Pharmacognostic Evaluation of DPSUU III IN Breast Tumor Growth By G0/G1 Cell Cycle Arrest

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ABSTRACT

Objective: Plant-inferred bioactive particles have been utilized to turn away or treat tumor for a considerable length of time. As of late plant blends were found to have significantly more grounded exercises than utilizing plant alone. In this study, we evaluated the anticancer impacts of DPSUU III which contains a mix of plant concentrates of Beta vulgaris, Syzygium cumini, Limonia acidissima. Material and Methods: After extraction from these plants, DPSUU III was granulated and powder was extricated with 80% ethanol, dried by rotating evaporator under vacuum and put away until utilize. DPSUU III was offered orally to mice at single measurements of 1000mg/kg/day alongside vehicle control and creatures were seen amid the initial 12 hr for any change in side effects of mobility, posture, amount of sustenance utilization and for mortality after MCF-7 cells were infused into mammary cushion of C57BL6 mice to create strong breast tumours. Then DPSUU III treated gathering was contrasted with the doxorubicin (Dox)-treated gathering. Results: The breast disease development was altogether stifled in mice treated with DPSUU III without loss of body weight and any symptoms while Dox-treated mice showed huge body weight reduction with little measure of toxicities. An expansion in life expectancy (ILS% = 62.80%) was seen in the DPSUU III - controlled gathering, contrasted with the tumor control bunch. DPSUU III shows hostile to proliferative movement against MCF-7 breast tumor cells through actuating G0/G1 cell cycle capture. DPSUU III hinders the declaration of cyclin D1, CDK4, CDK2 and prompts p21. Conclusion: DPSUU III could be a potential chemo preventive supplement to breast tumor patients.

Keywords: Breast cancer, DPSUU III, G0/G1, anti-cancer, cyclin D1.

INTRODUCTION

Recently, a number of bioactive molecules, including anti-tumor agents, have been originated from different plants. There is much practical and lucid evidence of the efficacy of plant mixtures in cancer patients. However, systematic preclinical assessment of these plants is necessary. Recently, plant mixtures were discovered to have even stronger activities than using plant alone.1 Beta vulgaris (B vulgaris) belongs to the Amaranthaceae, and it is commonly called “beet” in India. B vulgaris is a well-established medicinal plant that has been used to treat different human cancers in Asia. We have demonstrated that Beta vulgaris has anti-proliferative activities2 which shows tumor cell growth inhibition in human breast cancer cells. Syzygium cumini (S cumini), commonly referred as the Jammu in Odisha, India belongs to the Myrtaceae family. S cumini is found in the deep forests of India, Nepal, Korea, Eastern and Northern Europe, the USA and Canada. There have been various studies demonstrating its antioxidant3 anti-tumor and antimicrobial activities. Limonia acidissima (L. acidissima) is an indigenous plant of family Rutaceae that grows mostly in India,4 Sri Lanka and Nepal. L. acidissima is used in trauma.

DOI: 10.5530/ijper.51.1.10
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ditional Indian medicine for treating food and drug poisoning, diarrhoea, abdominal pain, hypertension, pruritus and liver dysfunction. Our group reported that the ethanol extract of *L. acidissima* exhibits anti-cancer activity and suppresses mammary carcinoma responses in mice.

We assessed various formulations of plant mixtures to pick the most viable one against MCF-7 human breast cancer cells, both in vivo. Based on preparatory results, we chose to further evaluate the effects of DPSUU III, a plant mixture, composed of *Beta vulgaris*, *Syzygium cumini*, and *Limonia acidissima*.

In the present study, we assessed the effects of plant mixture DPSUU III on the development of MCF-7 breast cancer cells in vivo.

