# *In vitro* Antioxidant and Cytotoxic Activities of New Herbal Ointments

**Sirisha Kalam[1*], Kasarla Soujanya[1], Porika Mahendar[2], Safia Begum[1]**

[1]Department of Pharmaceutical Chemistry, Vaagdevi College of Pharmacy, Ramnagar, Warangal-506001, Telangana, INDIA.
[2]Department of Biotechnology, Kakatiya University, Warangal-506009, Telangana, INDIA.

## ABSTRACT

**Objective:** The present study was aimed to investigate the possible effect of newer poly herbal ointment formulations against skin cancers. **Materials and Methods:** The newer polyherbal ointment formulations (PHF-I, PHF-II and PHF-III) containing varying proportions of *Zingiber officinale* (rhizome), *Curcuma longa* (rhizome), *Aloe barbadensis* (leaf), *Citrus aurantium* (peels), *Emblica officinalis* (fruit) extracts and castor oil (*Ricinus communis*) were evaluated for their *in vitro* antioxidant activity (by using DPPH radical scavenging activity, $H_2O_2$ radical scavenging activity, Hydroxyl radical scavenging activity, total phenolic content and total reducing power assay) and *in vitro* cytotoxic activity (MTT assay) against skin cancer cell lines A431(skin carcinoma), A375 (melanoma) using Cisplatin as standard. **Results:** All the formulations have shown significant antioxidant activity ($p<0.05$) and ($p<0.005$) when compared to standard. PHF–II was found to be more potent than PHF-I and PHF-III. From these results PHF-II was selected for cytotoxic activity. PHF-II was found to be nontoxic for normal skin cells (HaCaT) but was found to exhibit toxicity for skin cancer cells with $IC_{50}$ values of 35 $\mu$g/ml and 41.5 $\mu$g/ml respectively on A375 and A431 cell lines. These results were compared to that of the standard cisplatin ($IC_{50}=20$ $\mu$g/ml on A375 and $IC_{50}=9.01$ $\mu$g/ml on A431 cell lines). **Conclusion:** PHF-II containing *Aloe vera*, *Curcuma longa* and *Zingiber officinale* extracts in a relatively higher proportion exhibited the highest antioxidant and cytotoxic activities against skin cancer cell lines amongst the three formulations.

**Key words:** Antioxidant, Cytotoxicity, Polyherbal Formulation (PHF), Free Radicals, Absorbance, Ointments.

## INTRODUCTION

Free radicals are unstable molecules that include hydrogen atom, nitric oxide (NO) and molecular oxygen ($O_2$). These naturally occur in the body as a result of chemical reactions during normal cellular processes. Reactive oxygen species (ROS) include free radicals such as superoxide ions ($O_2^{\cdot}$) and hydroxyl radicals ($OH^{\cdot}$), as well as non free-radical species such as hydrogen peroxide ($H_2O_2$).[1] In living organisms ROS are produced in different ways, including normal aerobic respiration, stimulated polymorphonuclear leukocytes, macrophages and peroxisomes. These appear to be the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents and pesticides. Free radicals are harmful in nature.[2] In an attempt to stabilize, free radicals attack other molecules in the body potentially leading to cell damage and triggering the formation of another free radical resulting in a chain reaction.[3,4] These reactive oxygen species have been implicated in certain chronic and ageing diseases, including rheumatoid arthritis, inflammation, acquired immunodeficiency syndrome, heart disease, diabetes, cancer and neurodegenerative diseases.[5-10] Antioxidants cause protective effect by neutralizing free radicals, which are toxic byproducts of natural cell metabolism.[11] The human body naturally produces antioxidants but the process is not 100 percent effective in case of overwhelming production of free radicals and that effectiveness also declines with age.[12] Increasing the antioxidant intake can prevent diseases and lower the health problems. Research is increasingly showing

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**Correspondence Address**
***Dr. Sirisha Kalam,***
Department of Pharmaceutical Chemistry, Vaagdevi College of Pharmacy, Ramnagar, Warangal-506001, Telangana, INDIA.
Tel: +91 9949024247,
Fax: +91 870 2544949
Email: ragisirisha@yahoo.com

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that antioxidant rich foods, herbs reap health benefits.[13] Cancer is a multi-step disease incorporating physical, environmental, metabolic, chemical and genetic factors, which play a direct and/or indirect role in the induction and deterioration of cancers. ROS are potential carcinogens because they facilitate mutagenesis and promote tumor formation and growth.[14] Skin cancer is a disease in which malignant (cancer) cells form in the tissues of the skin. Skin cancer begins in the epidermis. It can occur anywhere on the body, but it is most common in skin that is often exposed to sunlight, such as the face, neck, hands, and arms. There are several types of cancer that start in the skin.[15] Plant derived natural products such as flavonoids, terpenoids, and steroids, have received considerable attention in recent years due to their diverse pharmacological properties including antioxidant and anti-cancer activities.[16] From this viewpoint, the present study was carried out to evaluate the *in vitro* antioxidant and cytotoxic activities of some new poly herbal formulations. The poly herbal formulations contained Ginger (*Zingiber officinale*), turmeric (*Curcuma longa*), amla (*Emblica officinalis*), bitter orange (*Citrus aurantium*), *aloe vera* (*Aloe barbadensis*) and castoroil (*Ricinus communis*) all of which have been selected based on the ethnopharmacological studies.[17,18]

## MATERIALS AND METHODS

### Collection of plant materials

Ginger (rhizome), turmeric (rhizome), amla (fruits), bitter orange (peels), *aloe vera* (leaf) and castor oil were collected from the local retail market and authenticated by Dr. Vastavya S. Raju, Senior Professor, Plant Systematics Laboratory, Department of Botany, Kakatiya University, Warangal (TS).

