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Original Article

Antitumor and Antioxidant Effects of Flavonoid Fraction of *Citrus sinensis* peel Extract

Godishala Shirisha[1], Kiranmai Mandava*[2], Uma Rajeswari Batchu[2], Kesava Rao Thammana[2], Vijaya Laxmi Turpu[2]

ABSTRACT

Background: Cancer is one of the leading causes of death and globally the numbers of cases of cancer are increasing gradually. However, surgeries, chemotherapies have become safer, but these treatments have debilitating side effects. Flavonoids present in the human diet comprise many polyphenolic secondary metabolites with broad-spectrum pharmacological activities including their potential role as anti-cancer agents. **Objective:** The objective of the present study was to extract, orange peel flavonoids (Orange Peel Extract) and to screen anticancer potential of OPF. **Methods:** In the present study tryphan blue dye exclusion, clonogenic assay and nuclear damage studies by ethidium bromide staining were performed to estimate *in vitro* antitumor properties of Orange Peel Extract and subsequently *in vivo* studies also performed using the Dalton Lymphoma Ascites (DLA) tumor model in Swiss albino mice. **Results:** *In vitro* studies revealed the moderate toxicity, high regenerative capacity of Orange Peel Extract and also showed changes in nuclear morphology similar to that of apoptotic cells which is one of the important aspect of an anticancer drug. *In vivo* studies confirmed the anticancer activity of Orange Peel Extract and has increased the average life span of treated animals and restored the antioxidant enzyme levels and hematological parameters to normal which was comparable to that of standard methotrexate. **Conclusion:** Overall, these findings have proved that out of the two doses (50mg/kg bw and 200mg/kg bw) employed for the study lower dose (50mg/kg) was found to be more effective than higher dose (200mg/kg). Hence flavonoid fraction of orange peels can be the better alternative to treat cancer.
Key words: Dalton lymphoma ascites, Ethidium bromide, Methotrexate, Orange peel flavonoids, Tryphan blue.

Godishala Shirisha[1], Kiranmai Mandava*[2], Uma Rajeswari Batchu[2], Kesava Rao Thammana[2], Vijaya Laxmi Turpu[2]

[1]Department of Pharmaceutical Biotechnology, JSS College of Pharmacy, Ootacamund, Tamil nadu 643001, INDIA.
[2]Department of Pharmaceutical Chemistry, Bharat Institute of Technology, Ranga Reddy District, Telangana 501510, INDIA.

Correspondence

Dr. Kiranmai Mandava

Department of Pharmaceutical Chemistry, Bharat Institute of technology, JNTUH, Telangana, 501510, INDIA.

Phone no : (+91) 9052001390

E-mail: gchaitra.kiran@gmail.com

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INTRODUCTION

Cancer is continuing to be one of the leading causes of death in developing and even in developed countries. It is a proliferative disorder which involves transformation, proliferation, angiogenesis, dysfunction of apoptosis, invasion and metastasis. Owing to the disease burden of cancer enormous therapies have designed for treatment. Conventional chemotherapy treatment could cause adverse and toxic side effects on normal cells during the course of treatment and fails to serve the ultimate purpose of curing cancer. In recent years, great attention has been paid to the discovery of anticancer bioactive compounds of natural origin due to their ability to produce a health benefit with negligible side effects. Cancer chemoprevention by use of natural or synthetic substances and its prevention through dietary intervention has become an important issue. Flavonoids have important effects on cancer chemoprevention and chemotherapy. Flavonoids are a group of about 4000 naturally polyphenolic compounds, found universally in plant origin. According to the differences in functional groups and their relative positions of the 15-carbon skeleton (aglycons), flavonoids are classified into several subgroups including the following: flavone, flavanone, flavonol, isoflavonoid, anthocyanidin and chalcones.[1] Flavonoids are widely present in the genus Citrus (family Rutaceae).[2] Many mechanisms of action of flavonoids have been identified, including carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis and differentiation, inhibition of angiogenesis, antioxidation and reversal of multidrug resistance or a combination of these mechanisms.[3]

