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# Antiproliferative Effect of *Rhus Succedanea, Rheum Emodi* and *Gardenia Gummifera* in Prostate PC-3 Cells

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## Abstract
In the present study antioxidants activity of *Rhus succedanea, Rheum emodi* and *Gardenia gummifera* determined by SOD activity which is increased and suggests that it exhibit highest superoxide anion-scavenging ability with an inhibition rate of more than 90 percent and has an efficient protective mechanism in response to ROS, in Anti-inflammatory assay lipoxygenase activity which is dose dependent response by displaying inhibitory potential at an $IC_{50}$ of 25.58µg/ml, in hyaluronidase inhibition assay the results shows that the activity is dose-dependent manner with $IC_{50}$ of 29.34µg/ml and the phytochemical analysis by GCMS analysis shows the presence of myristic acid vinyl ester, vinyl decanoate, tridecanoic acid which act as chemo preventive agents against prostate cancer.

**Keywords**
Antiproliferation, *Rhus succedanea, Rheum emodi, Gardenia gummifera*, prostate PC-3 cells

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## INTRODUCTION
Polyherbal formulation system of medicine is a global one and has existed from time immemorial. Approximately 80% of global population still depends on herbs for primary health care necessities. Lethal side effects and the lack of appropriate remedies in the modern system of medicine for many chronic diseases has re-opened the gates for herbal drugs. Hence, our study aimed to understand the synergistic effects of poly herbal formulation comprising three constituent herbs (*Rhus succedanea, Rheum emodi* and *Gardenia gummifera*) on apoptosis in prostate PC-3 cancer cells.

Today, chemotherapy is the most frequently used treatment in breast and other cancers. However, this method of treatment is not selective for cancer cells and often leads to the destruction of normal cells. Most of the current anticancer drugs are derived from plants, which act in different ways ultimately leading to the activation of apoptosis in cancer cells. To compensate for the limitations and toxicity of chemotherapy, Chinese herbal medicines and other alternative strategies are being developed. The herbal formulations have been standardized according to the WHO guidelines.

The anti-proliferative and anti-tumour effects of PC-SPES, a herbal preparation, have been documented. PC-SPES administered as a food supplement caused a dramatic decrease in prostate-specific antigen levels in some prostate

cancer patients with advanced disease [1]. PC-SPES is composed of eight Chinese herbs this mixture contains eight recognized and active anti-neoplastic compounds where PC-SPES had been widely used for prostate cancer and PC-SPESII has been used in a phase I trial for prostate cancer [2].

EquiguardTM is a dietary supplement comprising standardized extracts from nine herbs, the in-vitro use of EquiguardTM significantly reduced cancer cell growth, induced apoptosis, suppressed expression of the androgen receptor (AR) and lowered intracellular and secreted prostate specific antigen (PSA) and almost completely abolished colony-forming abilities of prostate cancer cells [3].

Plant based drugs are thus a ray of hope in cancer treatment in the coming years [4]. Hwangryunhaedok-tang (HRT; Oren-gedoku-to is the Japanese name) is one of the most famous traditional herbal medicines being used in Asian countries. HRT contains four kinds of herb that include *Coptis japonica, Scutellaria baicalensis, Phellodendron amurense* and *Gardenia jasminoides*. HRT has been clinically used in Korea for anticancer effects [5] and [6] reported that stigmastane-type steroids which were isolated from the resinous exudates of *Commiphora mukul* were evaluated for their antiproliferative activities against four human cancer cell lines, the study showed that enforce the PC3 cell cycle arrest in the G2/M phase and induce the apoptosis of PC3 cells by activation of Bax, caspases 3 and 9 and by inhibition of Bcl-2.

[7] determine the anti-cancer properties of four selected curcumin analogues, on the cytotoxicity, proliferative and apoptotic effects on androgen-independent human prostate cancer cells (PC-3 and DU 145). Initial cytotoxicity screening showed MS17 has the highest cell inhibitory effect, which has high selectivity index value for further analysis on the morphological and biochemical hallmarks of apoptosis. Fluorescence microscopy analysis revealed apoptotic changes in both treated prostate cancer cells. Relative caspase-3 activity increased significantly at 48 h in PC-3 and 12 h in DU 145 cells. Highest enrichment of free nucleosomes was noted at 48 h after treatment with MS17.