### Preparation of extracts

Extracts were prepared in order to study their antioxidant and cytotoxic activities. Ginger rhizomes were washed thoroughly and cut into small pieces and shade dried at room temperature before being pulverized with an electric grinder. The powdered ginger was extracted with ethanol using Soxhlet apparatus for 48 h. The extract was concentrated under reduced pressure to yield a semi-solid mass of ethanol ginger extract (EGE).[19]

The whole leaves of *Aloe vera* were cut into thin pieces, put onto a glass plate, lyophilized in vacuo for 2 days, and then ground into a fine powder for further use. 50 ml of 80% ethanol (v/v) was added to 1 g of lyophilized *Aloe vera* powder in a round flask, the sample was sonicated for 30 min and then filtered, and the residue was washed twice with 10 ml of ethanol. The filtrate was combined onto a flask that was weighed previously with a constant weight and concentrated to dryness at 30°C by rotary evaporation in vacuo. Then, the flask containing the dried ingredients was weighed, and the weight difference between the empty and sample flask was recorded as the mass of solid in the *Aloe vera* extracts.[20]

Dried amla pulp weighing 15 g was powdered and soaked in 50 ml of absolute ethanol and kept in 250 ml sterile conical flasks at 37°C with shaking at 120 rpm for 24 h. The content was filtered through Whatman No. 1 paper. The extract was concentrated under reduced pressure to yield a semi-solid mass.[21]

Citrus fruits were peeled off and the peels were washed thoroughly with running tap water and cut into small pieces and dried under mild sun light. Dried peels were grounded into fine powder. Ethanol extract was prepared by taking 5.0 g of powdered material in a container along with 50 ml of ethanol and kept for 1 week with periodic shaking. The solution was filtered and the filtrate was collected and the filtrates were pooled. The final extracts were passed through Whatman filter paper No.1. The pooled ethanol extracts were concentrated by rotary vacuum evaporator at 40°C and the collected extracts were stored at 4°C in an air tight bottle.[22]

These extracts were used as the test material throughout this study. Turmeric rhizomes were grounded into fine power, and the powder was directly used as test sample. Castor oil which was purchased from the market was directly used as sample.

### Reagents

Ethanol, EDTA (ethylenediamine tetra acetic acid), ferric trichloride, foline-ciocalteu's reagent, hydrogen peroxide, deoxyribose, TCA (trichloro acetic acid), TBA (thiobarbituric acid) were purchased from Sisco Research Laboratories Pvt. Ltd, Mumbai. DPPH (1,1-diphenyl-2-picryl hydrazyl) radical and MTT reagents (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium Bromide), ascorbic acid, BHT (butylated hydroxyl toluene) and cisplatin were purchased from Sigma Aldrich, Mumbai. PEG4000, PEG600 were purchased from Loba chemicals Pvt Ltd., Mumbai and gallic acid was purchased from QualiKems Fine Chem Pvt. Ltd.

**Cell lines:** The normal skin cells (HaCaT) and skin cancer cells A431 (skin carcinoma), A375 (melanoma) were obtained from Cell Repository, National Centre for Cell Sciences (NCCS), Pune, India.

### Phytochemical studies

Collected extracts were subjected to various chemical tests for the preliminary determination of phytoconstit-

uents (Table 1). All extracts were mixed with equal proportion of alcohol and water (to get a hydro-alcoholic sample), before subjecting them to various chemical reagents.

## Preparation of formulations

After preparation of extracts and phytochemical studies, the next step was to formulate the polyherbal preparations. The concentrations of various extracts in the formulations are presented in Table 2. An ointment with water soluble base was of first choice due to their ease of preparation and also ease of cleaning after application. Polyethylene Glycol (PEG) Ointment base, a mixture of PEG 4000 and PEG 600 was found to have sufficient consistency in the ratio 3:7 respectively, and thus suitable for ointment preparation. Three formulations were prepared by Fusion method. The prepared formulations were then evaluated by various parameters e.g. consistency, stability etc.[23] Different concentrations of PHF-I, PHF-II and PHF-III were prepared in double distilled water containing 1% w/v carboxy methyl cellulose as a solution to determine the antioxidant and cytotoxic activities.