### MATERIALS AND METHOD

#### Preparation of DPSUU III

DPSUU III, a dietary supplement, made from *Beta vulgaris* (B vulgaris), *Syzygium cumini* (S cumini), *Limonia acidissima* (L. acidissima) was prepared by researchers of the Dept. of Pharmacy, Utkal University [11]. The initial set of *Beta vulgaris*, *Syzygium cumini*, *Limonia acidissima* plants were collected in the flowering period, between June and August 2014, from several regions of India. From the air-dried, plants the food supplements were ground to a fine powder with a grinder. The powder was extracted with 80% ethanol (EtOH) for 48h. The residue was extracted at room temperature and filtered again. The yield of the extracts were 7%, 12% and 18% respectively. We have considered the proportions ratio for the full and final formulations are 22:32:46 respectively. The extracts were dried by a rotary evaporator under vacuum at 40°C and stored at −20°C until use.

DPSUU III, the mixture of all the three plant extracts, was disintegrated in water and used for the animal experiments.

#### Experimental Animals

Female C57BL6 mice at 6 weeks of age were purchased from Jena Farm, Cuttack, Orissa and acclimatized under the controlled conditions for 1 week before the experiment. The animals were fed with commercial rodent feed from Lipton India, Mumbai, India. Food and water were provided to the animals. The protocols of experiment on animals were approved by the Institutional Animal Ethical Committee (Regd. No.:990/C/06/ CPCSEA), Utkal University, Vani-Vihar, Bhubaneswar, Odisha, India.

#### Acute Toxicity Test

DPSUU III was given orally to mice (06 mice/group) at single doses of 1000 mg/kg/day along with the vehicle control. Animals were observed during the first 12h for any alteration in the symptoms of mobility, posture, the amount of food consumption and for mortality. Mice were measured daily and observed for fourteen days following treatment.

#### Tumor Growth Analysis in Vivo

The inhibitory effect of DPSUU III on breast cancer tumor growth was investigated in an animal model. Tumors were induced as previously described. Experiments were performed in six groups: normal control group, saline injection; tumor control group, MCF-7 (2 × 10⁵ cells/mouse) injected in mammary pad; DPSUU III treated group, MCF-7 (2 × 10⁵ cells/mouse) injected in mammary pad; DPSUU III oral administered group, MCF-7 (2 × 10⁵ cells/mouse) injected in mammary pad; and Doxorubicin (Dox) (Sigma, Mumbai, India) intraperitoneally injected group, MCF-7 (2 × 10⁵ cells/mouse) (n = 7 per group). DPSUU III (300 mg/kg/day) and doxorubicin (1 mg/kg/day) were administered three days before MCF-7 breast cancer cell transplantation until sacrifice, respectively. Body weight was measured once every three days. The number of surviving animals was examined every day. Tumor was analysed on Day 14 following MCF-7 breast cancer cell transplantation. Mice were sacrificed 14 days following cell inoculation. Tumor tissues were collected for further analysis. Tumor was imaged with a digital camera (Canon, India).

#### Cell Culture

The MCF-7 breast cancer cell line was obtained from NCCS, Pune and cultured in MEM (Invitrogen Co., Mumbai, India) containing 10% (v/v) fetal bovine serum,100 unit/mL penicillin and100 µg/mL streptomycin in an atmosphere of 95% air and 5% CO₂ at 37°C.

#### Cell Proliferation Assay

Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan), as described previously. Cells (1 × 10⁴ cells/well) were incubated with the indicated concentrations in the absence or presence of samples for the indicated times. The cell proliferation rate (%) was calculated as the absorbance of sample-treated cells divided by the absorbance of control cells (n = 3). The cell viability of the control group was calculated as 100%.
Flow Cytometric Analysis of DNA Content for the Cell Cycle

MCF-7 breast cancer cells (5 × 10^5 cells/mL) in 6-well plates were incubated in the presence or absence of DPSUU III extract for the indicated time. After cell harvest, cells were fixed with 70% (v/v) ice-cold ethanol and then incubated with a staining solution containing 0.2% (w/v) NP-40, RNase A (30 μg/mL) (ILS, BBSR, Odisha, India) and propidium iodide (50 μg/mL) (Sigma, Mumbai, India) in a phosphate-citrate buffer (pH 7.2). Cellular DNA contents were analysed by flow cytometry, using a Becton Dickinson laser-based flow cytometer (Sigma Aldrich, Mumbai, India). At least, 10,000 cells were used for each analysis, and the results were displayed as histograms. Stained cells were run on a FACS Calibur (Sigma Aldrich, Mumbai, India) with an excitation wavelength of 488 nm and an emission wavelength of 585 nm. Histograms were analysed using Cell Quest software (Sigma Aldrich, Mumbai, India) to determine the cell cycle distribution.

