Indian Journal of Traditional Knowledge
Vol 19(2), April 2020, pp 435-441

The modulatory effect of septilin on cytotoxicity of cisplatin in a human breast adenocarcinoma cell line

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Received 11 February 2019; revised 23 December 2019

Cisplatin (Csp) is a recurrently used chemotherapeutic drug but its use is inadequate due to undesirable adverse effects. In search of alternative medicine more attention has been given to phytochemicals. Septilin (Spt), a polyherbal drug and its therapeutic potential is huge but there is a scarcity of studies on its cytotoxic potential on cancer cells. The current study was designed to examine the effects of Spt in combination with Csp on human breast adenocarcinoma (MCF-7) and normal human breast epithelial (MCF-10A) cell lines. Cell viability for Spt treated cells was studied using MTT assay. IC_{50} value of Csp on MCF-7 cells was found to be 10 µg/mL at 24 h. This dose was further used to study the combined effects of Csp with Spt on MCF-7 and MCF-10A cell lines. Maximum cytotoxicity of Spt on MCF-7 cells was observed at Spt 5 µg/mL. The mechanism of Spt induced cytotoxicity was studied using apoptosis assay. Spt did not show any cytotoxic effects on MCF-10 A normal human breast epithelial cells, indicating Spt has no effect on normal cells. Our findings suggest that Spt can be used in combination with an anticancer drug Csp to increase its efficacy and/or to minimize its side effects on normal cells.

Keywords: Apoptosis, Cisplatin, Cytotoxicity, MCF-7, MCF-10A, Septilin

IPC Code: Int. Cl.[20]: A61K 38/00, A61K 39/395

Breast cancer is one of the major causes of morbidity and mortality among women worldwide[1,2]. Csp is among the most effective chemotherapeutic drugs widely used to treat ovarian, testicular, breast, bladder, neck, lung, sarcomas, blood vessels and bone cancers[3,4]. In cancer cells, the cytotoxicity of DNA alkylating agent Csp is due to the formation of adducts and inter/intrastrand cross links. Because of drug resistance and side effects other platinum-containing chemotherapeutic drugs like carboplatin, nedaplatin, oxaliplatin and others have also been used[5]. Csp is also used in combination with natural agents such as osthol, honey bee venome, anvirzel, vinblastine and bleomycin to produce synergistic or additive effects in death of cancer cells, without causing undesirable effects on normal cells[6].

Chemotherapy, radiotherapy, hormone therapy, immune therapy and surgery are found to be an effective modality for cancer treatment and prevention[7,8]. Due to the side effects of conventional cancer treatment therapies, it is necessary to search newer effective chemotherapeutic agent with potent activity and minimal side effects[9]. In recent days, more importance has been given for plant derived components as pharmacological agents. Plant based components are rich in nutritional and medicinal value[10]. Septilin is an ayurvedic herbo-mineral preparation from himalaya herbal health care, consisting of extracts and powders from *Maharasanadi qoath*, *Tinospora cordifolia*, *Rubia cordifolia*, *Glycyrriza glabra, Emblica officinalis*, *Moringa pterigosperma* (*Moringa oleifera*), *Balsamodendron mukul* and Shankha bhasma[11]. In the conventional health practices, Spt is being used as antibacterial, anti-inflammatory and as an immunomodulator. The herbal constituents of Spt are rich in phytochemicals including tannin, sugars, alkaloids, flavonoids and proteins[12]. Our previous study gave some insight into the protective effects of Spt against an anticancer agent cyclophosphamide induced cytogenetic damage on mice bone marrow cells[13]. Based on the earlier reports available on the

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pharmacological activities of Spt, prompted us to evaluate its cytotoxic effects and possible mechanism in induction of cell death in human breast adenocarcinoma cells.

Materials and methods

Chemicals

Spt, procured from the Himalayan drug company, India (Batch No.- 37301126B), was prepared in Dulbecco's Modified Eagle medium (DMEM). Csp (Lot No.: MKBN7276V), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), Ethidium bromide (EtBr) and Acridine orange (AO) were obtained from Sigma-Aldrich, (USA). DMEM, Fetal bovine serum (FBS) and Gentamycin were acquired from HiMedia, India.

Cell line and cell culture

MCF-7 and MCF-10A cell lines were obtained from National Centre for Cell Sciences (NCCS; Pune, India) and stock cultures were preserved in liquid nitrogen. Cells were cultured in 75 cm^2 flasks containing DMEM supplemented with 10% FBS, 0.15% sodium bicarbonate, 2.5 mL of antibiotic-antimycotic solution and 2 mL of gentamycin (80 µg/mL) and incubated in 5% CO_2 (95% humidified air) at 37^{0}C (NuAire, Plymouth, MN, USA).