## Measurement of antioxidant activity

### DPPH Radical Scavenging Activity[24]

The free radical scavenging activity of the formulations is based on the scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. Different concentrations of formulations (0.1 ml) were added to 3 ml of a 0.004% methanolic solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percentage inhibition activity was calculated from the equation:

$$\% \ Inhibition = (A0\text{-}At) \ / \ A0 \times 100;$$

where $A_0$ was the absorbance of the control (blank, without formulations) and $A_t$ was the absorbance in the presence of the formulations/standard.

The inhibition curve was prepared and $IC_{50}$ value was calculated.

### Reducing power activity[24]

Different concentrations of formulations were mixed with 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixtures were incubated for 20 min at 50°C. After incubation, 2.5 ml of 10% trichloro acetic acid were added to the mixtures, followed by centrifugation at 650×g for 10 min. The upper layer (5 ml) was mixed with 5 ml of distilled water and 1 ml of 0.1% ferric chloride and the absorbance of the resultant solution were measured at 700 nm.

### Total Phenol Content Determination[24]

To measure total phenol content of formulations, 1 mg of each formulation and standard gallic acid (20, 40, 60, 80, 100 µg/ml) were mixed with 500 µl of the Folin–Ciocalteu reagent and 1.5 ml of 20% sodium carbonate. The mixture was shaken thoroughly and made up to 10 ml using distilled water. The mixture was allowed to stand for 2 h. Then the absorbance at 765 nm was determined. These data were used to estimate the phenolic contents using a standard curve obtained from various concentration of gallic acid.

$$Absorbance = 0.001 \times Gallic\ acid\ (\mu g) + 0.0033$$

### Hydrogen peroxide-scavenging activity[24]

The different concentrations of formulations were dissolved in 3.4 mL of 0.1 M phosphate buffer (pH 7.4) and mixed with 600 µL of 43 mM solution of hydrogen peroxide. The absorbance value (at 230 nm) of the reaction mixture was recorded after 10 min interval against blank solution containing the extract without $H_2O_2$.

$$\% \ Inhibition = [(A_0\text{-}A_1)/A_0] \times 100$$

Where $A_0$ was the absorbance of the control and

$A_1$ was the absorbance of the extract and standard.

### Hydroxyl radical-scavenging activity[24]

One milliliter of the final reaction solution consisted of aliquots (500 µl) of various concentrations of the formulations, 1 mM $FeCl_3$, 1 mM EDTA, 20 mM H, 1 mM L-ascorbic acid, and 30 mM deoxyribose in potassium phosphate buffer (pH 7.4). The reaction mixture was incubated for 1 h at 37°C, and further heated in a boiling water bath for 15 min after addition of 1 ml of 2.8% (w/v) trichloroacetic acid and 1 mL of 1% (w/w) thiobarbituric acid. The color development was measured at 532 nm against a blank containing phosphate buffer.

$$\% \ Inhibition = [(A_0\text{-}A_1)/A_0] \times 100$$

Where $A_0$ was the absorbance of the control and

$A_1$ was the absorbance of the extract and standard.

### Measurement of cytotoxic activity by MTT assay[25]

The assay detects the reduction of MTT [3-(4, 5-dimethylthiazolyl)-2, 5-diphenyl-tetrazolium bromide] by mitochondrial dehydrogenase to blue formazan product, which reflects the normal functioning of mitochondria and hence the cell viability. The experiment was conducted as described by Mosmann (1983). The normal skin cells (HaCaT) and skin cancer cells A431 (skin carcinoma), A375 (melanoma) were seeded in 96-well plates at a density of $1\times10^4$ cells/well in 100 µl DMEM (Dulbecco's Modified Eagle's Medium) with

**Table 1: Phytochemical studies of various extracts**

| Sl. No | Chemical list | *Curcuma longa* | *Zingiber officinale* | *Aloe vera* | *Citrus aurantium* | *Emblica officinalis* |
|---|---|---|---|---|---|---|
| 1 | Alkaloids | - | - | - | + | + |
| 2 | Carbohydrates | - | + | - | + | + |
| 3 | Saponins | - | - | + | - | + |
| 4 | Proteins | - | + | + | - | - |
| 5 | Amino acids | - | + | + | - | - |
| 6 | Steroids | - | + | + | + | + |
| 7 | Terpenoids | + | + | + | + | + |
| 8 | Glycosides | - | + | - | + | + |
| 9 | Flavonoids | - | + | + | + | + |
| 10 | Tannins | - | + | - | + | + |
| 11 | Phenols | + | + | + | + | + |

[(+) = present, (-) = absent].

