International Journal of Medical and Health Research
ISSN: 2454-9142
Received: 01-11-2018; Accepted: 03-12-2018
www.medicalsciencejournal.com
Volume 5; Issue 1; January 2019; Page No. 01-05

Cytotoxic and apoptotic effects of Pancha Paasana Chendhuram, a herbo-mineral Siddha medicine, against human cervical cancer cells (HeLa cells)

Venkataraman Manjari[1*], Rajagopal Pattarayan[2]
[1] Department of Nanjunoolum Maruthuva Neethi Noolum, National Institute of Siddha, Tambaram Sanatorium, Chennai, Tamil Nadu, India
[2] Department of Kulandhai Maruththuvam, Velumailu Siddha Medical College, Kancheepuram, Tamil Nadu, India
[*]Corrresponding Author: Venkataraman Manjari

Abstract
Siddha system of medicine finds important place in the management chronic ailments including cancer. I*n vitro* studies using a variety of cancer cell types have shed light on the mechanistic insights into the anticancer effect of various regimens including Siddha medicines. Pancha Paasana Chendhuram (PPC) is a herbo-mineral formula claimed to possess significant anticancer activity in Siddha system. In the present study, PPC was investigated for its apoptosis mechanism using human cervical cancer cells. PPC up-regulated Bax and p53 and down-regulated BCl2 mRNAs expression in human cervical cancer cells (HeLa cells).

Keywords: Pancha Paasana Chendhuram, cytotoxic, apoptosis, anti-cancer, siddha medicine, herbo- mineral

1. Introduction
Cervical cancer is the fourth most prevalent malignant disease affecting women, causing 10-15% of all female cancer-related deaths worldwide [1]. It is the most common malignancy with an estimated 500000 new cases and 250000 deaths annually worldwide [2]. The availability of vaccines and screening tests has reduced incidence and mortality rates of cervical cancer. About 85% of new cases are found in less developed countries, as the most of patients are diagnosed in the third or fourth stage of malignancy, where late diagnoses adversely affects the treatment. About 9/10 cervical cancer deaths occur in under developed countries [3,4]. Although chemotherapy, surgery and radiotherapy are the major therapies for the patients of cervical cancer, they impart severe toxic effect and failed to prevent metastasis. As like other cancers, metastasis and re-occurrence remains the main causes of mortality. Therefore, there is a pressing requirement to develop novel options for the early diagnosis and treatment of cervical cancer [5].
Plants are the major sources of potentially bioactive compounds and used in the treatment of cancer from ancient times [6]. Moreover, active phytochemicals are ubiquitous in nature, having less or no side effects than the currently used chemotherapeutic agents and can be taken as a part diet [7,8]. Plant-derived compounds hold great potential for further testing in clinical trials because they are target specific towards cancer cells but showed no toxicity to normal cells [8]. Siddha medical system is the first and foremost system, that possesses treatment for different life threatening diseases including cancer. It helps not only to alleviate the symptoms of this disorder, but to cure also. In the Siddha literature, cancer is mentioned in the name of *putru* (undetermined growth) which gives the direct meaning 'Termite mound' because of its proliferative nature and classified as *Arpudham* (spectacular tumors) and *Vanmeegam* (precarious tumors) [9].
Siddha medicine are obtained from 3 major divisions- Plant kingdom, Inorganic compounds (IOC) and Animal kingdom. IOC are further divided into Metals (Ulogam)-12, Minerals (Karasaram)-64, Hydrochemicals (Uparasam)-24 and Toxins (Paasanam)-120. IOCs are usually made into preparations such as parpam, chendhuram, chunnam, padhangam, kattu, kalangu *etc* [10]. Chendhuram (herbo-mineral formulations) are very vital in Siddha Medicine because (i) challenges incurable diseases, (ii) effective even in minimal dose, (iii) increased bioavailability, (iv) elevated therapeutic efficacy, (v) enhanced shelf life as compared to plant products and (vi) greater adoptogenicity. (ie) the same drug can be successfully used for various diseases [11].
Cellular *in vitro* studies using a variety of cancer cell types have offered wealth of mechanistic insights into the anticancer effect of various Siddha medicines. Pancha Paasana Chendhuram (PPC) is one among the numerous cancer drug formulations mentioned in Siddha system. In the present study, PPC was investigated for its apoptosis mechanism in HeLa cells.