Assessment of cytotoxicity - MTT assay

The cytotoxicity of Csp and Spt on MCF-7 and MCF-10A cells was measured using MTT assay as per the method described by Mosmann[14]. Concisely, cells at density of 5×10^3 cells (100 µL/well) were seeded in 96-well plates and incubated at 37^{0}C in 5% CO_2 for 24 h. After incubation, the medium was replaced with the medium containing varying concentration of Csp (1.0, 2.5, 5.0, 10 and 20 µg/mL) and Spt (0.5, 1.0, 2.5, 5.0 and 10 µg/mL). After 24 h of the treatment, cells were washed with phosphate buffered saline (PBS) and incubated for 4 h with 20 μL (1 mg/mL) of MTT at 37^{0}C. The formazon crystals formed in each well after incubation were dissolved in 100 μL of DMSO and absorbance was read at 570 nm using multi well spectrophotometer (Tecan, Austria). Each treatment was completed in triplicates and cell viability was calculated. The percentage of viable cells were used to determine the IC_{50} value, which is the concentration of Csp inhibiting 50% of MCF-7 cell growth compared with that of untreated cells (GraphPad Software, Inc., CA, USA). The obtained IC_{50} value of Csp was further used for combined treatment studies.

Apoptosis assay

Morphological changes in cells were determined by employing AO/EtBr dual staining method. For apoptotic assay, exponentially growing cells were seeded at the density of 10^6/plate in 6 cm plates and incubated at 37^{0}C in a 5% CO_2 atmosphere for 24 h. After incubation, media was aspirated and cells were treated with Csp (10 µg/mL) and Spt (5 µg/mL) and incubated for 24 h. After incubation, cells were collected by centrifugation at 1000 rpm for 5 min. Collected cells were washed with PBS and stained with AO/EtBr (1:1 v/v) dye. 100 stained cells from each treatment group were scored under fluorescence microscope at 400 X magnification and were classified as live cells (L), early apoptotic cells (EA), late apoptotic cells (LA) and necrotic cells (N) as per the method of Aithal *et al.,*[10]. Live cells have intact plasma membrane and hence, allow only AO to enter them which makes their nuclei fluoresce green under microscope. EtBr can only permeate if cell membrane integrity is lost; therefore, EA, LA and N cells were observed under fluorescence microscope as condensed yellowish-green nuclei, condensed red nuclei and as red nuclei respectively.

Statistical analysis

Statistical significance of the data was analysed by employing one way ANOVA and Dunnett's post hoc tests using Graph Pad Prism 5 (GraphPad Software, Inc., CA, USA) and data were expressed as mean±SE. Differences with a *p*-value of 0.05 or lower were considered to be statistically significant. Significance of obtained results were designated by $p<0.05$ (significant), $p<0.01$ (very significant) and $p<0.001$ (highly significant).

Results

Cytotoxic effects

In MCF-7 cells treated with Csp, a concentration dependent decrease in the viability of cells was observed. An IC_{50} value of Csp on MCF- 7 cells at 24 h time interval was found to be 10 µg/mL (Fig. 1). The cytototoxic potential of Spt on MCF-7 cells at 24 h treatment interval was also studied. In MCF-7 cells were treated with varying concentrations of Spt (0.5, 1.0, 2.5, 5.0 and 10 µg/mL), a dose dependent reduction in the viability of human breast adenocarcinoma cells at all treatments was noticed.

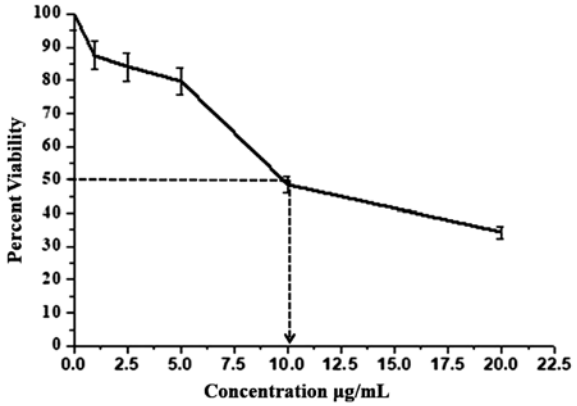

Fig. 1 — An IC_{50} curve for MCF-7 cells treated with cisplatin at 24 h. (IC_{50} value–10 µg/mL)

Table 1 — Effect of septilin and cisplatin on MCF-7 cells at 24 h

Concentration (µg/mL)	% MCF-7 cell inhibition	
	Spt	Spt+Csp (10 µg/mL)
0.5	4.31±0.012	55.4±0.487
1.0	4.62±0.058	60.9±0.796
2.5	5.49±0.069	67.0±0.198
5.0	10.1±0.080	72.4±0.731
10.0	20.9±0.096	69.5±0.986

Values are mean±SE, (n=3).