Immunoblot Analysis

Immunoblotting analysis was performed as described previously. Total protein (25 μg) was separated by electrophoresis on a 12% SDS-PAGE polyacrylamide gel and then electrophoretically transferred onto polyvinylidene fluoride membranes (PVDF) (Sigma Aldrich, Mumbai, India). The membranes were incubated with 5% skim milk solution followed by incubation with primary antibody. The membranes were washed in a 1 × TBS-T buffer and incubated with HRP-conjugated secondary antibodies (Sigma Aldrich, Mumbai, India, 1:5000) for 1–2h. The immunoreactive bands were detected using the enhanced chemiluminescence western blotting detection system (Biosesang, Seoul, Korea).

Statistical Analysis

Data are expressed as means ± standard error of the mean (SEM). Differences between groups were determined by one-way ANOVA followed by Dunnett’s t-test. Significant differences were considered from *p<0.05, **p<0.01, ***p<0.001.

RESULT AND DISCUSSION

The body weight and general appearance of every mouse was measured and observed every day as evidence of systemic toxicity. 

In the acute toxicity study, dosages up to 1,000 mg/kg did not exhibit any mortality or any signs of toxicity (e.g., changes in body weight and the sum of food consumption) after oral administration of a single dose (Figure 1). This dosage might be considered as the no observed adverse effect level (NOAEL) for DPSUU III.

We inspected whether DPSUU III could suppress tumor growth in vivo. Mammary padinfusion of MCF-7 into C57BL6 mice developed into solid breast tumors. Then quantity of saline, DPSUU III, Doxorubicin listed in Table 1 were injected into tumor bearing C57BL6 mice. Either DPSUU III or saline was orally administered three days before tumor cell implantation (Figure 2). The mean tumor weights in DPSUU III treated mice (300 mg/kg/day) were significantly inhibited compared to those of the tumor control group. Doxorubicin (Dox) (1mg/kg i.p., daily, for 17 days), used as the positive control, also inhibited tumor development and tumor weight significantly (Figure 3). Doxorubicin (Dox) is one of the most broadly used antitumor drugs against a lot of solid tumors, including breast cancer. Although Dox had been demonstrated to exert robust antitumor activity, its effectiveness was often confined by drug-resistance and dose-dependent side effects, particularly Dox-induced cardiomyopathy. This study demonstrated that the 300 mg of DPSUU III/kg/day regimen lowered the tumor weight, comparable to the doxorubicin (Dox)-treated group.

The body weights of the DPSUU III-treated mice were comparable to those of the normal control group (Figure 4), showing that there were no critical side effects during medication. Information indicate that breast cancer growth was significantly suppressed in mice treated with DPSUU III without loss of body weight, while the Dox-treated mice indicated significant body weight loss. Based on the observation of body weight (Figure 4), a small amount of host toxicities were observed after Dox treatment. The effects of DPSUU III on the survival rate of mice are listed in Table 2.

Table 1: Melanoma model in 7-week-old female C57BL6 mice

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Treat</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>7</td>
<td>DW (5 ml/kg/d)</td>
<td>Survival, Body weight, Tumor weight</td>
</tr>
<tr>
<td>Tumor control</td>
<td>8</td>
<td>DW (5 ml/kg/d)</td>
<td></td>
</tr>
<tr>
<td>Tumor + DPSUU III</td>
<td>7</td>
<td>300 mg/kg/d (i.p.)</td>
<td></td>
</tr>
<tr>
<td>Tumor + Doxorubicin</td>
<td>7</td>
<td>1 mg/kg/d (i.p.)</td>
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Table 2: Effect of DPSUU III on the survival rate of mice

<table>
<thead>
<tr>
<th>Group</th>
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<th>Final</th>
<th>Death</th>
<th>Survival Rate (%) at day 14</th>
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<tr>
<td>Normal control</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Tumor control</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>57</td>
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<tr>
<td>Tumor + DPSUU III</td>
<td>7</td>
<td>6</td>
<td>1</td>
<td>86</td>
</tr>
<tr>
<td>Tumor + Dox</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>100</td>
</tr>
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Figure 1: The effect of DPSUU III on body weight and food consumption in the acute toxicity study. – (Scheme of the acute toxicity study.)

