The Implication of a Novel Herbal Formulation in Reversal of Drug-Resistance for Cancer Treatment

Umrao Ankita[1,2], Rao Gururaj A[3], Rao S.G.A.[2], Dasgupta Debjani[1] and Rao Jyothsna A[2*]

[1]School of Biotechnology and Bioinformatics, D.Y. Patil University, Navi Mumbai, India
[2]Sri Raghavendra Biotechnologies Pvt Ltd., Bangalore, 560064, India
[3]International Stemcell Services Ltd, Bangalore, 560027, India
*Corresponding author : jyotrao@gmail.com

Abstract

Traditional medicines or herbal medicinal compounds are being increasingly considered as useful complementary and alternative treatments for cancer. A large number of *in vitro* and *in vivo* studies have reported the beneficial effects of herbal medicines alone and in combination with conventional therapeutics. We developed a poly-herbal formulation Swastharakshak® (SR033), exhibiting anti-tumorigenic property. Although, majorly 5-Fluorouracil (5FU) resulted in necrosis of HeLa cells, however in combination with SR033, the majority of cells underwent apoptosis with an increase of 30%, SR033 alone was considered as an experimental control. Moreover, 5FU and PTX resistance were reversed by SR033 with $2.5×10^3$ and $0.17×10^3$ fold, respectively, in resistant HeLa-R cells. We observed a 2 fold decrease in free radicals when treated with SR033 as compared to untreated controls; however, 1.2 fold decrease was observed in SR033 + 5FU groups in comparison to 5FU alone.These results demonstrated that SR033 acts synergistically with 5FU, along with chemo-protective and 5FU resistance reversal property. Hence, SR033 is a potential herbal formulation that could be used effectively with 5FU as a combination therapy for cancer.

Key words: Herbal medicine, Cancer, Drug-resistant cell line, 5-Fluorouracil, Integrative therapy, Drug Resistance Reversal.

Introduction

Cancer is one of the most common non-communicable diseases and a leading cause of death worldwide. It is a second deadly disease with an estimate of 9.6 million deaths worldwide in 2018 (1). Moreover, low- and middle-income countries are at higher risk and they count approximately 70% of the cancer deaths (1). Even though chemotherapy is central to clinical management of cancer, failure in chemotherapy is not uncommon, mainly due to the dose-limiting toxicities, which is also associated with the occurrence of drug resistance. Apart from the modern technologies such as the use of nanoparticles, dendrimers to deliver chemo drug, use of natural compounds/ products to use as adjuvant enhancing chemo drug activity and to counteract drug resistance may be beneficial (2).

Cancer cells have increased levels of oxidative stress as compared to their normal counterparts. Maintaining reactive oxygen species (ROS) homeostasis is crucial as it promotes cell proliferation and differentiation at a moderate level (3, 4), whereas causes oxidative damage at higher levels (5). Currently, 313 drugs have been approved by the Food and Drug Administration to treat cancer. At least 40% of them can induce oxidative stress (6) due to their non-selective nature. Therefore, manipulating ROS levels by redox modulation is a way to selectively kill cancer cells without causing significant toxicity to normal

cells (7) by the presence of some exogenous factors, such as drugs, radiations and diet (8). ROS mediated mechanisms also provide resistance towards many anti-cancer agents (9). Additionally, the generation of endogenous ROS in combination with the drugs which initially induce ROS production may contribute to a decrease in the sensitivity to these drugs in long-term treated cancer cells (10-12). Due to these reasons, the survival rate of cancer patients is not significantly encouraging. To face this complex situation, many researchers are turning to find out an alternative treatment plan which can be used as therapeutics, as an adjuvant treatment besides other treatments, or as chemo-preventive agents (13-16), that can avoid and/or minimize the risks of unusual side effects and improve the efficiency of conventional treatment approaches (17). This constitutes a major step towards cancer management, which is holistic and comes under the ambit of integrative oncology. Various herbal compounds and their formulations are being used for effective cancer treatment, among these, epigallocatechingallate (EGCG) and curcumin are widely used.