**Table 2: Composition of poly herbal formulations (I, II and III)**

| Name of extracts | % concentration of extract used in formulations | | |
|---|---|---|---|
| | PHF-I | PHF-II | PHF-III |
| *Aloe vera* | 1% w/w | 3% w/w | 2% w/w |
| *Citrus aurantium* | 3% w/w | 1% w/w | 2% w/w |
| *Curcuma longa* | 1% w/w | 3% w/w | 2% w/w |
| *Emblica officinalis* | 3% w/w | 1% w/w | 2% w/w |
| *Zingiber officinale* | 1% w/w | 3% w/w | 2% w/w |
| *Castor oil* | 3% w/w | 1% w/w | 2% w/w |

**Table 3: Scavenging effects of poly herbal formulation and standard ascorbic acid on DPPH radical. The data represents percentage inhibition of DPPH radical**

| Concentration (µg/ml) | PHF-I % inhibition | PHF-II % inhibition | PHF-III % inhibition | Ascorbic acid % inhibition |
|---|---|---|---|---|
| 10 | 2.57 ± 0.42 | 14.54 ± 0.37 | 9.39 ± 0.42 | 37.17 ± 0.12 |
| 25 | 9.84 ± 0.21 | 30.6 ± 0.41 | 23.33 ± 0.21 | 55.58 ± 0.53 |
| 50 | 25.60 ± 0.22 | 89.69 ± 0.21 | 85.30 ± 0.22 | 75.42 ± 0.21 |
| 75 | 84.84 ± 0.2 | 92.12 ± 0.42 | 89.24 ± 0.2 | 90.01 ± 0.41 |
| 100 | 90.30 ± 0.21 | 94.39 ± 0.2 | 90.75 ± 0.2 | 94.17 ± 0.32 |
| 125 | 91.96 ± 0.2 | 96.21 ± 0.21 | 92.42 ± 0.21 | 97.38 ± 0.32 |
| $IC_{50}$ (µg/ml) | 70 | 30 | 35 | 22 |

Values are means of three determinations ± standard deviation. Values followed by dunnet test. Results are significant ($^{a}P < 0.05$) from one another.

**Table 4: Total reducing effect of poly herbal formulations and standard ascorbic acid on $Fe^{3+}$**

| Concentration (µg/ml) | PHF-I Absorbance | PHF-II Absorbance | PHF-III Absorbance | Ascorbic acid absorbance |
|---|---|---|---|---|
| 10 | 0.22 ± 0.005 | 0.26 ± 0.02 | 0.25 ± 0.01 | 0.27 ± 0.01 |
| 25 | 0.37 ± 0.015 | 0.41 ± 0.01 | 0.39 ± 0.05 | 0.41 ± 0.05 |
| 50 | 0.45 ± 0.01 | 0.5 ± 0.01 | 0.47 ± 0.02 | 0.5 ± 0.05 |
| 75 | 0.49 ± 0.005 | 0.51 ± 0.005 | 0.50 ± 0.005 | 0.52 ± 0.005 |
| 100 | 0.55 ± 0.005 | 0.62 ± 0.005 | 0.58 ± 0.005 | 0.66 ± 0.02 |
| 125 | 0.7 ± 0.01 | 0.76 ± 0.03 | 0.72 ± 0.01 | 0.75 ± 0.01 |

Values are means of three determinations ± standard deviation.

**Table 5: Total phenolic content of poly herbal formulations**

| Concentration | Absorbance | Total phenolic content (mg GAE/g dry sample) |
|---|---|---|
| PHF-I (100 µg/ml) | 0.027 | 35.42 ± 0.14 |
| PHF-II (100 µg/ml) | 0.032 | 42.57 ± 0.23 |
| PHF-III (100 µg/ml) | 0.029 | 38.28 ± 0.16 |

Values are means of three determinations ± standard deviation. Values followed by dunnet test. Results are significant ($^cP< 0.005$) from one another.

**Table 6: Scavenging effects of poly herbal formulation and standard ascorbic acid on $H_2O_2$ The data represents percentage inhibition of $H_2O_2$**

| Concentration (µg/ml) | PHF-I %inhibition | PHF-II %inhibition | PHF-III %inhibition | Ascorbic acid %inhibition |
|---|---|---|---|---|
| 10 | 14.21 | 16.22 | 9.34 | 16.03 |
| 25 | 26.39 | 33.74 | 26.82 | 49.05 |
| 50 | 37.05 | 40.39 | 34.14 | 60.37 |
| 75 | 48.22 | 70.52 | 49.18 | 68.86 |
| 100 | 55.31 | 83.24 | 57.72 | 77.35 |
| 125 | 73.17 | 83.9 | 60.16 | 88.67 |
| $IC_{50}$ | 77 | 60 | 75 | 25 |

Values are means of three determinations ± standard deviation. Values followed by dunnet test. Results are significant ($^cP< 0.005$) from one another.

**Table 7: Scavenging effects of poly herbal formulation and standard BHT on hydroxyl radical.The data represents percentage inhibition of hydroxyl radical**

| Concentration (µg/ml) | PHF-I %inhibition | PHF-II %inhibition | PHF-III %inhibition | BHT %inhibition |
|---|---|---|---|---|
| 50 | 54 | 65.8 | 70.5 | 66.94 |
| 100 | 56.36 | 69.33 | 71.6 | 70.48 |
| 250 | 62.26 | 70.5 | 78.77 | 72.84 |
| 500 | 68.16 | 71.6 | 78.77 | 78.74 |
| 750 | 78.77 | 81.13 | 81.13 | 85.83 |
| 1000 | 87.02 | 90.56 | 88.2 | 91.73 |
| $IC_{50}$ | 49 | 43 | 40 | 42 |

Values are means of three determinations ± standard deviation. Values followed by dunnet test. Results are significant ($^cP< 0.005$) from one another.

