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Evaluation of Anti Cancer Activity of Polyherbal Formulation

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ABSTRACT

Cancer is multipathological disease that is being considered immortal in today's global medicine world. Therefore, the present study was aimed to develop and evaluate polyherbal formulation for its anticancer activity. Four polyherbal formulations namely SJT ONC (1-4) were prepared by mixing of different proportion of extracts of stem bark of *Tecomella undulata*, *Bauhinia variegata*, *Oroxylum indicum* and leaves of *Indigofera tinctoria*. All the four formulations were evaluated for their cytotoxic effect using MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay on cell lines of human colon adenocarcinoma (Caco-2) and human breast adenocarcinoma (MCF-7). Amongst all four polyherbal formulations, SJT ONC-1 (1000 $\mu\text{g mL}^{-1}$) showed significant cytotoxicity ($p < 0.001$) on the Caco-2 (63.82%) and MCF-7 (74.18%) cell lines as compared to control. Further, SJT ONC-1 was evaluated for its apoptotic activity using cell death detection ELISA PLUS assay and it showed significant increase ($p < 0.01$) in DNA fragmentation of Caco-2 (14.83 fold) and MCF-7 (3.22 fold) compared to control group. Moreover the antitumor activity of SJT ONC-1 (300 mg kg^{-1} , p.o.) was evaluated against Dimethylbenz (a) anthracene induced mammary tumors in rats fed with high fat diet. The result suggested the ST ONC-1 significantly decrease the tumor volume and the effect was comparable to the standard drug 5-fluoro uracil. Thus, the SJT ONC-1 could render prospective candidate for the therapy of cancer.

Key words: Polyherbal formulation, MTT assay, cell death detection ELISA PLUS, Caco-2, MCF-7

INTRODUCTION

Cancer was most dreaded disease of 20th century and doing so in 21st century also. Cancer is characterized by uncontrolled growth and local tissue invasion with sometimes distant metastases of abnormal form of body's cell (Dashora *et al.*, 2011). Breast cancer is globally ranked 2 amongst all cancer in terms of morbidity and mortality associated with it, with 40,000 death per year (Aini *et al.*, 2008). Breast cancer is highest affecting population in India with 12.1% incidence and 8.5% mortality whereas colon cancer has incidence (3.1%) and mortality (4.1%) (Anonymous, 2008).

Unfortunately, the current therapeutic modalities for advanced disease are limited. The available therapies for the cancer till the date include, surgery, radiation therapy, chemotherapy, hormonal therapy and alternative therapy but the currently available therapy has very serious side-effects (Sasaki *et al.*, 2002). Besides these side effects most of, these drugs are patent protected and of very high cost for common man's reach. Therefore, there is an urgent need for safe and

improved pharmaceutical or medicinal preparations for use in the treatment of cancer (Tripathi, 2003). Nowadays, cancer research is going on towards traditionally used herbal remedies in efforts to discover new therapeutic agents which are devoid of side effects associated with the current therapeutics modalities (Sunilson *et al.*, 2009).

SJT ONC (S.J. Thakkar Oncology) is a proprietary herbal blend. The name derives from SJT, for institute and ONC, word for Oncology. The preparation includes four different herbs, stem bark of *Tecomella undulata* Seem, *Bauhinia variegata* Linn, *Oroxylum indicum* Vent and leaves of *Indigofera tinctoria* Linn. So produced formulation can be able to act on multiple targets, produce synergistic effects and give safe and effective therapy for cure of cancer. Natural compounds are themselves having such properties and also having anti cancer activity at lower concentration when they are used in combination due to synergism (Bisht *et al.*, 2011). These all selected plant drugs individually reported for their anticancer activity. *T. undulata* is reported to have apoptosis activity with activation of FAS (cell surface receptor protein of the TNF family also known as CD95), Fas associated protein with death domain (FADD), caspase 8, caspase 3/7 and fragmentation of DNA (Ravi *et al.*, 2011). *B. variegata* is reported to have cytotoxic, chemo preventive activities (Pandey and Agrawal, 2009). *O. indicum* is reported to have cytotoxic activity and DNA damage protective activity (Kumar *et al.*, 2010; Uddin *et al.*, 2003). *I. tinctoria* leaves are reported to have blocked cell cycle progression in G0/G1 phase, down regulation of the activity of cyclin E dependent kinase and chemo-preventive (Kameswaran and Ramanibai, 2008). Therefore, the present work was undertaken to evaluate anticancer activity (*in vitro* as well as *in vivo*) of the polyherbal formulation SJT ONC.