Epigallocatechingallate, one of the most phenolic catechins present in green tea, exhibit chemo-protective and chemo-preventive action against various cell lines (18) by inhibiting the interaction of tumor promoters (ligands) with their receptors on the cell membrane, known as "Sealing effects of EGCG" (19). It also acts synergistically with anti-cancer drugs in tertiary cancer prevention (20-23). Furthermore, in combinational therapy, the presence of EGCG significantly increased the bioavailability of tamoxifen (24), 5-fluorouracil (25), and doxorubicin (26) and also showed synergistic enhancement of anti-cancer activity against human cancer cell lines (27-29). Moreover, it is suggested that from clinical trial based studies of breast cancer, EGCG shows protection against the toxic effects of chemotherapy (30). Another compound, curcumin is a polyphenol found in turmeric has broad pharmacological activities, including anti-

inflammatory, anti-oxidative, and anti-tumour effects (31). Also, it has been found that curcumin can reverse drug resistance in tumour cells from gastric, hepatic and cervical cancers as well as other malignancies (32,33).

We have developed a poly-herbal formulation, SR033 and have been tested against 5FU sensitive HeLa and resistant (HeLa-R) cell line. SR033 (patent applied, FSSAI approved manufactured by Sri Raghavendra Bio-technologies Pvt Ltd, Bangalore) is a composition of five herbal compounds which are well established anti-oxidative, anti-cancer, chemo-preventive, chemo-protective, anti-proliferative and anti-inflammatory (34-38). The formulation shows chemo-protective, chemo-sensitization, apoptotic and anti-oxidative properties against cancer cell lines.

Materials and methods

Maintenance of cells: Human cervical cancer cell line HeLa (drug-sensitive) was obtained from NCCS, Pune. Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher) in a humidified incubator at 37ÚC with 5% CO_2. Trypsin digestion with 0.25% trypsin solution was used for cell passaging. 5FU sensitive and resistant HeLa cells (HeLa and HeLa-R, respectively) were continuously cultured in 0 μg/mL and 2 μg/mL of the drug, respectively. *In vitro* experiments were carried out at 80% cell confluency and confirmed in at least three independent experiments, each performed in triplicates.

Development of resistant cells: Stably resistant cells to 5-Fluorouracil (5FU) were developed in our laboratory, as described earlier(39) with slight modifications.HeLa cells were exposed to an initial dose of 0.1 μg/mL 5FU and surviving cells were cultured for 10 days and two passages. The cells were exposed to increasing concentrations of 5 FU to 0.5 μg/mL, 1.0 μg/mL and 2.0 μg/mL (40). The surviving resistant cells were named as HeLa-R. These cells were also tested for paclitaxel (PTX) resistance.

Detection of cytotoxic effects of SR033, 5FU and PTX on HeLa and drug-resistant HeLa-R cells: Single-cell suspensions of HeLa and HeLa-R cells were prepared in DMEM medium at log phase of growth. Each well of 96-wells plate was seeded with 100 µL/well (2×10^4 cell/mL) of the cell suspension, and the plate was incubated for 24 h to allow cell adhesion. Different concentrations of Swastharkshak® (SR033) ranging 0, 20, 10, 5, 2.5, 0.625, 0.31, 0.1 mg/mL SR033 (100 µL in each well) were added to drug-sensitive and drug-resistant cells for 24 h and 48 h. Similarly, 5FU was added to HeLa and HeLa-R cell lines at different concentrations, ranging 0.015, 0.031, 0.062, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 µg/mL and 53, 106, 312, 625, 1250, 2500, 5000 µg/mL, respectively and PTX was added at concentrations 1.57, 3.13, 6.25, 12.5, 25.0, 50.0 $\times10^{-3}$ µg/mL to both the cell lines. These drugs were added individually to both cell lines to determine their IC_{50} value. The culture medium was aspirated and fresh medium was added with MTT dye to respective wells and incubated for 4 h. The complete solution was aspirated and DMSO was added to dissolve Formazan crystals. The plate was incubated for 30 minutes and values were measured using a microplate reader at OD_{545}. The IC_{50} of SR033 was calculated. IC_{50} value determines the concentration of drug at which 50% of cells survive. Drug Resistance Index (DRI) of 5FU on HeLa-R cells was calculated using the formula: IC_{50} of resistant cells/ IC_{50} of sensitive cells (41).