[8] Reported that Cabernet Sauvignon and Rombola wine inhibited the androgen-insensitive human prostate cancer cells (PC-3) here the antioxidant polyphenols can act as chemopreventive agents against prostate cancer and Ibrahim [9] studied that antiproliferative effects of *Morus nigra, the* study evaluate the phenolic composition and antioxidant activity of dimethyl sulfoxide extract of *M. nigra* (DEM) and to investigate, the probable cytotoxic effect in human prostate adenocarcinoma (PC-3) cells together with the mechanism involved. Ascorbic acid and chlorogenic acid were the major phenolic compounds detected at HPLC analysis. DEM arrested the cell cycle of PC-3 cells at the G1 phase, induced apoptosis via increased caspase activity and reduced mitochondrial membrane potential.

In our study, the apoptotic process mediated by anti-proliferative activity of polyherbal extract in colorectal (HCT116) and prostate cancer (PC-3) cell lines will be evaluated. The anti-proliferative activity of PHF1through apoptosis will be substantiated by cytotoxicity, DNA fragmentation, inhibition of survival colonies (clonogenic assay) and cell cycle arrest at subG0/G1 and G2/M phase studies. In consonance with the issues raised, the present study was undertaken to study the antiproliferative, antioxidant and anti-inflammatory activity of polyherbal formulations, molecular characterization and its effect on cancer cells.

## MATERIALS AND METHOD

### Antioxidant activity of polyherbal extract:

**SOD activity:** SOD activity was performed according to the method described in the kit (SOD Assay Kit, 19160-1KT-F, Sigma-Aldrich).

**Materials:** WST-1 working solution [(2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl) - 2H tetrazolium, monosodium salt]: 1ml of WST-1 solution was diluted with 19ml of buffer solution.

**Enzyme working solution:** The enzyme solution was centrifuged for 5s, mixed by pipetting add 15µl of the enzyme solution was diluted with 2.5ml of the dilution buffer.

**SOD Solution (for assay monitoring, if necessary):** SOD was diluted with dilution buffer to prepare SOD standard solution as follows:

**Method:** About 5 × 105 RAW 264.7 cells were grown per 35-mm dish for 24h. Later, cells were treated with different concentrations of the test solution (0, 3.13, 6.25, 12.5, 25, 50, 100µg/ml). After 18h of treatment with the test solution, the cells were washed with PBS and harvested using a cell scrapper. Collected cells were centrifuged at 2000 × g for 10 min at 4°C. The obtained pellet was homogenized in cold 20mM HEPES buffer, pH 7.2, containing 1mM EGTA 210mMm mannitol and 70mM sucrose. It was centrifuged at 1500 × g for 5 min at 4°C. The supernatant was collected for the assay. SOD activity was performed according to the kit method. Later, 200µl of WST working solution was added to each well and mixed. Then, 20µl of the dilution buffer was added to each blank 2 and blank 3 wells. Finally, 20µl of the enzyme working

solution was added to each sample and the blank 1 well and then mixed thoroughly. The plate was incubated at 37°C for 20min. Absorbance was read at 450nm using a spectrophotometer (Shimadzu, UV-1800, Japan). The SOD activity was calculated for the percentage of inhibition rate using the following formula.

$$\text{SOD activity} = \frac{[(\text{Ablank1-Ablank3}) - (\text{A Sample} - \text{Asample2})]}{(\text{Ablank1- Ablank3})} \times 100$$

**Anti-inflammatory assay:**

**Lipoxygenase inhibition assay:**

**Materials:** Soybean lipoxidase, Activity 1, 12,000 units per mg of solid (E.C. 1.13.11.12), stored at 0°C.

**Enzyme stock:** 5mg of the enzyme was dissolved in 10ml of cold borate buffer (pH 9.0) and linoleic acid, and stored at 0°C.

**Reference standard:** Indomethacin (stored at room temperature).

Boric acid (1.23g) was suspended in 25ml of distilled water. The pH is adjusted to 9.0 using NaOH and made up to 100ml with distilled water.

**Preparation of working solution:** 1785µl from the enzyme stock was made up to 10ml to obtain 10,000 units/ml. 50mg of linoleic acid was dissolved in 5 ml distilled water containing 50µl Tween-20 and made up to 50ml with distilled water.