2. Materials and Methods
2.1 Chemicals and cell media
Dulbecco's Modified Eagles Medium (DMEM), Phosphate Buffered Saline (PBS), fetal bovine serum (FBS), 0.25% trypsin EDTA, antibiotics (penicillin, streptomycin), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Ethidium Bromide (EtBr), Acridine Orange (AO), stain were obtained from Hi-media Lab Ltd., Mumbai, India. Protease inhibitor cocktail, bovine serum albumin (BSA), acrylamide, 2-mercaptoethanol, sodium dodecyl sulphate (SDS), N,N,N0,N0-tetramethylene diamine (TEMED), were purchased from Sigma-Aldrich (USA).

2.2 Ingredients of Pancha Pasaana Chendhuram
Minerals: Lingam (Red sulphide of mercury), Pooram (Mercury sub chloride), Velllai pasanam (White arsenic),

Thalagam (Arsenic trisulphide), Manosilai (Arsenic disulphide), Gandhagam (Sulphur), Gaantham (Magnetic oxide of Iron).

Herbal juices for grinding: Kuppaimeni (Acalypa indica), Vetrilai (Piper betel), Paruthi (Gossypium hirsutum), Vellarugu (Enicostema oxillare), Thulasi (Ocimum sanctum), Veliparuthi (Pergularia daemia), Poduthalai (Lipia nodiflora).

2.3 Procurement and collection of raw drugs

Lingam (Red sulphide of mercury), Pooram (Mercury sub chloride), Vellai pasanam (White arsenic), Thalagam (Arsenic trisulphide), Manosilai (Arsenic disulphide), Gandhagam (Sulphur), Gaantham (Magnetic oxide of Iron) are procured from K.RamasamyChetty Country drug shop, Rasappa street, Park town, Chennai.

Kuppaimeni (*Acalypa indica*), Vetrilai (*Piper betel*), Paruthi (*Gossypium hirsutum*), Vellarugu (*Enicostema oxillare*), Thulasi (*Ocimum sanctum*), Veliparuthi (*Pergularia daemia*), Poduthalai (*Lipia nodiflora*) are collected from forest area Bairnaykanpatty-Dharmapuri Dt and Mathuranthagam- Kanchipuram Dt.

2.4 Identification and Authentication

All the mineral raw drugs are identified and authenticated by Department of chemistry, Siddha Central Research Institute, Arignar Anna Govt. Hospital Campus, Arumbakkam, Chennai-600106. All the herbals are identified and authenticated by Prof. Dr.P.Jeyaraman, PhD, Plant anatomy research center, No.4, 2nd street, Sakthi Nagar, West tambaram, Chennai-600045.

2.5 Preparation Procedure of Pancha Paasana Chendhuram

Purified raw drugs - Red sulphide of mercury (*lingam)*, arsenic disulphide (*manosilai)*, magnetic oxide of Iron (*Kaantham)* arsenic tri sulphide (*thaaram*), sulphur (*gandhagam*), mercuric sub chloride (*pooram*), white arsenic (*velllai pasanam*), and were ground with herbal juices Acalypa indica (*kuppaimeni*), Piper betel (*vetrilai)*, Gossipium herbaceum (*paruthi*), Enicostema axillare (*vellarugu*), ocimum sanctum (*thulasi)*, Pergularia daemia (*veliparuthi*), and Lipia nodiflora *(poduthalai)* one after one another for 12 h till a semisolid consistency is attained. Then made into villai (tablet) and dried under shade. Dried villai were placed on the betel leaves in the mud vessel. Then the mud vessel was surmounted an equal mud vessel and sealed it clay plaster winded it for seven times. After drying, the whole set up was mounted on a stove and heated it in a mild flame (deepam pol) for 12h and allowed to cool. Then this by product was ground with *Kuppai meni* (Acalypa indica.Linn) juice for 12 hrs again drying, heating and cooling process were continued. This process was repeated for another two times to get final product as chendhuram. Then the final chendhuram was collected from the mud vessel and ground well. After that the chendhuram was stored in air tight container for further experimental use.