MATERIALS AND METHODS

The present study was conducted from 1st November-2010 to 15th May-2011 (the duration of study was seven and half months).

Plant material: Dried stem barks of *T. undulata*, *O. indicum* and dried leaves of *I. tinctoria* were procured from the Prashant pharmaceuticals, Rajpipla, Gujarat, India. *B. variegata* was collected in the month of March from the Junagadh, Gujarat, India. The plant material was first identified by comparing their morphological and microscopical characters with description given in the different standard texts and floras (Robert and Henry, 2007; Prajapati *et al.*, 2007; Anonymous, 2007). Further the plant material was identified and authenticated by Prof. Vishal Muliya, Botany Department, Christ Science College, Rajkot, Gujarat, India. The Voucher specimen of *B. variegata* (SJTPC 32) was deposited in the department of pharmacology, S. J. Thakkar Pharmacy College, Rajkot. Stem bark of *B. variegata* was dried under the sun. All the plant material were reduced to powder (60 mesh) separately and stored in airtight containers at room temperature. Stem bark of *T. undulata* was extracted by chloroform while stem bark of *B. variegata*, *O. indicum* and leaves of *I. tinctoria* were extracted with methanol separately. All the above mentioned extracts were mixed in different proportion and prepared four polyherbal formulations, SJT ONC-1, SJT ONC-2, SJT ONC-3 and SJT ONC-4.

Example 1: *Tecomella undulata* stem bark is 50%; *Bauhinia variegata* stem bark is 17%; *Indigofera tinctoria* leaves is 17% and *Oroxylum indicum* stem bark is 16%.

Reagents/chemicals and Kit: Eagle's minimum essential medium with 2 mM L-Glutamine, 1 mM sodium pyruvate, nonessential amino acid and 1.5 g L⁻¹ sodium bicarbonate (AL 047S); Fetal bovine serum, USDA Approved (RM 9952); Trypsin EDTA (0.25% trypsin, 0.038% EDTA in hank's BSS and phenol red without Ca⁺⁺ and Mg⁺⁺⁺) (TCL 048); Penicillin; Streptomycin (A 001); Amphotericin B (A011); Dimethyl sulfoxide (MB058); MTT [3-{4,5-dimethylthiazol-2-yl}-2,5-diphenyltetrazolium bromide] (RM1131) were purchased from Himedia bioscience (Mumbai, India). Dimethylbenz [a] anthracene (D3254) was purchased from Sigma Aldrich (USA). Cell death detection ELISA PLUS kit (Version 10.0) purchased from Roche Diagnostics (Mannheim, Germany). Other reagents were of analytical grade and purchased from Merck chemicals.

Cell lines: Human colon adenocarcinoma (HBT-37) Caco-2 and Human breast adenocarcinoma MCF-7 were purchased from National center for cell science, Pune university campus, Pune, India. Cell lines were maintained and cultured in 80% complete medium containing eagle's minimum essential medium supplemented with 2 mM L-glutamine, Earl's BSS, 1.0 mM Na pyruvate, 20% fetal bovine serum, penicillin (100 units mL⁻¹), streptomycin (100 µg mL⁻¹) and amphotericin B (1000 µg mL⁻¹). The cells were harvested at ~80% confluency and sub cultured in the complete growth medium.

Animals: Virgin female Sprague dawley rats (200-250 g, age 35 days) were used. They were housed in plastic cages with filter tops under controlled conditions of a 12 h light/ 12 h dark cycle, 50% humidity and 28°C. The animals were fed standard rat chow and water *ad libitum*. The experimental protocol No. SJT/031/2011 was approved by Institutional Animal Ethical Committee (IAEC) constituted under CPCSEA.