Determination of apoptosis using Hoechst staining: Hoechst staining was performed for qualitative and quantitative analysis of apoptosis in HeLa cells with SR033 alone, 5FU alone and SR033 in combination with 5FU. HeLa cell suspension (0.3×10^6 cells) was cultured in 35 mm dishes for 24 h to allow cell adhesion. Cells in each dish were treated with 2 mL DMEM culture medium (supplemented with 10% FBS), containing medium alone, SR033 (IC_{50}), 5FU (IC_{50}) and SR033 (IC_{50}) + 5FU (IC_{50}). After cells were cultured for 48 h, the culture medium was aspirated and cells were washed with 1X Phosphate buffered saline (PBS). Cells were then trypsinized, the pellets were spread and air dried completely on grease-free slides. The slides were treated with paraformaldehyde (4%, pH=7.4) prepared in 1X PBS for 30 minutes and then dipped in a coupling jar containing 1X PBS, three times. Each time fresh PBS was added to coupling jars. The slides were dried and Hoechst stain was added in the dark (42). The stain was further washed by using 1X PBS and slides were observed using a fluorescence microscope.

Drug resistance reversal effect of SR033 on drug-resistant HeLa-R cells: Single cell suspension of HeLa-R cells in log phase growth was prepared in DMEM and incubated in 96 wells plate (100 µL/well at 2×10^4 cells/mL) for 24 h to allow cell adhesion. Cells were treated with 600 µg/mL SR033 (100 µL) and different concentrations of 5FU (4, 2, 1, 0.5, 0.25, 0.12, 0.06, 0.03, 0.01 µg/mL) along with untreated controls. After cells were cultured for 48 h, the culture medium was aspirated and fresh medium with MTT reagent (5 µg/mL, 100 µL for each well) was added to each well. The plate was then incubated for 4 h. The complete solution was aspirated and DMSO was added to dissolve Formazan crystals. The plate was incubated for 30 minutes and values were measured using a microplate reader at OD_{545}. The drug resistance reversal ratio was calculated according to the following formula: IC_{50} in blank control group/ IC_{50} in the reversed group (43). Similarly, the experiment was performed to determine the drug reversal effect by SR033 (600 µg/mL) with respect to Paclitaxel (0.04, 0.08, 0.17, 0.35, 0.70, 1.41, 2.83, 5.66 $\times10^{-3}$ µg/mL).

Determination of Superoxide Dismutase (SOD) in HeLa-R cells : HeLa-R cells were seeded in 35 mm dishes and treated with IC_{50} values of SR033 and 5FU in different groups after 24 h incubation. Cells were trypsinized and the lysate was prepared by using RIPA lysis buffer (Thermo Scientific) (44). The supernatant was collected and the assay was performed using Cayman's Superoxide Dismutase detection kit (45). The per

cent inhibition was plotted as a function of final SOD activity (U/mL). The unknown samples' absorbance was later mapped to its corresponding SOD activity using the formula, Percent inhibition by SOD= [(control absorbance - sample absorbance)/ control absorbance]×100, where one unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The values were interpreted in terms of percent free radicals.

Statistical analysis: GraphPad Prism 5.0 was used to analyze and represent graphical data. One way ANOVA was performed, followed by Turkey's test. The experimental data are expressed as mean and standard deviation. Statistical significance was achieved when $p<0.05$.

Results and Discussion

Cytotoxic effect of SR033, 5FU and PTX on HeLa and drug-resistant HeLa-R cells: To understand the cytotoxic effect of SR033 on HeLa and HeLa-R cells, MTT assay was performed and the following analysis was undertaken at different concentrations and time points. IC_{50} of SR033 on HeLa cells was 500 µg/mL at 24 h and 600 µg/mL at 48 h. The IC_{50} of SR033 on HeLa and HeLa-R cells at 24 h was 500 µg/mL and 1200 µg/mL, respectively (Figure 1A and B). IC_{50} of 5FU and PTX was also determined by MTT assay to calculate DRI of HeLa-R. The IC_{50} of 5FU on HeLa and HeLa-R cells was 2 µg/mL and 5000 µg/mL (Figure 1C), respectively, with DRI of $2.5×10^3$. The IC_{50} of PTX on HeLa and HeLa-R cells was $5.96×10^{-3}$ µg/mL(Figure 1D) and $13.430×10^{-3}$ µg/ mL, respectively, with DRI of 2.25.In several studies, IC_{50} of 5FU ranges from 2-5 µg/mL on various cancer cell lines; moreover, this dose of 5FU is not effective on 5FU resistant cells (43,46,47). IC_{50} values of 5FU on a colon cancer cell line, HT-29 was exceptionally higher (39 µg/ mL) compared to other cell lines[48].In a recent study, it was reported that DRI of HCT-8 cells is 74.12(43). DRI obtained in this study is significantly higher than that reported earlier, higher DRI would be impactful for testing the effectiveness of any chemo-sensitizing compound. This study also reveals the multi-drug resistance of HeLa-R cells, as cells were also found resistant to PTX, in addition to 5FU. IC_{50} values obtained in this experiment were used to further determine drug resistance reversal by SR033.