**Table 8: Absorbance and % viability of A375 and A431 cell lines, by poly herbal formulation-II**

| Concentration (µg/ml) | Absorbance of A 375 cell lines | Absorbance of A431 cell lines | % viability of A375 cell lines | % viability of A431 cell lines |
|---|---|---|---|---|
| 10 | 0.53 ± 0.03 | 0.41 ± 0.01 | 81.81 ± 0.03 | 79.53 ± 0.01 |
| 25 | 0.39 ± 0.02 | 0.34 ± 0.04 | 59.93 ± 0.02 | 66.79 ± 0.04 |
| 50 | 0.29 ± 0.04 | 0.21 ± 0.02 | 45.38 ± 0.04 | 40.92 ± 0.02 |
| 75 | 0.13 ± 0.04 | 0.07 ± 0.02 | 21.41 ± 0.04 | 13.77 ± 0.02 |
| 100 | 0.02 ± 0.02 | 0.11 ± 0.13 | 4.35 ± 0.02 | 22.58 ± 0.13 |
| Control | 0.65 ± 0.02 | 0.51 ± 0.08 | 100 | 100 |
| Cisplatin at a dose 20 µg/ml | 0.32 ± 0.02 | 0.12 ± 0.02 | 50 ± 3.51 | 24.13 ± 3.26 |

| Table 9: % Inhibition of A375 and A431 cell lines by poly herbal formulation-II | | |
|---|---|---|
| **Concentration µg/ml** | **% inhibition of A375 cell lines** | **% inhibition of A431 cell lines** |
| 10 | 18.1 | 21.5 |
| 25 | 41.1 | 34.3 |
| 50 | 65.7 | 60.1 |
| 75 | 79.6 | 87.3 |
| 100 | 96.7 | 78.5 |
| $IC_{50}$ | 35 | 41.5 |
| Control | 100 | 100 |
| Cisplatin at a dose 20 µg/ml | 49.7 | 75.9 |

Values are means of three determinations ± standard deviation. ($^bP< 0.01$)as compared with control using one way ANOVA followed by Dunnet test.

high glucose+FCS (fetal calf serum). After twenty-four hours of seeding, the medium was removed and then the cells were incubated for 3 days with DMEM with high glucose+FCS with the absence and/or the presence of various concentration of poly herbal formulations. After incubation, 20 µl of MTT reagent was added into each well. These plates were incubated again for 4 h in $CO_2$ incubator at 37°C. The resulting MTT-products were determined by measuring the absorbance at 570 nm using ELISA reader.

### The cell viability was determined using the formula

*Viability % = (optical density of sample/optical density of control) × 100*

### The % inhibition of cells was determined using the formula

*% Inhibition at concentration 'X' = [1–(absorbance average)/(control absorbance average)]×100*

$IC_{50}$ values were calculated as the concentrations that show 50% inhibition of proliferation on any tested cell line.

### Statistical Analysis

The data were presented as mean±standard deviation of three determinations. Statistical analyses were performed using a one-way analysis of variance. Multiple comparisons of means were done by the Dunnet test. A probability value of $p<0.05$, $p<0.01$ and $p<0.005$ were considered significant.

## RESULTS AND DISCUSSION

Herbs are staging a comeback and herbal 'renaissance' is happening all over the globe. Today, nearly 80% of the global population turns to plant derived medicines as their first line of defense for maintaining health and combating diseases.[26] This is due in part to the recognition of the value of traditional and indigenous pharmacopeias, the incorporation of some derived from these sources into pharmaceuticals.[27] The herbal products today symbolize safety in contrast to the synthetics that are regarded as unsafe to humans and environment.[28]

Literature revealed that the selected six herbs *Zingiber officinale*, *Curcuma longa*, *Aloe barbadensis*, *Citrus aurantium*, *Emblica officinalis* and castor oil have antioxidant and cytotoxic activities. Hence an attempt was made to formulate a polyherbal ointment, and to evaluate for its physical parameters, *in vitro* antioxidant and anticancer activities and compare the results with standard drugs like ascorbic acid and cisplatin.

Ethanolic extracts were prepared and phytochemical investigation of different extracts was performed. The details of qualitative chemical tests and phytoconstituents present in the extracts were shown in (Table 1).

In all formulations there was no considerable change in characters like color, odor, and consistency and there was no phase separation observed during the course of study. Also, no patches on rat skin were observed during skin irritant test.

### *In vitro* antioxidant activity

### DPPH radical scavenging assay

(Table 3) illustrates a significant decrease in the concentration of DPPH radical due to the scavenging ability of PHF-I, PHF-II, PHF-III and ascorbic acid. The scavenging effect of PHF-I, PHF-II, PHF-III and ascorbic acid on the DPPH radical decreased in the order of *ascorbic acid* > *PHF-II* > *PHF-III* > *PHF-I* (Figure 1). DPPH free radical scavenging activity of PHF-I, PHF-II, PHF-III increased with an increase in concentration. The $IC_{50}$ values of PHF-I, PHF-II and PHF-III were 70 µg/ml, 30 µg/ml and 35 µg/ml respectively. The $IC_{50}$ value of ascorbic acid was 22 µg/ml.