Cell proliferation by MTT assay: The yellow tetrazolium dye MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) is reduced by metabolically active cells into the intracellular purple formazan which can be solubilized and quantified by spectrophotometric means. 1×10⁴ cells were (Caco-2 , MCF-7) plated in 100 µL of complete medium per well in a 96 well plate. Three wells were left for blank controls. One hundred microliters of different concentration of the all four formulations (200, 400, 600, 800, 1000 µg mL⁻¹) dissolved in 0.5% DMSO (Dimethyl Sulphoxide) separately were added to 96 well plate. Further the method was performed according to previously described protocol (Roy *et al.*, 2002; Khorshid *et al.*, 2011).

In cell proliferation assay, amongst four formulations SJT ONC-1 was found to be most active so it was extended for further study.

Cell death detection ELISA plus assay: The cell death detection ELISA was used to evaluate the apoptotic and necrotic activities in the cells after incubation with SJT ONC-1 (200-1000 µg mL⁻¹) for a period of 24 h. After treatment, the cells were lysed to release cytoplasmic histone associated-DNA-fragments, an indicator of apoptosis. Cellular supernatants were collected for the measurement of cell lysis material, an indication of cell necrosis. Absorbance (ABS) was read at 405 nm. Higher ABS correlated with increased apoptosis. The data are reported as a percentage of the untreated control (McFadden *et al.*, 2003).

Dmba induced mammary tumors (Nesaretnam *et al.*, 1998): Female SD rats (150-200 g of 55 day age) were divided in following three groups:

Group 1: (Control): Vehicle treated (0.25% CMC Solution)

Group 2: (Test): SJT ONC-1 (300 mg kg⁻¹ body weight, p.o.)

Group 3: (Standard): 5 flourouracil twice a week (20 mg kg⁻¹, body weight, p.o.) after development of tumor

At the first day of treatment mammary tumors were initiated by intragastric administration of a single dose of 10 mg of DMBA in 0.5 mL of sesame oil. Simultaneously all the rats were fed high fat diet to accelerate the growth of tumor. High fat diet contained 28% casein, 40% dextrose, 20% coconut oil, 1% vitamin mix, 4.5% salt mix and 0.3% methionine. As mentioned above the mentioned treatment was given to all animals daily for 12 weeks. The animals were daily examine for signs of distress or pain, but were weighed and palpated for mammary tumors at weekly intervals. Three animals from standard and one animal from control group died within 4 weeks of DMBA administration. Twelve weeks later development of mammary tumors was confirmed by breast palpation. The animals examined for the existence of tumors. All grossly visible tumors were measured. Volume of tumor was calculated as (Pereira *et al.*, 2011):

$$\left(\frac{\pi}{6}\right) \times a^2 \times b$$

Standardization of SJT ONC-1

Estimation of phenolic content: The phenolic content was measured from the SJT ONC-1 formulation (Anonymous, 2002; Adesegun *et al.*, 2007). The standard curve was prepared using standard Gallic acid.

Estimation of flavonoids: The flavonoids were estimated by the aluminum chloride method (Bahorun *et al.*, 1996).

Statistical analysis: Data were expressed as Mean±SEM. Prism statistical analysis software (Graph pad software, version 5.0) was used for statistical analysis. Effects on cell proliferation and antiapoptosis were evaluated by one way ANOVA. Tukey's multiple comparison test was used to identify significant treatment effects (p<0.05) for MTT assays and cell death detection ELISA PLUS assay.

RESULTS

Cell proliferation by MTT assay: All the developed four formulations, SJT ONC-1, SJT ONC-2, SJT ONC-3 and SJT ONC-4 were evaluated for their cytotoxicity against human adenocarcinoma cell line of colon (Caco-2) and breast (MCF-7) cancer using MTT assay. Figure 1 shows that SJT ONC-1(36.18%) outperformed having lowest cell viability of Caco-2 cells than SJT ONC-2 (50.77%), SJT ONC-3 (50.63%) and SJT ONC-4 (44.27%) at dose of 1000 µg mL⁻¹. SJT ONC-1 (1000 µg mL⁻¹) also showed significant lowest cell viability (p<0.001) on MCF-7 (25.85%) (Fig. 2).