Spt at a concentration of 10 µg/mL on MCF-7 cells have shown 20.9% growth inhibition. In combined treatment groups, the maximum growth inhibition of MCF-7 cells (72.4%) was noticed at Spt 5 µg/mL+Csp 10 µg/mL concentration. The response of Spt on MCF-7 cells is clearly shown in Table 1 & Fig. 2.

Non-cancerous MCF-10A cells were also treated with media containing different concentrations of Spt (0.5, 1.0, 2.5, 5.0 and 10 µg/mL). Spt did not show any cytotoxic effects on normal MCF-10A cells, on the other hand increase in the proliferation of normal cells in a concentration dependent manner. Results indicated that, MCF-10A cells were less susceptible to the actions of the Spt. Spt in combination with Csp have minimised the toxic effect of Csp on normal cells and is evident by increased MCF-10A cell viability in combined treatment groups. However, the maximum inhibition of Csp induced cell toxicity on normal breast epithelial cells was observed at Spt 5 µg/mL concentration ($p<0.001$). Fig. 3 illustrates the cytoprotective effect of Spt on MCF 10A cells against Csp induced cytotoxicity

Apoptosis assay

Apoptosis assay was performed to determine the apoptotic morphological changes induced by Spt and

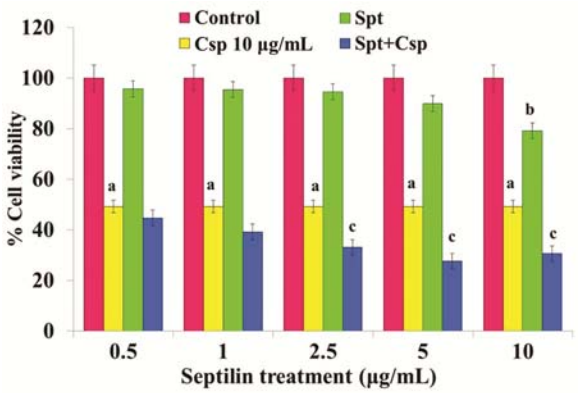

Fig. 2 — Effect of septilin and cisplatin on MCF-7 cell viability. Values are mean±SE, (n=3), One way ANOVA followed by Dunnett's post hoc test; [a]$p<0.001$ compared to control cells, [b]$p<0.05$ compared to control cells, [c]$p<0.01$ compared to control cells [d]$p<0.001$ compared to Csp treated cells, [e]$p<0.05$ compared to Csp treated cells, [f]$p<0.01$ compared to Csp treated cells.

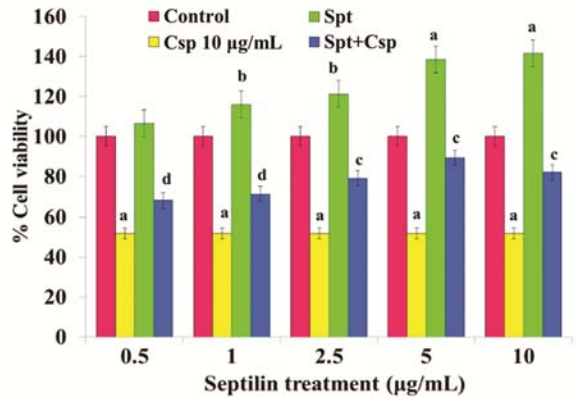

Fig. 3 — Effect of septilin and cisplatin on MCF-10A cell viability. Values are mean±SE, (n=3), [a]$p<0.001$ compared to control cells, [b]$p<0.01$ compared to control cells, [c]$p<0.05$ compared to Csp treated cells, [d]$p<0.01$ compared to Csp treated cells.

Csp on MCF-7 and MCF-10A cells using AO/EtBr staining method. The nuclei of untreated control cells were stained with acridine orange and were appear to be intact green in colour (Fig. 4 A). The cells treated with Csp (10 µg/mL), showed the presence of EA, LA and N cells with fragmented nuclei (Fig. 4 C).