2.6 Cell lines and treatment

HeLa cells were obtained from National Centre for Cell Science (NCCS), Pune, India and maintained as a monolayer culture in DMEM, supplemented with 10 % FBS, in a humidified atmosphere at 37 °C and 5% CO_2. Cells were counted on a Z2 Coulter Counter (Beckman Coulter, USA) and seeded in Petri dishes and 96-well culture plates at an appropriate concentration. After overnight incubation, cells were treated with a range of concentrations (2 - 1024 µg/ml) of PPC. 24h after treatment, control (0.1% DMSO in DMEM) and PPC exposure cells were collected and analyzed. Each experiment was repeated at least three times.

2.7 Cytotoxicity assessment

3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl-tetrazolium bromide (MTT) assay ws performed as per Mosmann (1983) method. Cells were plated in 96 well plates (8000 cells/well) for 24 h at 37°C. Then cells exposed to PPC and again incubated for 18 h. MTT solution was added to each, cells were then incubated for further 4 h at 37°C. OD was determined at 550 nm/670 nm. The cytotoxicity potential of PPC was calculated:

$$\% \text{ growth inhibitory rate} = \frac{\text{control} - \text{PPC}}{\text{control}} \times 100$$

2.8 Determination of apoptotic morphological changes

Acridine orange (AO) and ethidium bromide (EBr) staining were used to detect apoptotic cells affirmation[10]. The cells were cultured in 6-well plate ($3x10^4$/well) treated with different concentration of (30 and 100 µg/ml) PPC for 24 h. The cells were fixed in methanol:glacial acetic acid (3:1) for 30 min at 4 ºC. The cells were washed in PBS, and stained with 1:1 ratio of AO/EBr for 30 min at 37º C. Stained cells were washed with PBS and viewed under a floid cell imaging station (Invitrogen, USA). The number of cells showing features of apoptosis was counted as a function of the total number of cells present in the field.

2.9 PCR

PCR mixture (final volume of 20 µL) contained 1 µL of cDNA, 10 µL of Red Taq Master Mix 2x (Amplicon) and 1µM of each complementary primer specific for Bax, Bcl2, P53, Caspase 3 and β-actin (internal control) sequence. The samples were denatured at 94ºC for 5 minutes, and amplified using 35 cycles of 94ºC for 30 seconds, 49ºC for 30 seconds, and for Bax, Bcl2, P53 renaturation was set to 53ºC and for β-Actin the renaturation was set to 55º C for 30 seconds followed by a final elongation at 72ºC for 10 minutes. The optimal numbers of cycles have been selected for amplification of these two genes experimentally so that amplifications were in the exponential range and have not reached a plateau. Ten microliters of the final amplification product were run on a 2% ethidium-stained agarose gel and photographed.

3. Results

3.1 Effect of PPC on cell viability

MTT is reduced by living cells and the resultant formazan product is proportional to the cell viability. PPC treatment (2 - 1024 µg/ml for 24 h) of HeLa cells induced a dose-dependent cytotoxicity, with approximate LD50 observed at 63.91 µg/ml (Figure 1).