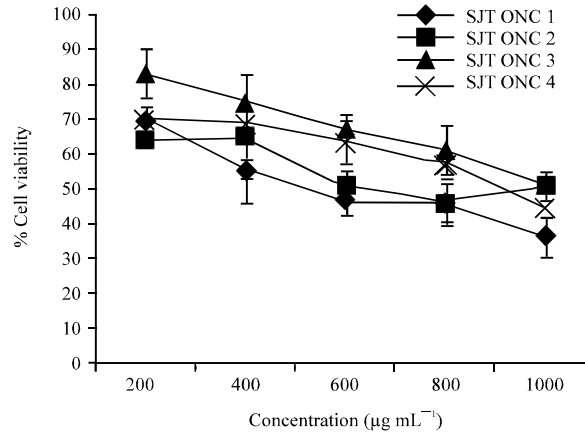


Fig. 1: Effect of the different polyherbal formulations on Caco-2 cell culture on cell Proliferation by MTT assay. Caco-2 cells were incubated in culture medium with SJT ONC 1-4 for 48 h. Cell viability was measured by MTT assay. Presented data are shown as relative cell number to control. All values represent Mean±SEM, n = 3

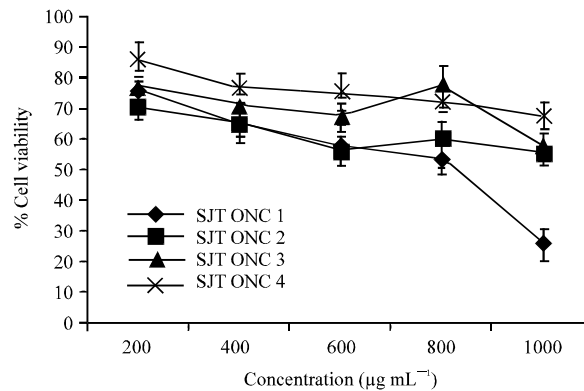


Fig. 2: Effect of the different polyherbal formulations on MCF-7 cell culture on cell Proliferation by MTT assay. Caco-2 cells were incubated in culture medium with SJT ONC 1-4 for 48 h. Cell viability was measured by MTT assay. Presented data are shown as relative cell number to control. All values represent Mean±SEM, n=3

Cell death detection ELISA PLUS: A cell death detection ELISA assay was used to determine whether the significant decrease in cell growth observed after treatment with SJT ONC-1 was the result of enhanced apoptosis in Caco-2 and MCF-7 cell lines. Figure 3 shows the maximum ($p < 0.001$) increase in apoptotic activity noted in the Caco-2 cells at a $600 \mu\text{g mL}^{-1}$ of SJT ONC -1, a 14.83 fold increase compared with the untreated control was observed. However the treated MCF-7 cells showed dose dependent apoptotic activity. SJT ONC-1 at a dose of $1000 \mu\text{g mL}^{-1}$ showed significant ($p < 0.05$) increase in apoptotic activity (3.22 fold) in MCF-7 as compared to control (Fig. 4).

Decreases in cellular necrosis were observed with both cell lines treated with SJT ONC-1. In the MCF-7 cells, decreased in necrosis were observed at all doses compared to untreated controls ranging from 200 to $1000 \mu\text{g mL}^{-1}$ (Fig. 4). While SJT ONC-1 did not show any decrease in necrosis of Caco-2 cells (Fig. 3).

