Chapter 5

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5. MECHANISM OF ANTICANCER ACTIVITIES OF

SOLANUM XANTHOCARPUM AND TRIBULUS TERRESTRIS

5.1. Introduction

Plant-derived natural phytochemicals and plant extracts have gained significant recognition in the management of several human clinical conditions since they can act on specific and/or multiple molecular and cellular targets. Plants have been a prime source of highly effective phytochemicals, which offer great potential in the fight against cancer by inhibiting the process of carcinogenesis through the up regulation of cytoprotective genes that encode for carcinogen detoxifying enzymes and antioxidant enzymes and through inhibition of aberrant cell signal transduction pathways.

The mechanism of possible herb-induced anticancer effect includes cell cycle distribution and apoptosis. Apoptosis is an important mechanism that balances cell division and cell death for controlling the tissue kinetics (Hengartner, 2000; Kerr et al., 1972). The underlying mechanism of the extract to inhibit cell growth was further investigated and demonstrated that the growth inhibition of A549 cells was induced by apoptosis which is one of the most common anticancer effects, a pathway previously reported with some other plant extracts (Cheng et al., 2004; Eom et al., 2008; Ma et al., 2008; Sa et al., 2008, Zhao et al., 2008). It is regulated by several effector proteins including caspase enzymes, such as caspase 3.

Apoptotic cell death is morphologically defined by chromatin condensation, nuclear fragmentation, shrinkage of cytoplasm and formation of apoptotic bodies. The apoptotic events were analyzed using various staining procedures to study the cytotoxicity and cell viability, extent of apoptosis, morphological changes, nuclear changes and DNA fragmentation, which occurs at the final stage of apoptosis. The development of human cancers is often mainly a consequence of deregulated cell cycle
control and/or suppressed apoptosis (Gryfe et al. 1997). Impairment of apoptosis is being related to cell immortality and carcinogenesis, the induction of apoptosis in neoplastic cells is therefore, important in cancer treatment (Kamesaki, 1998).

One of the current strategies for the treatment of human cancers is to activate the cellular apoptotic death program (Kaufmann SH and Earnshaw 2000, Toomey M and Simony, 2002). Currently applied radiation therapy and standard chemotherapeutic drugs kill some tumor cells through induction of apoptosis. Unfortunately, however, the majority of human cancers are resistant to these therapies (Harrison, 1995). It is therefore urgent to look for novel natural or synthetic apoptosis-inducing compounds as candidate antitumor agents. Apoptosis is induced in vitro by incubating the cells with chemicals such as antitumor agents/oxidants (Radar and Parma, 2011). Along this line, plant-derived compounds have great potential to be developed into anticancer drugs because of their multiple mechanisms and low side effects (Darzynkiewicz et al., 2000).

Previous studies of Solanum xanthocarpum and Tribulus terrestris revealed that these plants have high antioxidant potential, cytotoxicity and antitumor activity. In order to understand whether the cell death is due to apoptosis in the present investigation, the effect of the methanolic extract of these plants were tested on chromatin condensation and cell cycle analysis of MCF -7 cell line. Nuclear condensation analysis was done using Hoechst 33342 dye and cell cycle analysis was done by fluorescence activated cell sorter using dye Propidium Iodide.

5.2. Materials and methods

5.2.1. Nuclear condensation analysis

Nuclear condensation analysis was done using Hoechst 33342 dye. Hoechst 33342 (2'-[4-ethoxyphenyl]-5- [4 - methyl- 1-piperazinyl] - 2, 5' - bi-1 H-benzimidazole trihydrochloride trihydrate) is a cell-permeable DNA stain that is excited by ultraviolet light and emits blue fluorescence at 460-490 nm. Hoechst 33342 binds preferentially to adenine-thymine (A-T) regions of DNA. This stain binds into the minor groove of DNA and exhibits distinct fluorescence emission spectra that are dependent on dye: base pair ratios. Hoechst 33342 is used for specifically staining the nuclei of living or fixed cells.
and tissues. The cells require no permeabilization for labeling, but do require physiologic conditions since the dye internalization is an active transport process.