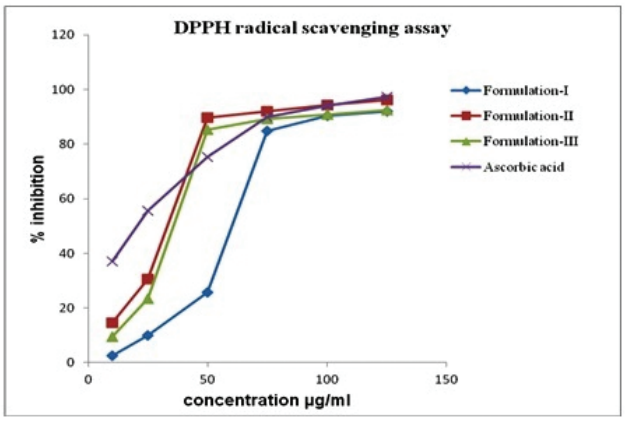


**Figure 1: Scavenging effects of poly herbal formulation and standard ascorbic acid on DPPH radical. The data represents percentage inhibition of DPPH radical**

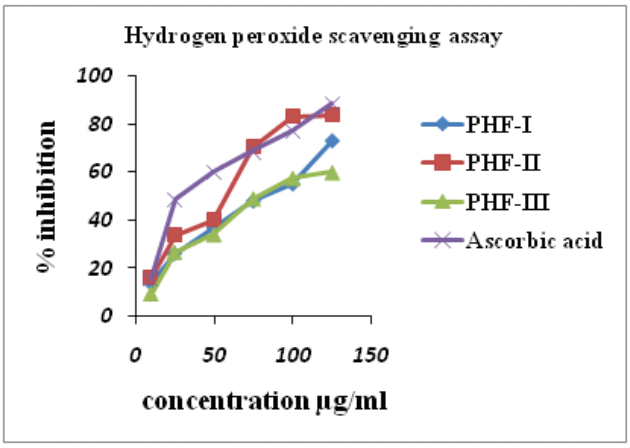


**Figure 4: Scavenging effects of poly herbal formulation and standard ascorbic acid on $H_2O_2$ radical. The data represents percentage inhibition of $H_2O_2$**

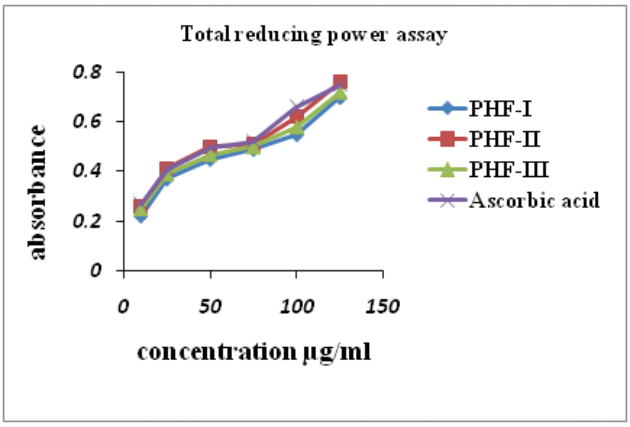


**Figure 2: Total reducing effect of poly herbal formulation and standard ascorbic acid on $Fe^{3+}$. Data represents the absorbance values of poly herbal formulation and ascorbic acid**

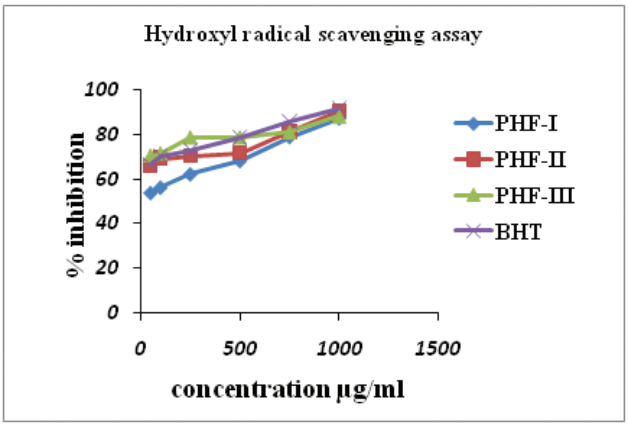


**Figure 5: Scavenging effects of poly herbal formulation and standard ascorbic acid on Hydroxyl radical. The data represents percentage inhibition of hydroxyl radical**