**Method:** To the test tubes, 0.8ml of test solution and reference standard (indomethacin, 300µg/ml dissolved in 3% methanol) of various concentrations (0.00, 1.25, 2.50, 5.00, 10, 20, 40, 80 and 160µg/ml) were added. Further, 0.1ml of 2M Borate buffer, pH 9.0, and 0.1ml of 500-1000 units Lipoxygenase enzyme (type 1 from soybean, EC No. 1.13.11.12) were added. The tubes were mixed and incubated at room temperature for 5min. Subsequently, 2.0ml of the substrate solution (50 mg of linoleic acid dissolved in 50µl Tween 20, then made up to 50ml with 2M borate buffer and diluted to a concentration of 166.6µg/ml with 2M borate buffer pH 9.0) was added and mixed well. The absorbance was measured for 4min at 234nm. Basal and vehicle control reaction was carried out without the test sample [10]. The percentage inhibition of lipoxygenase was calculated using the following formula.

$$\% \text{ Inhibition} = \frac{\text{OD of Control} - \text{OD of Sample}}{\text{OD of Control}} \times 100$$

**Hyaluronidase inhibition assay:**

**Materials:** Hyaluronidase from bovine testis, stored at - 200C (EC 3.21.35); sodium hyaluronate, stored at 2-8°C; disodium cromoglycate (Cromolyn), calcium chloride, sodium acetate, acetic acid, sodium hydroxide, hydrochloric acid, potassium tetra borate, P-dimethyl amino benzaldehyde.

Preparation of 0.1M acetate buffer (pH 3.8):

(A) Acetic acid (0.1M): 5.775ml of glacial acetic acid in 1000ml of deionized water.

(B) Sodium acetate (0.1 M): 8.2g of C2H3O2Na or 13.6g of $C_2H_3O_2Na.3H_2O$ in 1000ml.

Now, 44ml of solution A is mixed with 6 ml of solution B and made up to 100 ml with de-ionized water.

**Preparation of 67 mM P-dimethyl amino benzaldehyde (DMAB):** 10g of DMAB was dissolved in 100ml of glacial acetic acid containing 12.5% 10N HCl (85ml of conc. HCl in 100ml of water). 1ml of the above stock solution was diluted with 9ml of glacial acetic acid.

**Method:** The assay is performed according to the research of [11]. Hyaluronidase was activated by incubating 100µl hyaluronidase (4.15 mg/ml in 0.1M acetate buffer, pH 3.8) with 50µl sodium chloride (26.3 mg/ml in 0.1M acetate buffer pH 3.8) for 20min at 37°C. Following activation, the enzyme mixture was pre incubated with 200µl of test samples and reference standard at various concentrations (0.00, 1.25, 2.50, 5.00, 10, 20, 40, 80 and 160) for 20min at 37°C. Following pre incubation, 150µl of sodium hyaluronate (6 mg/ml in 0.1 M acetate buffer pH 3.8) was added and the reaction mixture was incubated at 37°C for 40min. The reaction was stopped by the addition of 0.1ml (0.4N) sodium hydroxide and 100µl (0.8 M) potassium tetraborate. This is followed by heating the mixture at 100°C for 3min. The mixture is cooled and 3ml of 67mM DMAB (P-dimethyl amino benzaldehyde) was added and incubated at 37°C for 20min. The absorbance was measured at 590nm. Controls were run, which were devoid of test samples. The percentage inhibition of hyaluronidase was calculated as follows.

$$\% \text{ Inhibition} = \frac{\text{OD of Control} - \text{OD of Sample}}{\text{OD of Control}} \times 100$$

**GCMS analysis:**

The methanolic extract of PHF1 was reconstituted in methanol (10mg/ml) and analyzed by GC-MS with National Institute of Standards and Technology (NIST) library matching to identify important compounds (Stein, 1999). GC-MS was performed on a Clarus 500 Perkin Elmer system gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument. The methanolic extract (1µl) was injected into GCMS in the split ratio of 10:1. The extracts were separated in Restek RtxR – 5 column (30meter × 0.25mm, 5% diphenyl/95% dimethyl polysiloxane), running in an electron impact mode at 70eV with helium as carrier gas at a constant flow of 1ml/min. The injector temperature was maintained at 280°C. Total GC running time was 60min. The oven temperature was programmed to an initial temperature of 45°C (isothermal for 5min) and subsequently increased at a rate of 5°C/min to 270°C, then ending with an isotherm of 270°C for 10 min. Mass spectra were taken at 70eV at 0.5-second scan interval and the fragment size ranged from 40 to 1000Da.