- Hoechst 33342 dye stock concentration of 1 mg/ml.
- Dilute Hoechst Intermediate Stock Solution to the target concentration for staining in DMEM. Concentration 1 µg/ml prepared in serum free Opti MEM.
- Drug addition was carried out in different cell lines with respective drugs for 24 hours incubation period.
- After the incubation period 100µl volume of Hoechst 33342 Working Solution was added to completely cover the sample in the 96 well tissue culture plates.
- Place aluminum foil over the sample to protect it from light and incubate at room temperature (37 °C) for 15-20 minutes.
- Samples were analyzed for nuclear condensation using fluorescent microscope with excitation by ultraviolet light.

5.2.2. Cell cycle analysis

The most commonly used dye for DNA content for cell cycle analysis is Propidium Iodide. Propidium Iodide is a red color stain which is used in cell cycle analysis to determine in which phase of the cell cycle, the arrest is occurring. It intercalates with major groove of DNA strand and produces highly fluorescent adduct which can be excited at 488 nm with a broad emission centered on 600nm. Since PI can also intercalate with RNA, it is necessary to treat cells with RNase for optimal DNA resolution.

The cells treated with drugs were collected after 24 hours, by trypsinization. After centrifugation the pellet was resuspended in 1ml of ice cold 1 X PBS. To the pellet after centrifugation, 0.3 ml of cold 1X PBS and 0.7 ml of 70 % ice cold ethanol was added. After 45-60 min. incubation on ice, the tubes were centrifuged and the pellet was washed with 1X ice cold PBS. The pellet was resuspended 5µl of 10mg/ml of RNase A (final conc.: 0.2-0.5 mg/ml) dissolved in 0.25 ml of 1X ice cold. After 30 minutes incubation at 37°C, 5µl of 1mg/ml PI stain was added and kept at dark for 5 minutes. Using a 0.75µm
filter, the suspension was filtered after diluting with 1X cold PBS. The tubes were then loaded for analysis in a Flow Cytometer.

5.3. Results

Under experimental conditions a decrease in proliferation rate was observed at 24 hour for *S.xanthocarpum* and *T.terrestris* extracts. Both extracts showed 100% inhibition at the concentration of 200 µg/ml (Fig.5.3.1, Fig.5.3.2.). By the staining with Hoechtle dye nuclear fragmentation and condensation was observed. In cell cycle analysis by propidium iodide, in G0 stage DNA fragmentation occurred but the result was not significant from the control (Fig.5.3.3.). In G1 phase and S phase of cell cycle there is decrease in DNA content. But at the G2 phase of the cell cycle, DNA content was 63.25%, 14.9% and 21.3% for control, *S.xanthocarpum, T.terrestris* treatments respectively. Both extracts had drastic effect in inducing apoptosis by blocking the transition of S phase to G2 phase and *S.xanthocarpum* was more effective than *T.terrestris* (Fig.5.3.4.).

5.4. Discussion

Over the past few years, flavonoids have been demonstrated to act on multiple key elements in signal transduction pathways related to cellular proliferation, differentiation, cell-cycle progression, apoptosis, inflammation, angiogenesis, and metastasis; however, these molecular mechanisms of action are not completely characterized, and many features remain to be elucidated (Ramos 2008). It has been shown that flavonoids can trigger apoptosis through modulation of several key elements in cellular signal transduction pathways linked to apoptosis. Earlier studies suggest that flavonoids may exert regulatory activities in cells through actions at different signal transduction pathways such as cyclin-dependent kinases, caspases, Bcl-2 family members, epidermal growth factor/epidermal growth factor receptor, phosphatidylinositol-3-kinase/Akt, mitogen-activated protein kinase (MAPK), and nuclear factor kappa B, which may affect cellular function by modulating genes or phosphorylating proteins (Gupta *et al.*, 2003). It has been shown repeatedly that antioxidants and their derivatives, especially flavonoides selectively induce apoptosis in cancer cells but not in normal cells in culture (Arabinda *et al.*, 2010).
Fig. 5.3.1. Nuclear condensation analysis on MCF 7 cell lines