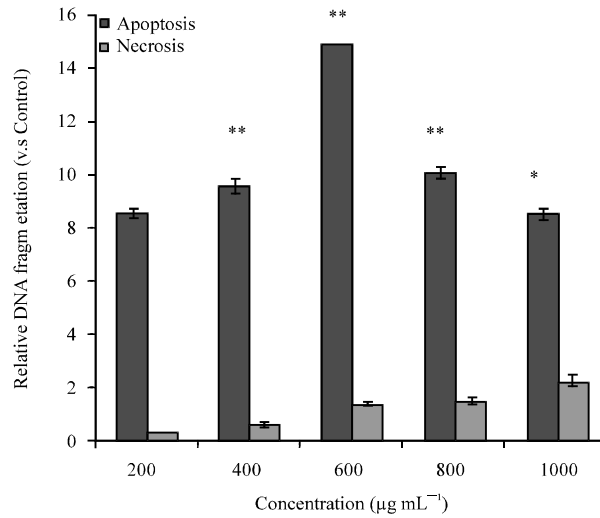


Fig. 3: Apoptotic effect of SJT ONC-1 on Caco-2 cells. DNA fragmentation in Caco-2 cells treated with SJT ONC-1 for 24 h. Relative DNA fragmentation was assigned the control to a value of 1.0. All values are represented as Mean=SEM, n=3 in each group $p \leq 0.01$ when compare with control

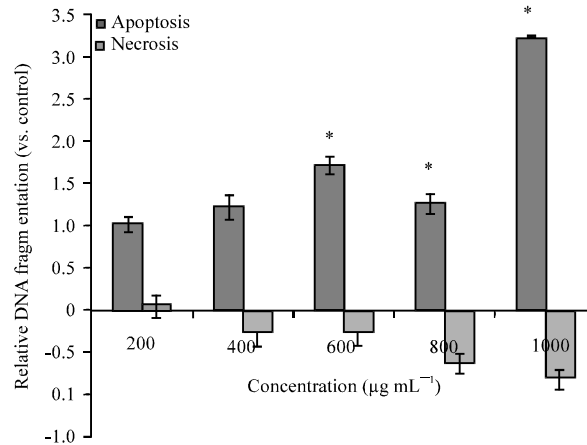


Fig. 4: Apoptotic effect of SJT ONC-1 on MCF-7 cells. DNA fragmentation in MCF-7 cells treated with SJT ONC-1 for 24 h. Relative DNA fragmentation was assigned the control to a value of 1.0. All values are represented as Mean=SEM, n=3 in each group $p \leq 0.01$ when compare with control

SJT ONC-1 (300 mg kg^{-1}) significantly reduced the tumor volume (6 CC^3) as compared to mammary cancer control rats (15 CC^3). 5-flourouracil produced maximum reduction in tumor volume (4 CC^3) (Fig. 5).

Standardization of SJT ONC-1: The polyherbal formulation SJT ONC-1 was estimated for its total phenolic and flavonoids. The total phenolic and flavonoids were found to be 2.805 and 0.347% w/w, respectively.

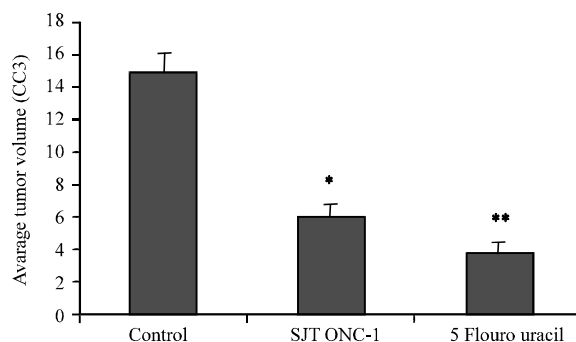


Fig. 5: Effect of SJT ONC-1 on DMBA induced mammary tumor. SJT ONC-1 and 5 fluoro uracil significantly reduced tumor volume as compared to control rat. All data are represented as Mean±SEM. * $p \leq 0.05$, ** $p \leq 0.01$ when compared to control

DISCUSSION

The components of polyherbal formulations, stem bark of *T. undulata*, *B. variegata*, *O. indicum* and leaves of *I. tinctoria* were reported individually to have potential anticancer activity. The plant materials were selected which have different mechanism and synergistic anticancer effect.

The prepared four formulations were screened for their cytotoxic activity on Caco-2 and MCF-7 cells. The result revealed that SJT ONC-1 showed significant decrease in cell viability of Caco-2 and MCF-7 cells and out performed.