**Statistical analysis:**

All the assays were performed in triplicates and the mean of two independent experiments was analyzed. IC50 values for antiproliferative activity was derived from a nonlinear regression analysis (curve fit) based on sigmoidal dose response curve (variable) and computed using Graph Pad Prism 5 (Graph Pad, San Diego, CA, USA).

**RESULTS**

**Determination of antioxidants of polyherbal extract by SOD activity:**

Superoxide dismutase (SOD) plays an important role in the elimination of reactive oxygen species (ROS) and protects cells against the deleterious effects of super oxide anion. SOD catalyzes the scavenging of superoxide anion into hydrogen peroxide and molecular oxygen. WST-1 upon reduction with superoxide anion produces a high water-soluble formazan dye and indirectly lowers the absorbance reading.Thus, the inhibition activity of SOD can be determined by measuring the superoxide anion-caused formation of water-soluble dye. The SOD activity of poly herbal formulation showed dose depended activity, with increased level of inhibition rate shown at higher concentration tested. One unit of SOD activity is defined as the amount of enzyme required to inhibit the reduction of nitro blue tetrazolium (NBT) by 50% under the specified conditions. The results have been expressed as units (U) of SOD activity/ml. Thus, the observed increase in SOD activity suggests that the polyherbal extract exhibited the highest superoxide anion-scavenging ability with an inhibition rate of more than 90 percent and has an efficient protective mechanism in response to ROS. Thus, polyherbal extract may act as an antioxidant by scavenging ROS and stimulating SOD activity (Figure 1).

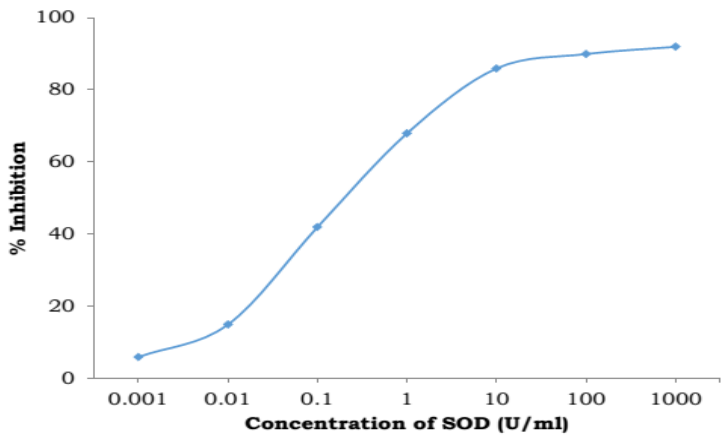


**Figure 1: Effect of polyherbal extract on SOD activity (0, 3.13, 6.25, 12.5, 25, 50, 100 µg/ml) in mouse macrophage cells.**

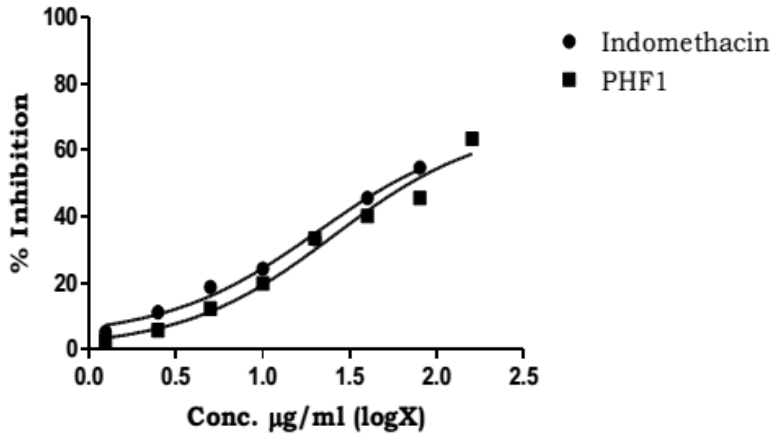


**Figure 2: Inhibitory effect of Indomethacin (+ve control) and relative effect of polyherbal extract on the activity of lipoxygenase.**