The results of AO/EtBr double stained MCF-7 cells treated with Csp and Spt at 24 h are shown in Fig. 5. In which, the apoptotic morphological changes induced by Csp and Spt were expressed in percentage. As indicated in figure it is clear that, in MCF-7 cells treated with Csp (10 µg/mL) live cells percentage was found to be decreased with gradual increase in the percentage of necrotic cells. Spt (5 µg/mL) treatment decreased the number of viable MCF-7 cells ($p<0.01$) without increasing the frequency of necrotic cells

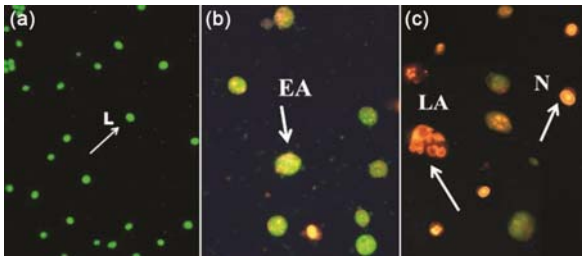

Fig. 4 — Nuclear morphological alterations in MCF-7 cells induced by septilin and cisplatin assessed using AO/EtBr staining A. Untreated control cells Live cells (L); B. Spt (5 µg/mL) treated cells showing more early apoptotic cells; C. Csp (10 µg/mL) treated cells showing more late apoptotic (LA) and necrotic (N) cells.

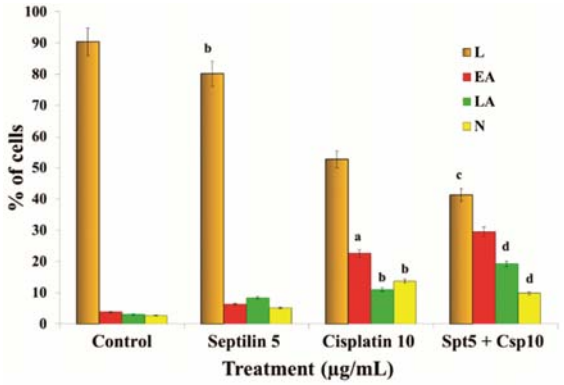

Fig. 5 — Percentage of viable, apoptotic and necrotic MCF-7 cells treated with septilin and cisplatin. Values are in mean±SE, (n=3), 100 cells scored from each treatment group. One way ANOVA followed by Dunnett's post hoc test; [a]p<0.001 compared to control cells, [b]p<0.01 compared to control cells, [c]p<0.05 compared to Csp treated cells, [d]p<0.01 compared to Csp treated cells. L - Live cells; EA - Early apoptotic cells; LA - Late apoptotic cells; N - Necrotic cells.

stained with red colour, when compared to Csp treated cells. Thus indicating, MCF-7 cell death occurred primarily through apoptosis rather than necrosis. These results are in agreement with the results of MTT assay showing the cytotoxic potential of Spt on human breast adenocarcinoma cells.

MCF-10A cells treated with Spt (5 µg/mL) did not show any nuclei morphological alteration compared to untreated control cells ($p<0.001$). Spt (5 µg/mL) enhanced the growth of normal breast epithelial cells as noticed by increased number of viable normal cells. MCF-10A cells treated with Csp revealed the presence of more number of early apoptotic, late apoptotic and necrotic cells when compared to untreated control MCF-10A cells ($p<0.001$). Spt (5 µg/mL) in combination with Csp (10 µg/mL) protected the normal MCF-10A cells against the cytotoxic effects of Csp significantly ($p<0.001$) compared to Csp alone treated cells (Fig 6).

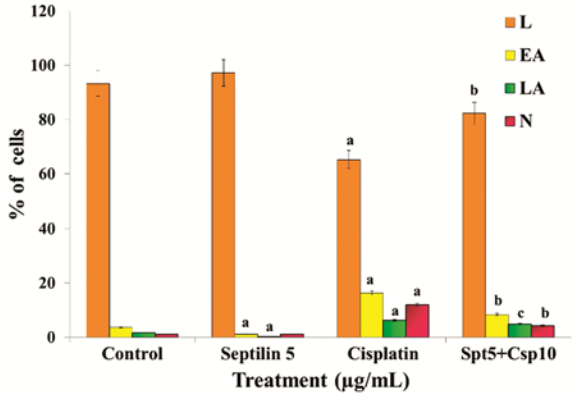

Fig. 6 — Percentage of viable, apoptotic and necrotic MCF-10A cells treated with septilin and cisplatin. Values are in mean±SE, (n=3), 100 cells scored from each treatment group. One way ANOVA followed by Dunnett's post hoc test; [a]p<0.001 compared to control cells, [b]p<0.001 compared to Csp treated cells. [c]p<0.01 compared to Csp treated cells. L - Live cells; EA - Early apoptotic cells; LA - Late apoptotic cells; N - Necrotic cells.

