odorattissimus*. Lane 1- 1 kb ladder, Lane 2- DNA from cells treated with 25 µg sample, Lane 3- DNA from cells treated with 50 µg sample, Lane 4- DNA from untreated.

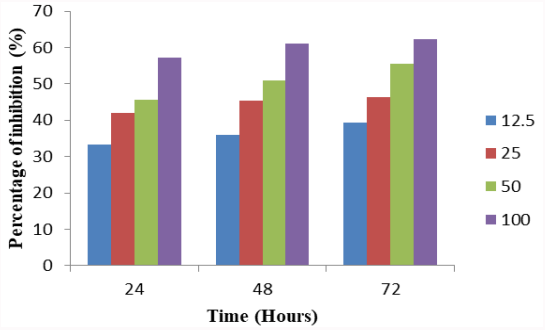

Figure 2: Cell growth inhibition for *Pandanus odorattissimus* methanol extracts against KB cell lines at 24 h, 48 h and 72 h.

The DNA fragmentation by agarose gel electrophoresis of methanolic extract of *Pandanus odoratissimus* (Figure 2). Lane 1 to 1 kb ladder, Lane 2-DNA from cells treated with 25 µg samples, Lane 3-DNA from cells treated with 50 µg samples, Lane 4-DNA from untreated.

Discussion

Apoptosis is a programmed cell death that specifically removes or eliminates unwanted or dead cells. Unlike shrunken cells, the characteristics of apoptotic cells include condensation of the cytoplasm and nucleus, aggregation of chromatin and formation of membrane-bound vesicles called apoptotic bodies [12]. In contrast, necrosis denotes a pathological activity. Necrosis is known to be pro inflammatory and is characterized by cell swelling that is often accompanied by chromatin condensation. Necrotic cells eventually developed cellular and nuclear lysis followed by inflammation [13], which would be disadvantageous for an anticancer agent.

Many plant derived cancer drugs have been clinically useful; include vinblastine irinotecan, topotecan and paclitaxel [14,15]. Various biological processes and biological interactions occur between flavonoids, phenolic compounds or polyphenols, with enzymes, proteins that make them toxic to the cell or inhibit the growth of cells [16].

The conventional anticancer drugs that are used act on both normal cells and tumor cell and cause brutal side effects and tumor resistance. Anticancer activities through apoptotic induction by herbs show no side effects [17]. Natural herbs can be used extensively to prevent and treat cancer. Caspases are responsible for the programmed cell death or apoptosis. It starts with the activation of intrinsic and extrinsic paths. Once activated, these destructive proteases systematically break down the cell to ensure its effective removal without damage to surrounding cells and tissues. Hence *Pandanus odoratissmus* helps by producing these caspases to induce apoptosis in the oral cancer cell line.

The current study showed that the presence of phytochemicals in selected plant leaf extract has an effective cytotoxic activity against oral cancer cells. The exact compounds responsible for the plant's anticancer activity will help in the search for new anticancer agents.

Conclusion

The mechanism that can represent a possibly viable approach for effective tumor treatment that has several advantages over conventional therapies and the more current "designer" approaches. The secondary metabolites of herbs are always promising with antioxidant and anticancer activity. The ability of apoptotic induction by *Pandanus odoratissmus* extract can be used in anticancer formulation. The cancer inhibition and antioxidative effects of certain medicinal herbs can therefore be used to treat trauma over a longer period of time, which is always very promising. Therefore, *Pandanus odoratissmus* can be used in the treatment of cancer.

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