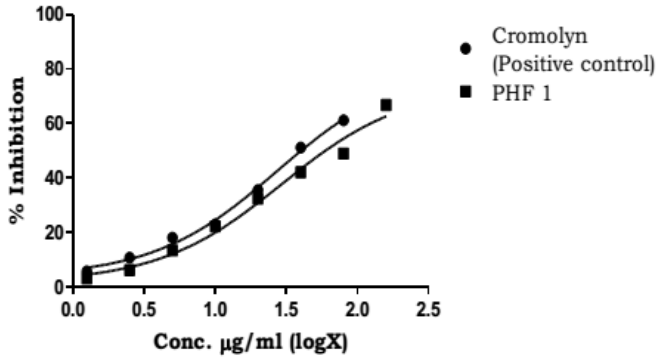


**Figure 3: Inhibitory effect of Cromolyn (+ve control) and relative effect of polyherbal extract on the activity of hyaluronidase.**

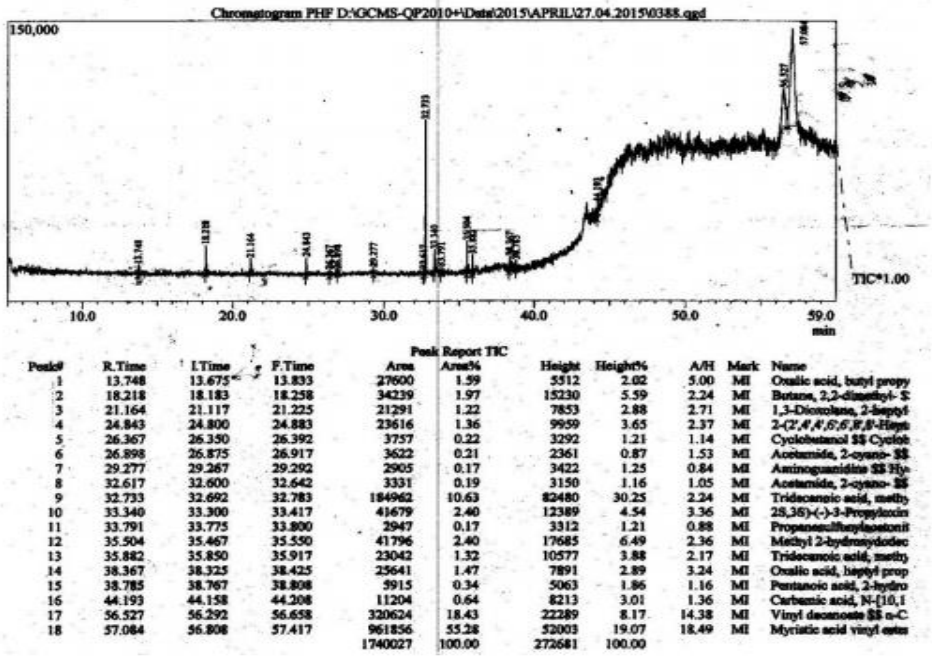


**Figure 4: GCMS analysis of polyherbal formulation.**

**Anti-inflammatory assay:**

**Lipoxygenase assay:** The anti-inflammatory activity measured through the inhibition of lipoxygenase activity by polyherbal extract shows a dose dependent response by displaying inhibitory potential at an IC50 of 25.58µg/ml, as compared to Indomethacin, a positive control with an IC50 of 20.70µg/ml(Figure 2). This study demonstrates that treatment with polyherbal extract has effectively inhibited the lipoxygenase.

**Hyaluronidase inhibition assay:** Results revealed that polyherbal extract inhibited the hyaluronidase activity in a dose-dependent manner with IC50 of 29.34µg/ml. Cromolyn, a known hyaluronidase inhibitor in vitro was used as the positive control. This also showed dose-depended inhibition with IC50 of 27.18µg/ml. This study demonstrates that treatment with polyherbal extract has effectively inhibited the Hyaluronidase (Figure 3).

**Gas chromatography mass spectroscopy (GCMS) analysis:**

The sample was subjected to GCMS and the total separated peaks are shown in Figure 4. The mass of the compounds and fragments recorded were matched with the values in the database of National Institute Standard and Technology (NIST) for identification of probable compounds present in the sample. Interpretation of the mass spectrum of GC-MS was done using the database of NIST that hasmore than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. Phytochemical analysis by GCMS analysis of polyherbal extract revealed the presence of myristic acid vinyl ester, vinyl decanoate, tridecanoic acid, etc.

**Conclusion:**

Here first time an attempt is made to know the effect of poly herbal formulation of *Rhus succedanea, Rheum emodi* and *Gardenia gummifera* for their antiproliferative properties on prostate cancer cells further studies are required to understand the molecular mechanism behind these protective effects.

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