Discussion

Csp is anticancer medication used to treat breast, ovarian, lung and neck cancer. Improving the efficacy of Csp is crucial for the survival of malignancy patients. Csp targets the cancerous cells through inducing cytotoxic effects however; some side effects can also be seen on normal cells[15,9,16]. In the present study, 24 h after the treatment of MCF-7 cells with Csp showed decrease in the percentage of viable cells in a dose dependent manner and is evident by the results of MTT assay (Fig. 1). Csp induced cytotoxicity on cancerous cells is may be due to the activation of extracellular stress response mitogen activated protein kinase (MAPK) cascade pathway. This pathway is important for cytotoxic action of Csp[17]. As we evaluated, the concentration of Csp required to inhibit 50% growth of MCF-7 cells was found to be 10 µg/mL (Fig. 1). Csp not only showed its cytotoxicity towards cancer cells but also, it showed its cytotoxicity on normal breast epithelial cells. In clinical practice, Csp is frequently given with other chemotherapeutic drugs to enhance the efficacy of Csp on killing cancerous cells[6,18]. Synergy between two or more drugs enhances the cytotoxic effect of Csp[19].

Now a days, interest in natural compound has been grown due to the cost and side effects of conventionally used pharmacological drugs like Csp[20,21]. The bioactive compounds with known antiproliferative and antioxidant activities can be used in combination with Csp to enhance its cytotoxic effect on cancerous cells without affecting the growth

of normal cells. In line of this, recently Mirmalek *et al.,*[22] compared the cytotoxic effects of hypercin and Csp on MCF-7 cells. Hypercin, a bioactive compound and its concentration needed for 50% inhibition of MCF-7 cells was found to be less compared to Csp. In our study, to evaluate the cytotoxic potential of Spt on human breast adenocarcinoma cell line we carried out the MTT assay. Spt is a polyherbal drug formulation and in our study it displayed its mild cytotoxic effect on MCF-7 cells. Even if, there is no much anti-proliferative effect of Spt alone on MCF-7 cells but, Spt in combination with Csp have showed their modulatory effect on Csp cytotoxicity in MCF-7 cells. Spt enhanced the efficacy of Csp on cancerous cells. The maximum MCF-7 cell inhibition by Spt (5 µg/mL) in combination with Csp was found to be 72.4% (Table 1). Also, Spt have shown its distinctive mode of action on cancerous and normal cells. As indicated in Fig. 3, Spt enhanced the proliferation of MCF-10A cells in a dose-dependent manner. The cytoprotective effect of Spt on MCF-10A cells was also noticed in combination of Csp wherein, it provided significant protection against the toxic effects induced by Csp on normal cells at all the concentration tested. The increased viability of normal breast epithelial cells treated with Spt suggests its different way of action on normal and cancerous cells. Consistently with the above conclusion, Omar *et al.,*[23] reported the antiproliferative and synergistic effects of bee pollen extract in combination with Csp on MCF-7 cell line and L929 normal cell lines. The solvent extracts of bee pollen extracts showed its antiproliferative effects only on cancer MCF-7 cells devoid of causing any cytotoxic effect on normal L929 cells. The differential effects of bee pollen extract on cancer and normal cells indicate its specificity towards cancer cells.

Based on the earlier reports available on the plant constituents of Spt, the possible cytotoxic effect offered by Spt is may be because of the presence of phytochemicals such as phenolics, tannins, saponins and terpenoids. There are few supporting reports on cytotoxicity of the plant components of Spt on MCF-7 cell lines. *M. oleifera* showed its cytotoxicity on MCF-7 cells as evident by reduction in number of viable cells compared to vehicle control group. The cytotoxic activity of this plant may be due to the existence of seed essential oil[24]. Rahaman *et al.,*[25] also revealed the cytotoxic potential of *M. oleifera* seed and leaf extract on MCF-7 cells with reduction in viable cells. The presence of Glucosinolates (sulphur containing) compound in *M. oleifera* was ascribed for this effect. β-glycyrrhetic acid a bioactive component present in the *Glycyrrhiza glabra* may attributed for its cytotoxic activity against MCF-7 cells[26]. Aqueous and hydro-alcoholic extracts of *T. cordifolia* induced the degenerative morphological changes in the MCF-7 cells[27]. Kumari and Jesudas,[28] evaluated the cytotoxic and anticancer potential of *T. cordifolia* against MCF-7 cells and this study revealed that, the presence of phenolic components in this plant was responsible for its antiproliferative potential.