Synergistic effect of SR033 and 5FU on apoptosis of HeLa cells: Apoptosis is programmed cell death and is programmed to occur in a sequentially with minimal inflammation or injury to the surroundings. Chemo-drug kills cancer cells by necrosis which also damages the nearby cells/ tissues(43,49), Curcumin and green tea extract inhibit cancer cell proliferation (49-52), eventually, cancer cell leads to apoptosis. In this study, apoptosis was qualitatively and quantitatively analyzed using Hoechst stain. Cell morphology in treated groups has been compared with media control (Figure 2A). Cells were considered necrotic and apoptotic on the basis of nuclear structure and its stain intensity. Cellular membrane was ruptured in necrotic cells, whereas was intact and brightly stained nucleus was observed in living cells. Blebs were formed and brightly stained fragmented DNA was observed in apoptotic cells. SR033 alone and 5FU alone groups are comparable with SR033 along with 5FU treated group. Cells treated with 5FU alone underwent necrosis. Cells treated with SR033 alone showed 45.9% apoptosis, whereas combination with 5FU showed 80%, which is a 30% increase in apoptosis rate (Figure 2B), showing its synergistic effect.Similar studies were performed in HCT-8 and EC9706 cells, where 15% and 20% increase in apoptosis was noticed when treated with the combination of curcumin and 5FU (43,46). SR033 showed a remarkable increase in apoptosis rate when compared with the above-reported data (43,46).
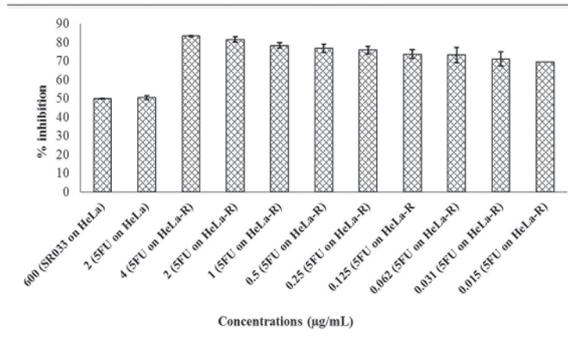
Drug resistance reversal effect of SR033 on resistant HeLa-R cells : Chemo-drugs like 5FU lead to resistance in cancer cells due to continuous dosage. Curcumin and green tea extract are known to exhibit drug resistance reversal properties (49-52). When 600 µg/mL SR033 (IC_{50} on HeLa) was added to HeLa-R with
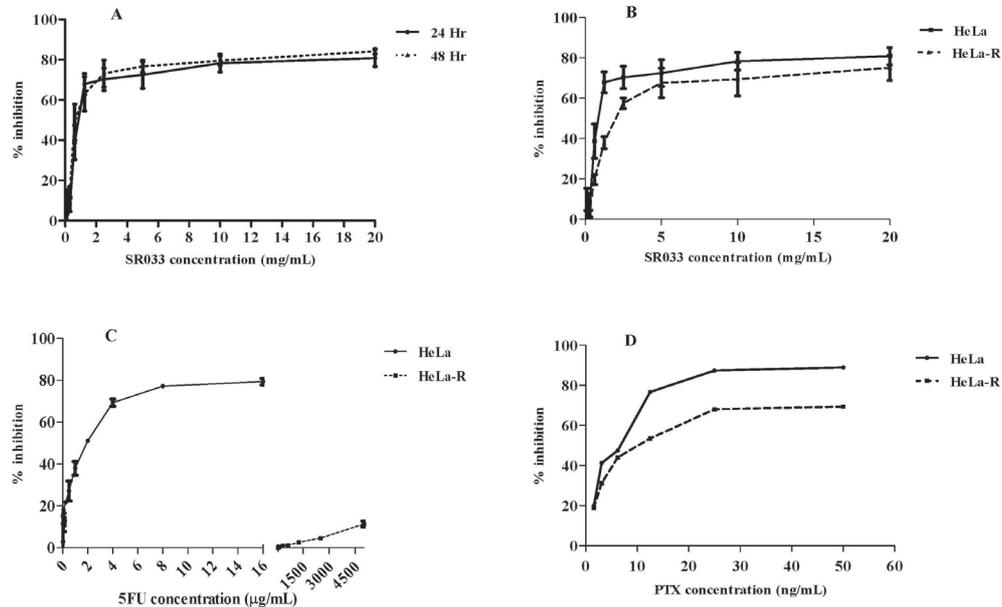
Fig. 1. Cytotoxicity assay for SR033, 5FU and PTX on HeLa and HeLa-R cells. A. Effect of SR033 on HeLa cells. B. Effect of SR033 on HeLa and HeLa-R cells. C. Effect of 5FU on HeLa and HeLa-R cells. D. Effect of PTX on HeLa and HeLa-R cells.
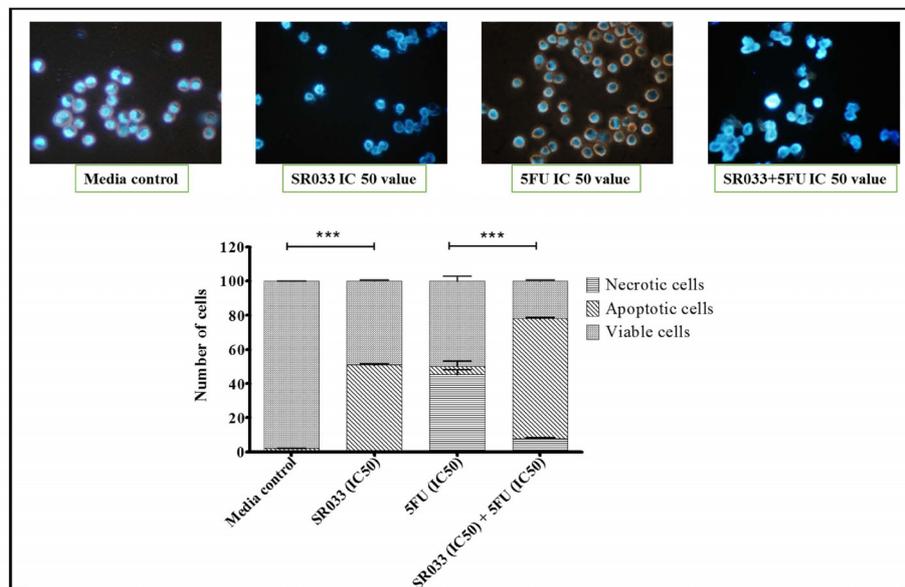
**Fig. 2.** Synergistic effect of SR033 and 5FU on HeLa cells. Hoechst staining of HeLa cells in different growth conditions (above panel). Below is the bar graph showing quantitative analysis of dead cells including apoptotic and necrotic cells and viable cells (cumulative result obtained from 10 fields of each group). $p<0.001$ (triple star) when compared media control with SR033 and 5FU with combination of SR033+5FU.