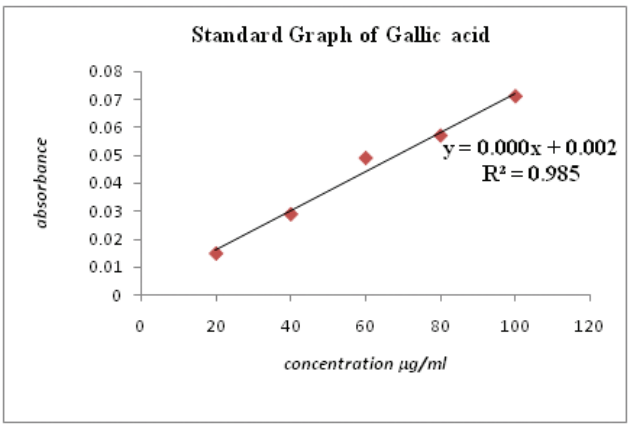


**Figure 3:Total phenolic content of Gallic acid**

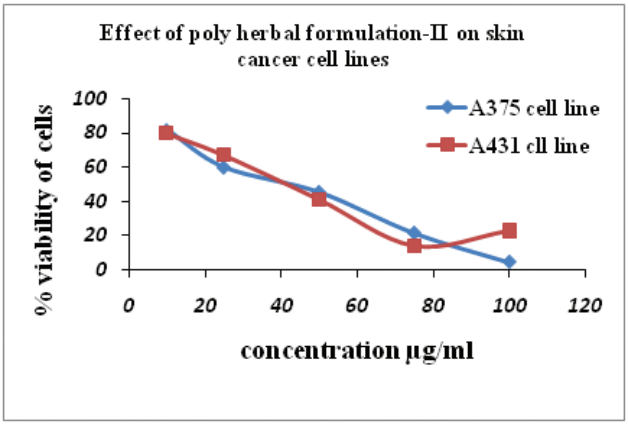


**Figure 6: Cytotoxic effect of poly herbal formulation-II on A375 and A431 skin cancer cell lines. Data indicates the % viability of A375 and A431 cell lines**

## Total reducing power assay

In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. PHF-I, PHF-II and PHF-III exhibited effective reducing power when compared to the standard (Ascorbic acid) by the potassium ferric cyanide reduction method (Table 4). Like the antioxidant activity, the reducing power increased with

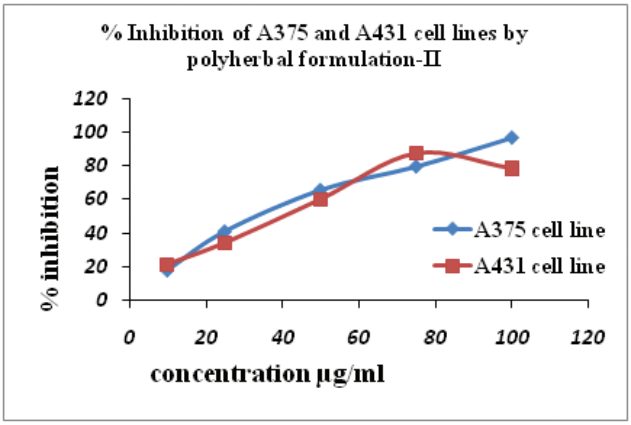


**Figure 7: Cytotoxic effect of poly herbal formulation-II on A375 and A431 skin cancer cell lines. Data indicates the % inhibition of A375 and A431 cell lines**

increase in the dose of poly herbal formulations. For the measurement of the reductive ability, the $Fe^{3+}$-$Fe^{2+}$ transformation was investigated in presence of the poly herbal formulations. Presence of reductants causes the reduction of the $Fe^{3+}$/ ferric cyanide complex to the $Fe^{2+}$ form. This $Fe^{2+}$ can be monitored by measuring the formation of perl's Prussian blue at 700 nm. PHF-II has shown highest reducing activity than PHF-I and PHF-III (Figure 2). It was found to be more potent than the standard.

## Total phenolic content

Total phenol compounds, as determined by Folin Ciocalteu method, are reported as Gallic acid equivalents i.e., the results expressed in terms of mg GAE/g dry sample with reference to standard curve (y=0.0007x+0.002, $R^2$=0.985) (Figure 3).[29] The total phenolic content varied from 35.42 ± 0.14 to 42.57 ± 0.23 mg GAE/g dry sample. It was observed that the total phenol content of PHF-II (containing 3% w/w each of *Aloe vera, Curcuma longa* and *Zingiber officinale)* was highest amongst the three formulations (Table 5). This may be due to the Curcumin I, Curcumin II and Curcumin III present in Curcuma longa and Gingerols and Shogoals present in *Zingiber officinale* which may explain for its high antioxidant potential. The results are consistent with earlier reports on *Zingiberaceae*s pecies (*Curcuma longa* and *Zingiber officinale*) as potential source of antioxidants due to their strong free radical scavenging ability in view of the high phenolic constitution.[30]

## Hydrogen peroxide scavenging activity

Scavenging activity of $H_2O_2$ by poly herbal formulations may be attributed to their phenolics, which can donate electrons to $H_2O_2$, thus neutralizing it to water. (Table 6) represents % inhibition of formulations and ascorbic acid on $H_2O_2$ (Figure 4). The poly herbal formulations are capable of scavenging $H_2O_2$ in a concentration dependent manner. The $IC_{50}$ values of ascorbic acid and PHF-I, PHF-II and PHF-III were found to be 25 µg/ml, 77 µg/ml, 60 µg/ml and 75 µg/ml. $H_2O_2$ scavenging activity of the formulations was in the order *ascorbic acid > PHF-II > PHF-III > PHF-I.*

## Hydroxyl radical scavenging activity

(Table 7) represents the % inhibition of PHF-I, PHF-II, PHF-III and BHT on hydroxyl radical. A concentration dependent inhibition against hydroxyl radical induced deoxyribose degradation was observed in the deoxyribose assay. Hydroxyl radical scavenging activity of the formulations was in the order *PHF-III>BHT>PHF-II >PHF-I* (Figure 5).