To understand the mechanism of cytotoxicity or cell death induced by Csp and Spt on cancer cells apoptosis assay was used. Apoptosis is one of the important genetically regulated programmed cell death that eradicates the physically damaged and abnormal cells. Cell death occurs either by apoptosis or necrosis. However, apoptosis was found to be more favourable because it does not trigger inflammatory response. In *in vitro* condition, due to lack of macrophages apoptotic cells will not be phagocytized instead cells undergo nuclear morphological changes and will be lysed. The effectiveness of anticancer drugs is measured by their ability to identify the cancer cells and to stimulate apoptosis[29]. Apoptosis is reported as one of the important mechanisms through which plants and bioactive compounds mediates anticancer effects[30].

In the present study, AO/EtBr dual staining method was followed to assess the mechanism of cytotoxicity induced by Spt and Csp on MCF-7 cells. In the AO/EtBr dual staining method, MCF-7 and MCF-10A cells treated with Csp showed increase in the percentage of necrotic cells in contrast to untreated control cells (Fig. 2). Also, it revealed the presence of cells with membrane blebbing, chromatin condensation and fragmentation (Fig. 4C). Csp induced apoptosis or necrosis is due the overload of intracellular reactive oxygen species. Csp is an alkylating agent and this kind of anticancer drug induces apoptosis/necrosis through oxidative stress mediated mechanism[10] Spt treatment augmented the cytotoxic effect of Csp on MCF-7 cells by increasing the percentage of early and late apoptotic cells compared to Csp treated cells ($p<0.001$). The reduction in the number of viable MCF-7 cells and presence of morphological apoptotic changes in the cells treated with Spt in combination with Csp indicates the cytotoxic and apoptotic potential of Spt

on human adenocarcinoma cells (Fig. 3). Cell death induced by Spt followed the sequence of early and late apoptotic morphological changes, representing cell death induced by Spt is through apoptotic pathway rather than necrotic (Fig 4B). This result is consistent with previous observations done by Ho *et al.,*[31] in which vanillin, a bioactive compound induced apoptotic pathway mediated cell death on human colorectal cancer cells.

The mechanism of action of Spt on MCF-7 cancer cells is not known however; earlier report had suggested that, plant extract induced apoptosis may be due to activation of several cellular pathways participating in induction of apoptotic and necrotic cells. This conclusion was supported by the work done by Padma *et al.,*[32] in which, mangiferin a natural polyphenol component induced apoptosis in rhabdomyosarcoma cells was through sustained oxidative stress. Cytotoxic and apoptotic effect of *Flueggea leucopyrus* on human endometrial carcinoma cells was due to imbalance between oxidative stress and antioxidant status[30]. Bhavana *et al.*[33] showed potential of *Croton bronplandianus* leaf extract induced apoptotic mediated cytotoxicity on lung cancer cell line A549. The polyphenolic compound present in this leaf extract induced the apoptosis in cancer cells by activating series of signalling pathway linked to apoptosis. Behzad *et al.*[34] revealed the cytotoxic and apoptotic potential of *Primula auriculata* extract on HT-29 human colon adenocarcinoma cells, the presence of phenolic compounds like flavonoids and saponins may attributed for its cytotoxic and apoptotic effects. Based on the above observations the possible apoptotic effect of Spt on MCF-7 cells is may be due to the enhanced oxidative stress and due to prooxidant activity of phytochemicals present in it.

Conclusion

The current study determined the potential role of Spt in enhancing the efficacy of Csp induced cytotoxicity on human breast adenocarcinoma cells without inducing any cytotoxic effect on normal breast epithelial cells. The current findings also highlight that, Spt induced cytotoxicity on cancer cells is through normal apoptosis mechanism rather than necrotic pathway. In view of the present findings, Spt can be used in chemotherapy treatment as an adjuvant to enhance the efficacy of Csp. Further studies may require to determine the molecular mechanism underlying the observed cytotoxic effects of Spt with Csp on cancerous cells.

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