Fig. 3A. Drug reversal property of SR033 on resistant HeLa-R cells. SR033 and 5FU were used alone at IC50 obtained from HeLa cells. Drug reversal effect was observed with SR033 IC50 on HeLa in combination at various doses of 5FU below IC50.
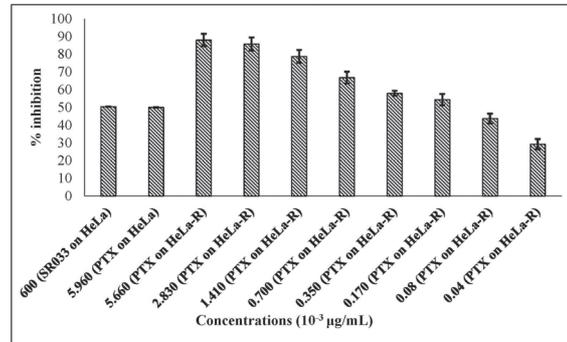
Fig. 3B. Drug reversal property of SR033 on resistant HeLa-R cells. SR033 and PTX were used alone at IC50 obtained from HeLa cells. Drug reversal effect was observed with SR033 IC50 on HeLa in combination at various doses of PTX below IC50.
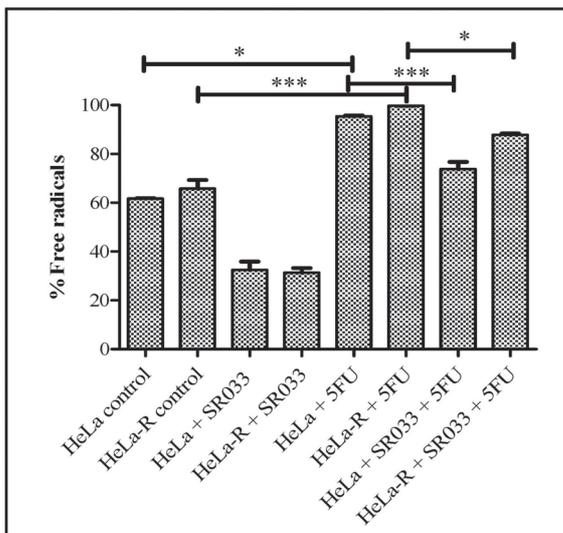
Fig. 4. Effect of SR033 on percent free radicals produced in combination with chemo-drug, 5FU. $p<0.001$ as shown with triple asterisk (***) and $p<0.05$ shown as single asterisk (*).