The cytotoxic effects of SJT ONC-1 may be mediated by alterations in apoptosis a genetically regulated form of cell death. So, further SJT ONC-1 was evaluated for its apoptotic activity. Reductions in apoptotic activity are a hallmark of many malignancies (Thompson, 1995). In addition, the apoptotic response to DNA damage becomes more variable with age thus contributing to the development of degenerative diseases. In particular, the susceptibility to cancer is increased owing to a decline in cell response to apoptotic cells. The study revealed that SJT ONC-1 significantly increased the apoptotic activity of Caco-2 and MCF-7 cells. Apoptosis occurs via two major different activation pathways in principle (Green and Reed, 1998). One pathway involve change in mitochondrial trans membrane potential, leading to the release of cytochrome c. Cytochrome c then binds the apoptosis activating factor-1 and procaspase 9, resulting in the activation of caspase 9 by proteolytic cleavage. The other pathway start with death receptor ligation or Fas/FasL interaction, followed by oligomerization of the receptor, recruitment of Fas-associated death domain protein (FADD) and activation of caspase-8 (Ashkenazi and Dixit, 1998). Both caspase-9 and caspase-8 are defined as initiator caspase and can in turn activate caspase-3, the executor of apoptosis (Stennicke and Salvesen, 1998). *T. undulata* the major component of SJT ONC-1 is reported to have caspase 8 and caspase 7 stimulatory activities (Ravi *et al.*, 2011). Such a mode of action may also responsible for significant apoptotic effect of SJT ONC-1. Further the flavonoid fraction of *I. tinctoria* reported to arrest cells in G_0/G_1 phase which is a preceding event of the apoptosis (Kameswaran and Ramanibai, 2008) and it supports the results of increasing mono oligonucleosomes by SJT ONC-1. From the literature review, *O. indicum* stem bark extract is reported to possess cytotoxic and DNA damage protective activity. Thus it protects normal cells from cytotoxic effect. Thus it shows selective cytotoxicity against cancer cell line compared to normal cells (Kumar *et al.*, 2010). As the SJT ONC-1 contains *O. indicum* which may attribute to increase the selectivity of SJT ONC-1 towards cancer cells which is the major lacuna of chemotherapeutic agent.

SJT ONC -1 conferred apoptosis in Caco-2 and MCF-7 cancer cells can be promising anticancer therapeutics candidates, this was validated from our *in vivo* study using DMBA induced mammary tumor in rats. DMBA-induced mammary gland tumor in rodents has been widely used as an animal model for breast cancer in humans (Guzman *et al.*, 1988). DMBA is an indirect acting carcinogen, but mammary epithelial cells are capable of metabolizing it to the active proximal carcinogens (Freedman *et al.*, 1990). Thus, mammary epithelial cells efficiently activate DMBA. Data of the present experiment indicate that the control group treated with only DMBA and daily fed with atherogenic diet showed significant increase in tumor volume and appearance of first tumor was at 4.5 weeks of the DMBA treatment. There is now a body of evidence implicating dietary fat in the development of DMBA-induced mammary cancer in rats. Several mechanisms have been put forward to explain the enhancement of rat mammary carcinogenesis by dietary fat including alterations to the immune system, prostaglandin synthesis, membrane fluidity, metabolic processes and cell sensitivity to hormones or growth factors. Dietary fat can either dissolved or retain compound in it and thus could increase levels of environment pollutants in the body and leads to cancer (Welsch, 1987). The SJT ONC-1 treated animals showed significant decrease in tumor volume and supported the result of *in vitro* anticancer activity.

The observed anticarcinogenic effect of SJT ONC-1 may be due to the presence of *B. variegata* bark which is reported to contain flavonoids and lectins. Flavonoids which have been shown to have anticarcinogenic activity (Hirano *et al.*, 1989) and lectin reported to produce structural variation of the cell envelope (Sammour and El-Shanshoury, 1992) and supports the results.

Further, SJT ONC-1 was found to be rich in phenolics (2.805%) and flavonoids (0.347%) which are reported to possess anticancer activity (Hirano *et al.*, 1989; Sammour and El-Shanshoury, 1992). This may attributed the anticancer activity of SJT ONC-1.

CONCLUSION

The developed polyherbal formulation, SJT ONC-1 showed significant effect on cancer cells (*in vitro* and *in vivo*) by selectively increasing cytotoxicity through apoptosis without causing toxicities and could render prospective candidate for the therapy of cancer. Further clinical trial will be of use in establishing it as a potential anticancer formulation.

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