(a) Control
(b) S. xanthocarpum 200 µg/ml
(c) Control
(d) T. terrestris 200 µg/ml
Fig. 5.3.2. Cell cycle distribution of MCF 7 cell lines after 24 h treatment. Control (A) with *S*. *xanthocarpum* (B) *T*. *terrestris* (C) methanolic extract. Cell phase distribution was determined by PI staining and FACS analysis. X axis represents DNA content and Y axis represents cell count. P1 represents cells which have been stained and detected via PI staining. Constitute 34.3% of cells. P2. Cells undergoing apoptosis by entering G0 cell cycle arrest. P3-G1 phase will have largest population of cells with minimum DNA content. P4-S phase have minimum population of cells with high DNA content. P5-G2 phase...
Fig 5.3.3. Cells undergoing apoptosis at different stages of cell cycle.
Plant steroids have been thoroughly described for their pharmacological properties, and special attention has been given to their potential for cancer chemoprevention, especially as apoptosis inducers. Apoptosis is considered to be the major process responsible for cell death in various physiological events. Diosgenin, a plant steroid, altered cell cycle and induced cell cycle arrest in different human cell lines by p53 and p21 activation. The mechanism of action was caspase 3 dependent, but also this molecule caused a nuclear localization of apoptosis inducing factor with a fall of mitochondrial membrane potential (Sandra et al. 2001).

Two types of cell death, necrosis and apoptosis, have been discriminated based on cell morphology (Wyllie 1991). Apoptosis is characterized by alterations of nucleus morphology organelle relocalization, and cell fragmentation whereas necrosis is characterized by cellular swelling, organelles alterations, and leakage of the cellular component (Francisco et al., 2006). There is a further proposition that during apoptosis there is DNA cleavage at the matrix attachment region where loops of chromosomal DNA are attached to the loops of the nuclear matrix resulting in chromatin condensation (Anton 1999). The effects of methanolic extract of S. xanthocarpum and T. terrestris on nuclear morphology in MCF-7 human breast cancer cell lines were analyzed using the membrane permeable nuclear dye Hoechst 33258 which stains both healthy and membrane disrupted cells. In extract -treated wells, apoptotic cells clearly showing nuclear condensation and fragmentation could be observed (Fig.5.3.1., Fig. 5.3.2.), indicating that the extracts reduced cell viability mainly via induction of apoptosis. Apoptosis with DNA fragmentation induced by quercetin (flavonoid) has also been demonstrated in a number of tumor and non-tumor cell types (Kuo et al. 2004). The pattern of nuclear condensation and fragmentation by the extract indicated that reduced proliferation rate was mainly due to apoptosis.

The present study is focused on ascertaining the potential mechanism by which the drugs inhibited MCF-7 human breast cancer cell proliferation rate. For this, the effects of the drugs, S. xanthocarpum and T. terrestris methanolic extracts in cell cycle distribution were evaluated. PI staining was employed to test the anticancer effect of extract in MCF-7 cancer cell lines to evaluate the nuclear changes of apoptosis, by fluorescence activated cell sorter. In cell cycle analysis after 24 h treatment with the
extract in G0 phase DNA fragmentation was observed. There is no significant variation in S and G1 phase, but in G2 phase of cell cycle significant inhibition is observed when compared to control. The steroid content of *S.xanthocarpum* and flavonoid content of *T.terrestris* may be the factors for cell cycle arrest and induction of apoptosis in these plants. Our data provide experimental evidence on antiproliferative and cytotoxic effects of these extracts on human breast cancer cells MCF -7. Furthermore, these extracts exerted their cytotoxic effects to eliminate cancerous cells by specific apoptotic cell death. Our studies demonstrate that the two extracts possesses potent anti-cancer activity and have the potential as therapeutic, due to the presence of steroids in *S.xanthocarpum* and flavonoids in *T.terrestris*. Further studies are necessary to identify the active ingredient(s) in these extracts that mediate their anti-proliferative and pro-apoptotic activities.