Figure 2: Experimental design of the effect of DPSUU III or doxorubicin (Dox) on MCF-7 Breast Cancer xenograft in an animal model.

Figure 3: The effect of DPSUU III on tumor growth in MCF-7 breast cancer-bearing mice. Representative mice were administered with DPSUU III, doxorubicin (1 mg/kg/day) or DW (distilled water). Mice were sacrificed on Day 14 after MCF-7 inoculation.

Figure 4: Tumor weight changes on MCF-7-inoculated mice treated with DW, DPSUU III (300 mg/kg/day) and Dox (1 mg/kg/day). Data were obtained from the three independent experiments and were expressed as the means ± SE.

Figure 5: Effect of DPSUU III on body weight of the mice was measured during treatment & the mean body weights before DPSUU III treatment in each group are expressed as 100%. The body weight of the following days is expressed as the ratio of the body weight to the initial body weight. Data were obtained from the three independent experiments and were expressed as the means ± SE. One-way ANOVA was used for the comparison of multiple group means, followed by Dunnett’s test (**p< 0.01 vs. Tumour control)

Figure 6: Anti-proliferative effects of DPSUU III extracts against MCF-7 breast cancer. Cells were treated with the indicated concentrations of DPSUU III extracts for 72 h. Cell viability was compared with the control, and the 4 statistically distinctive levels are denoted by: *p< 0.05,** p< 0.001. Data were acquired from the three independent experiments and are expressed as the means ± SE.
The survival rate of mice treated with DPSUU III was delayed compared to that of the tumor control group. Survival rates were 57% and 86%, for the DW (distilled water) - and DPSUU III -treated group, respectively. A Kaplan–Meier plot illustrates that the survival rate of DPSUU III extract-treated mice was increased when compared to tumor control animals. The maximum survival rate was observed in the DPSUU III administered group, as indicated in (Figure 5). The percentage increase in life span = \( \frac{T - C}{C} \times 100 \). Where \( T \) and \( C \) are the percent of surviving mice in the treated and tumor control groups. An increase in life span (ILS%= 50.88%) was gotten in the DPSUU III administered group, compared to the tumor control group. The body weights of the DPSUU III -treated mice were comparable to those of the normal control group (Figure 5), showing that there were no critical side effects during medication. Information indicated that breast cancer growth was significantly suppressed in mice treated with DPSUU III without loss of body weight, while the Dox-treated mice indicated significant body weight loss. Based on the observation of body weight (Figure 5), a small amount of host toxicities were observed after Dox treatment.

Next, we assessed if the mixture of plants likewise could inhibit the growth MCF-7 cancer cells in vitro. As seen in (Figure 6), DPSUU III uniquely suppressed proliferation of MCF-7 cells at 250 and 500 \( \mu \)g/mL (Figure 6) (\( p < 0.05 \) vs control).0,8,11,12