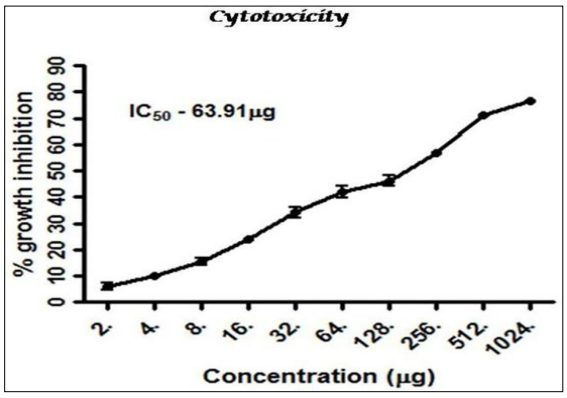

Fig 1: Cytotoxic effect of PPC on HeLa cells by MTT assay. Data are presented as mean ± SD of each of three replicates (n = 3).

3.2 Apoptotic morphological changes

Acridine orange/Ethidium bromide staining reveals distinctive characteristic of apoptotic morphology in HeLa cells. This method discriminates viable cells with uniform bright green nuclei and non-viable cells with orange to red nuclei. The results obtained from AO/EB staining are presented in Figure 2 A, B, C, D. Control cells fluoresced brightly with green nuclei and showed normal morphology. In contrast, at 30 and 100 µg/ml PPC exposure revealed a dose dependent orange luminescent apoptotic body formation and the percentage of apoptotic cells were found to be 32 ± 1.5% and 65.8 ± 3.2 % when compared to 11 ± 0.5% in control ($p<0.05$) HeLa cells.

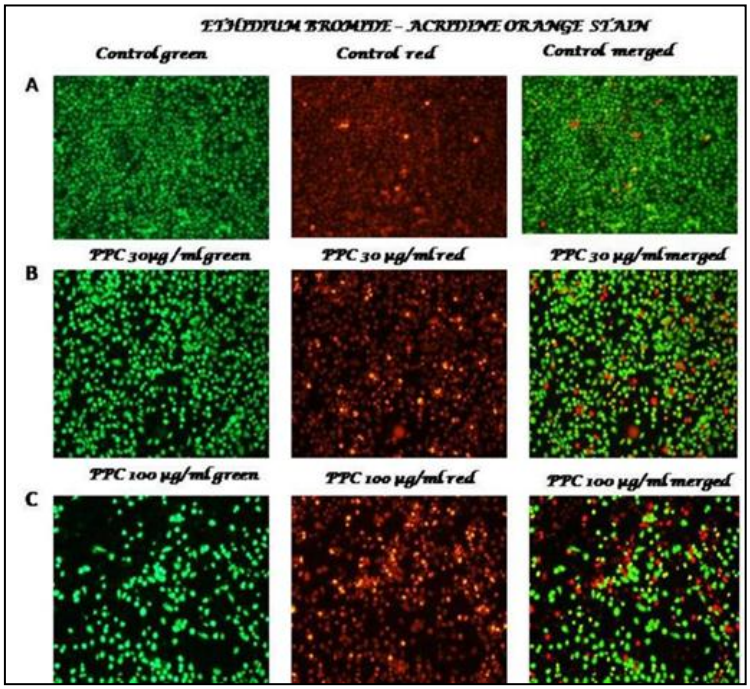

Fig 2: Photomicrograph showed the PPC induced apoptosis. (A) Control: White arrow showing normal cells, (B) 160 µg/ml: White arrow showing cells in early apoptotic phase, (C) 320 µg/ml: Yellow arrow showing cells in late apoptotic phase whereas white arrow represents necrotic cells, (D) 320 µg/ml – 1: White arrow showing cell lines in necrotic phase.

3.3 Effect of PPC on mRNA expression pattern of apoptotic markers

Bcl-2 and Bax are important members of cytoplasmic proteins engrossed in apoptosis. To analyze the anti-cancer effect of PPC on HeLa cells, we determined the mRNA expression of these pro- and anti-apoptotic markers. (Figure 3) depicts the expression of Bcl-2, p53 and Bax proteins by RT PCR analysis. PPC up-regulated Bax and p53, on the other hand it down-regualted the BCl2.