various low concentrations of 5FU, the inhibitory effect of SR033 + 5FU was significantly high. Treatment of HeLa-R cells at IC50 values of HeLa cells in the combination of SR033 (600 µg/mL) with 5FU (2 µg/mL) and PTX ($5.96×10^{-3}$ µg/mL) resulted in 80% and 88% death of HeLa-R,

respectively (Figure 3A, 3B). It is noteworthy to achieve $2.5×10^3$ and $0.17×10^3$ fold drug resistance reversal to 5FU and PTX, respectively. In earlier studies, 2 and 3.71 fold reversal of 5FU has been shown in resistant HCT116 and HCT-8 cells by curcumin, respectively (43, 53) Although, a study by Tian *et al* (2012) could not show significant 5FU resistance reversal in Esophageal squamous cell carcinoma by curcumin (54). The reversal ratio with SR033 was comparatively higher when observed with the above-reported data of curcumin (43, 53).

SR033 increases SOD in HeLa and HeLa-R cells: Superoxide Dismutase was analyzed for the combination of SR033 and 5FU to reveal the chemo-protective action of SR033. A high SOD value indicates the lower oxidative stress in the sample, i.e. the SOD activity (U/ml) will be higher if free radicals have been suppressed (55) and the higher per cent inhibition by SOD enzyme signifies lower oxidative stress and free radicals. The results obtained show significantly low free radicals for both HeLa and HeLa-R cells when treated with SR033 (Figure 4). In effect, the study showed that the usage of 5FU drug alone generates free radicals. The increase in free radicals in 5FU groups has been significantly ($p<0.001$) subsided by SR033 in the groups

treated with SR033 + 5FU, hence displaying its antioxidant capability. However, in earlier reports, use of 5FU with Irinotecan resulted in a 3 fold decrease in free radicals (56), whereas no significant increase was observed withbetulin-3, 28-diphosphate (57). In this study, we observed 2 fold decrease in free radicals when treated with SR033 as compared to untreated controls, however, 1.2 fold decrease was observed when compared 5FU alone groups with SR033 + 5FU groups. The effect of SR033 in combination to 5FU showed comparable results to above-mentioned reports (56,57). While the data does show that SR033 alone significantly reduces the number of free radicals, the practical application of using SR033 alone to treat cancer is not feasible (58).Similar studies were also performed earlier (59,60).

Conclusion

Since 5FU is used as one of the common chemo-drugs for cancer treatment, but toxicity and resistance is the major drawback of this drug (61,62). Nowadays, application of herbal compounds as complementary and alternative medicine/ adjuvants is increasing to overcome chemo-toxicity and resistance. Thus, the combinatorial approach with herbal compounds can be adopted to reduce the toxic effects of chemotherapy as well as combating drug resistance (50-54,56-60). In the present study, we have tested a poly-herbal formulation, SR033in combination with 5FU to reduce toxicity, to increase its efficiency and to prevent oxidative damage. Our study revealed a reversal of $2.5×10^3$and $0.17×10^3$ folds chemo-resistance by the synergistic effect of SR033 with 5FU and PTX, respectively, where $IC_{80}$and IC_{88} has been achieved with IC_{50} values by combining SR033 with 5FU and PTX, respectively, which implies that SR033 increases the efficacy of the drug at lower doses. Also, SR033 decreases the oxidative stress thus averting the collateral damage caused due to chemotherapy. Thus, the study concludes SR033 as a strong candidate for cancer treatment in combinational therapy with 5FU.

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