## *In vitro* Cytotoxic activity

As shown in (Table 8 and Table 9) poly herbal formulations were found to express cancer cell inhibitory activity against A431, A375 skin cancer cell lines when tested at concentrations of 10-100 µg/ml (Figure 6 and Figure 7). PHF-II exhibited the lowest cell viability on A375 (4.35%) and A431 (13.7%) at a dose of 100 µg/ml and 75 µg/ml respectively. The % cell viability decreased with increase in concentration of PHF-II. PHF-II was found to be almost two fold less potent against A375 cell lines ($IC_{50}$=35 µg/ml) and four fold less potent against A431 cell lines ($IC_{50}$=41.5 µg/ml) than Cisplatin having $IC_{50}$ values of 20 µg/ml and 9.01 µg/ml against A375 and A431 respectively. However the results are significant (**p<0.01) as compared to control.

# CONCLUSION

Poly herbal formulations (PHF-I, PHF-II, PHF-III) have shown significant antioxidant and free radical scavenging activities comparable to the reference compounds ascorbic acid and butylatedhydroxy toluene (BHT). Major antioxidant components present in the extracts seem to be phenolic and flavonoids based on the phytochemical studies. PHF-II containing *Aloe vera, Curcuma longa* and *Zingiber officinale* extracts in a relatively higher proportion exhibited the highest antioxidant and cytotoxic activities against skin cancer cell lines amongst the three formulations. Hence, further mechanistic studies can be carried out for PHF-II.

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## CONFLICTS OF INTEREST

The authors hereby declare that none of them have any financial/commercial obligations or conflicts with any individual or organization.

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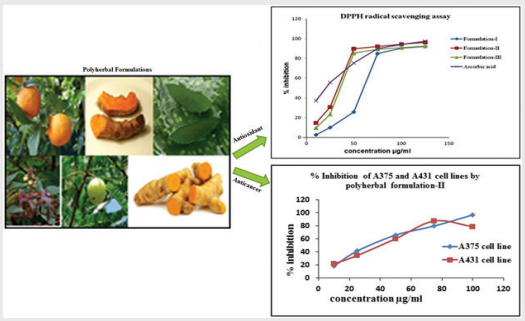
## PICTORIAL ABSTRACT




## SUMMARY

- PHF–II was found to be more potent anti-oxidant than PHF-I & PHF-III.
- PHF-II exhibited significant anticancer activity ($p<0.01$) with $IC_{50}$ values of 35 $\mu$g/ml and 41.5 $\mu$g/ml respectively on A375 (melanoma) and A431 (skin carcinoma) cell lines.
- PHF-II was found to be almost two fold less potent against A375 cell lines and four fold less potent against A431 cell lines than Cisplatin having $IC_{50}$ values of 20 $\mu$g/ml and 9.01 $\mu$g/ml against A375 and A431 respectively.
- Newer poly herbal formulations could be considered as potent antioxidants and cytotoxic agents.

## ABBREVIATIONS USED

**BHT:** Butylated hydroxyl toluene; **DPPH:** 1,1-Diphenyl-2-picryl hydrazyl; **EDTA:** Ethylenediamine tetra acetic acid; **$IC_{50}$:** Inhibitory Concentration in 50% population; **MTT:** 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium Bromide; **PEG:** Polyethylene glycol; **PHF:** Polyherbal formulation; **ROS:** Reactive oxygen species; **TBA:** Thiobarbituric acid; **TCA:** Trichloro acetic acid.

## About Authors

**Kalam Sirisha:** Persued Her M.Pharm (2004), Ph.D. (2010) from University College of Pharmaceutical Sciences, Kakatiya University, Warangal, Telangana. She is presently working as Associate Professor & Head, Department of Pharmaceutical Chemistry, Vaagdevi College of Pharmacy, Warangal. She was awarded Career Award for Young Teachers (CAYT) from All India Council of Technical Education (AICTE), New Delhi. Presently she is working for the development of new molecules from natural and synthetic origin for cancer, tuberculosis and diabetis mellitus. She has hands on experience in instrumental analytical techniques like UV-Visible, IR, NMR, HPLC etc.

**Mahendar Porika:** Obtained his M.Phil and Ph.D from Department of Biotechnology, Kakatiya University, Warangal, Telangana in 2007 and 2010, respectively. He was awarded with Postdoctoral Fellowship by University Grants Commission, New Delhi. Currently, he is working as a lecturer in the same Department. His current research interests include cancer detection, inhibition and role of telomerase in cancer and under treatment.