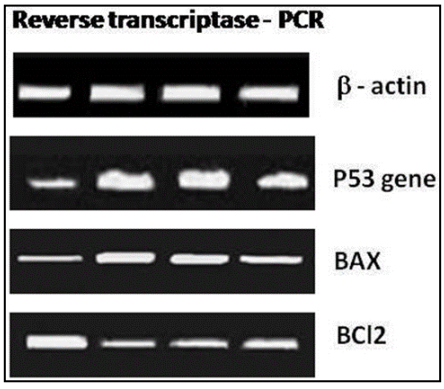

Fig 3: Dose dependent effect of PPC on mRNA expression pattern of apoptotic markers Bax, Bcl 2 and P53 in control and experimental groups. β-actin mRNA was used as housekeeping gene for the normalization of mRNA expressions. Quantification graphs Values are expressed as mean ± SD.

4. Discussion

Our results show that cytotoxicity was greatly increased in PPC treated HeLa cells, which was shows its cytotoxic potential. Reduction of the tetrazolium salt MTT to a blue formazan product is widely used for assessing cell survival. The reduction is mainly catalyzed by dehydrogenases localized in the mitochondria of viable cells [13]. Mitochondria indicates the viability and non-viability of the cells by playing a central role by controlling the cellular energy metabolism, contribution of reactive oxygen species (ROS), and release of apoptotic factors into the cytosol [14]. The results of MTT assay suggested a direct and dose dependent anti-cancer potential of PPC against HeLa cells.

Our results provided supportive microscopic evidence for apoptotic property of PPC in AO/EB staining. Acridine orange is a permeable dye which stains viable cells which will be uniformly green. During apoptosis, cell membrane becomes permeable to EtBr and the cells turn orange red after EtBr intercalating with DNA. Imbalance between deoxyribonuclease and the enzymes responsible to maintain DNA integrity occur which results in chromatin condensation and resultant cell death during apoptosis [15]. Following PPC exposure, cells succumbed to apoptosis indicating its anti-carcinogenic property.

In an apoptotic cell, Bad protein displaces Bax and binds to the antiapoptotic members Bcl-2 and Bcl-xL [16]. Bax and Bcl-2 are involved in the regulation of caspase-3 mediated apoptosis [17]. Numerous studies have shown that Bcl-2, as a negative regulator of cell death in the Bcl-2 family members, protects cells against suffering from apoptosis induced by various stimuli in a wide variety of cell types [18], whereas Bax as a positive regulator of cell death promote or accelerate cell death. Elevations in pro-apoptotic proteins, such as Bax, are believed to stimulate mitochondrial generation of ROS and contribute to neural cell death in neurodegenerative diseases. Moreover, over expression of Bcl-2 disrupts the pro-apoptotic proteins of Bax and prevent the mitochondrial release of cytochrome c, thereby inhibiting the activation of caspases cascade and apoptosis [19]. The ratio of Bcl-2 of Bax determines the survival or death of neurons following an apoptotic stimulus. PPC exposure enhanced the expression of Bax and diminished the expression of Bcl-2 in HeLa cells and thereby could induce the apoptosis. It may induce the loss of $\Delta\Psi$m and increases the mitochondrial permeability and the release of cytochrome c from the mitochondria, thereby activating caspase-9/3 and ultimate cell death. p53, also known as TP53 or tumor protein is a gene that codes for a protein that regulates the cell cycle and hence functions as a tumor suppression. It is very important for cells in multicellular organisms to suppress cancer. PPC administration enhanced the expression of p53 indicating its anti-cancer effect.

5. Conclusion

From the data it is clear that Pancha Paasana Chendhuram is cytotoxic to human cervical cancer cells (HeLa cells). It produces cytotoxicity by initiating apoptosis through up-regulation of Bax, p53 and down-regulation of BCl2 mRNA expression.