To explain the mechanism of cell growth inhibition by the DPSUU III plant complex, we determined its effect on cell cycle distribution. Exposure of MCF-7 cells to the DPSUU III plant complex resulted in the improvement of the G0-G1 fraction, which was accompanied by a decrease in the S phase (Figure 7). The rates of the G0-G1 population in DPSUU III-treated cells were 60.03% ± 0.56%, 66.12% ± 1.77% and 65.76% ± 0.03% at concentrations of 0, 250 and 500 \( \mu \)g/mL respectively. Thus, DPSUU III induced growth inhibition of MCF-7 cells is correlated with the G0-G1 phase cell cycle arrest. Eukaryotic cell cycle progression includes successive activation of CDKs with corresponding regulatory cyclins.9 As seen in (Figure 8), the G1-S transition is regulated by complexes of cyclin D and CDK4 or CDK 6 and cyclin E and Cdk2. The cell cycle is precisely controlled by a family of proteins called cyclin-dependent kinases (CDKs), which are positively regulated by cyclins (A, B, D and E) and negatively directed by cyclin-dependent kinase inhibitors (CDKIs).13 The G1-phase progression requires the presence of cyclin D-CDK4/6 complexes, and the G1/S phase transition needs cyclin E-CDK2 complexes. To delineate the molecular mechanism of cell cycle regulation by DPSUU III, we evaluated the expression of CDKs and cyclins in MCF-7 cells.
DPSUU III mediated G0/G1 arrest, we determined its effect on the key proteins involved in G0/G1 phase arrest. As can be seen in (Figure 8), DPSUU III treatment brought about a rapid and marked decrease in the expression level of cyclin D1, CDK4 proteins in MCF-7 cells. The DPSUU III treated MCF-7 cells indicated the decreased level of cyclin D1 and CDK4 protein expression, whereas the level of cyclin E was not adjusted in this cell line at the 48h time points (Figure 9). An increase in the expression of p21\(^{5,14}\), a cyclin dependent kinase inhibitor (CDKI), was observed in MCF-7 cells by DPSUU III treatment (Figure 9). p21 is known to negatively regulate the G1 transition. Therefore, the reduction of the G1 phase cell cycle-related proteins of cyclin D1, CDK4 and CDK2.\(^{5,14}\) DPSUU III might account for the G0/G1 phase arrest. The p21 protein is a well-known CDKI that inhibits the activation of cyclin-CDK4 complexes. Thus, DPSUU III functions as a regulator of cell cycle progression at G1.

**CONCLUSION**

This is the primary examination exhibiting that DPSUU III applied hostile to malignancy movement that is practically identical to Dox against breast cancer *in vivo*. The 300 mg DPSUU III/kg/day regimen decreased tumor weight, practically identical to the doxorubicin (Dox) -treated gathering, alongside expanded life expectancy. We additionally found an inhibitory impact of DPSUU III on the multiplication of MCF-7 breast disease cells. DPSUU III prompted G0/G1 cell cycle capture by smothering the declaration of cyclin D1 and CDK2 and instigating p21. These data prescribe that DPSUU III may be utilized as a chemopreventive\(^4\) agent against breast growth.

**ACKNOWLEDGMENTS**

We the authors are owe our sincere thanks to HOD, UDPS, Utkal University, Odisha, India for his timely suggestion. Moreover we acknowledge our thanks to UGC, (University Grant Commission) New Delhi, India110002 for their financial grants provided.

**CONFLICT OF INTEREST**

The author have no conflict of interest to declare.

**ABBREVIATION USED**


**REFERENCES**


SUMMARY
- In this study, we determined the anticancer effects of DPSUU III which consists plant extracts of Beta vulgaris, Syzygium cumini, Limonia acidissima.
- DPSUU III was given orally to mice at single doses of 1000mg/kg/day along with vehicle control and it was compared to the doxorubicin (Dox)-treated group.
- DPSUU III exhibits anti-proliferative activity against MCF-7 breast cancer cells through inducing G0/G1 cell cycle arrest.
- DPSUU III inhibits the expression of cyclin D1, CDK4 and CDK2 and induces p21.
- Hence the breast cancer growth was significantly suppressed in mice treated with DPSUU III without loss of body weight and any side effects while Dox-treated mice indicated significant body weight loss with small amount of toxicities.
- So DPSUU III could be used to treat breast cancer

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Cite this article: Pradhan D, Dasmohapatra T, Tripathy G. Pharmacognostic Evaluation of DPSUU III IN Breast Tumor Growth By G0/G1 Cell Cycle Arrest. Indian J of Pharmaceutical Education and Research. 2017;51(1):70-6.