6. Conflict of Interest

Authors declare no conflict of interest

7. References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin. 2016; 66:7-30.
2. Fitzmaurice C, Dicker D, Pain A, Hamavid H, Moradi-Lakeh M, *et al.* The Global Burden of Cancer 2013. *JAMA Oncol.* 2015; 1:505-527.
3. Tay SK. Cervical cancer in the human papillomavirus vaccination era. *Curr. Opin. Obstet. Gynecol.* 2012; 24:3-7.
4. De Vuyst H, Alemany L, Lacey C, Chibwesha CJ, Sahasrabuddhe V, Banura C, *et al.* The burden of human papillomavirus infections and related diseases in sub-saharan Africa. *Vaccine*. 2013; 31:32-46.
5. Li A, Gu Y, Li X, Sun H, Zha H, Xie J, *et al.* S100A6 promotes the proliferation and migration of cervical cancer cells via the PI3K/Akt signaling pathway. *Oncol Lett.* 2018; 15:5685-5693.
6. Cragg GM, Newman DJ. Plants as a source of anti-cancer agents. J Ethnopharmacol. 2005; 100:72-79.
7. Cornblatt BS, Ye L, Dinkova-Kostova A, Erb M, *et al.* Preclinical and clinical evaluation of sulforaphane for chemoprevention in the breast. Carcinogenesis. 2007; 28:1485-1490.
8. Greenwell M, Rahman PKSM. Medicinal Plants: Their Use in Anticancer Treatment. Int. J Pharm Sci Res. 2015; 6:4103-4112.
9. Rajalakshmi S, Musthafa MM, Isai M, Sathiyarajeswaran P. A Comparative study to evaluate the anti-cancer activity of siddha drugs "Veera Rasa Padhangam" and "Panchamuga Chendhuram" with the standard drug taxol. Int. J Curr. Res. Chem. Pharm. Sci. 2017; 4:29-33.
10. Rajalakshmi P, Sudeer RG, Vadivel V, Sahayam CS, Brindha P. Analytical studies on Annabethi Chenthuram, A Siddha Herbomineral Formulation. Indian Journal of Pharmaceutical Sciences. 2018; 79:987-993.
11. Natrajan S, Kannnan M, Sathyarajeswaran P, Anandhan T. High Order Pharmaceutical Forms in Siddha System of Medicine Heritage Amruth – *FRLHT*, 2011.
12. Karthikeyan S, Kanimozhi G, Prasad NR, Mahalakshmi R. Radiosensitizing effect of ferulic acid on human cervical carcinoma cells in vitro. *Toxicol In vitro.* 2011; 25:1366-1375.
13. York RD, Yao H, Dillon T, Ellig CL, Eckert SP, McCleskey EW, Stork PJ. Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature 1998: 392:622-626.
14. Beal MF. Energetics in the pathogenesis of neurodegenerative diseases. *Trends Neurosci.* 2000; 23:298-304.
15. Pirnia F, Schneider E, Betticher DC, Borner MM. Mitomycin C induces apoptosis and caspase-8 and -9 processing through a caspase-3 and Fas-independent pathway. *Cell Death Differ*. 2002; 9:905-914.
16. Yang ZJ, Chee CE, Huang S, Sinicrope FA. The role of autophagy in cancer: therapeutic implications. *Mol. Cancer Ther.* 2011; 10:1533-1541.
17. Reed JC. Cytochrome c: can't live with it--can't live without it. *Cell* 1997; 91:559-562. DOI: 10.1016/S0092-8674(00)80442-0

18. Korsmeyer SJ. Bcl-2 initiates a new category of oncogenes: regulators of cell death. Blood. 1992; 80:879-886.
19. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. *Annu. Rev. Cell Dev. Biol.* 1999; 15:269-290.