Fruits by-products such as seeds, peels, stems, barks and leaves usually been thrown into an environment which causes serious disposal problem in food and agriculture industries. Therefore, extensive researches have been carried out worldwide in order to minimize the above stated problem. *Citrus sinensis* (Orange, Rutaceae) fruit peels are beneficial to human health. Orange peels have been used in food, drug and cosmetic products. However, the overall demand of orange peels is of insignificant as applications have not been widely explored and recognized. The major constituents of orange peels include flavonoids such as polymethoxy flavonoids; terpenoids, such as limenene and linalool. These

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flavonoids are found to have antioxidant,[4] antimutagenic,[5] antiallergic, anti-inflammatory and antimicrobial[6] activities. The objective of the present study was to evaluate *in vitro* and *in vivo* antitumor effects of orange peel flavonoids including the estimation of antioxidant enzyme levels.

MATERIALS AND METHODS

Minimum essential medium (MEM), tryphan blue, crystal violet, ethidium bromide, ethylene diamino tetraacetic acid (EDTA), triton –X 100, trypsin, hank's balanced salt solution (HBSS), phosphate buffer saline (PBS), bovine serum albumin (BSA) were procured from Hi Media, Mumbai. Hydrogen peroxide (H_2O_2) was obtained from Sigma Aldrich Co., St. Louis, USA. Trichloroacetic acid, thiobarbituric acid were procured from Rankem chemicals, New Delhi, India.

Collection and extraction of orange peels

Fresh oranges were procured from the Ootacamund municipal market, Ooty, Tamilnadu, India. The dried peels were powdered (50g) and extracted using methanol:water (9:1) in the first step and followed by methanol:water (1:1) in the second step. The filtered two extracts were combined and concentrated until methanol is evaporated.

Isolation of flavonoids

The extracts prepared in the above step were defatted by using n-hexane in a separating funnel and repeated several times. The aqueous layer containing flavonoids was separated and vacuum dried under reduced pressure at 60°C±1°C and stored in an airtight container until further use.[7]

Determination of total flavonoid content

Total flavonoid content was determined according to the modified method of Chang *et.al.* Briefly, an aliquot of 0.5 ml of sample (1 mg/ml) was mixed with 1.5 ml of methanol, 0.1 ml of 1% aluminum chloride and 0.1 ml of potassium acetate solution (1 M). In the mixture, 2.8 ml of distilled water was added to make the total volume of 5 ml. The mixture was vortexed and absorbance was measured at 415 nm using UV-Visible spectroscopy (Shimadzu 1800) and calculated. Quercetin was used as standard.[8-9] The calibration curve was prepared by using standard quercetin at concentrations of 6.25 to 100 mg/ml in methanol.

In vitro antitumor studies

Maintenance of cell lines

Vero normal cell lines, L-929 cancer cell lines used in the present study were procured from National Centre for Cell Sciences, Pune, India and Dalton lymphoma ascites (DLA) tumor cells were procured from Amala Cancer Institute, Amala Nagar, Trissur, Kerala, India. Healthy adult mice were obtained from the animal house, JSS College of Pharmacy, Ootacamund, India and maintained under standard environmental conditions (22-28°C, 60-70% R.H, 12 h D/L cycle). The DLA tumor cells were maintained intra peritoneally *in vivo* in Swiss albino mice. All the experiments were conducted as per the guidelines of CPCSEA, Chennai, India (Approval No. JSSCP/IAEC/M.Pharm/PH.BIOTECH/01/2006-07).

Short term toxicity studies

The studies were carried out in DLA cells using tryphan blue dye exclusion assay[10-11] DLA cells were cultured in the peritoneal cavity of mice and bulged cells were withdrawn between 10-15 days using sterile syringe. Thereafter cells were washed thrice with 0.1ml of phosphate buffered saline (PBS, 0.2M, pH 7.4) and adjusted to cell count of $2X10^6$ cells/ml with Hank's balanced buffer saline (HBBS). Subsequently the diluted cell suspensions were exposed to 100µl of various concentrations of Orange Peel Extract (and methotrexate (standard) and the final volume was adjusted to 1ml with PBS. The cell culture was incubated at 37°C for 3h. Thereafter dye exclusion test was carried out mixing equal quantities of drug treated cells and tryphan blue (0.4%) and left for a min. Then the viability of the cells was recorded using haemocytometer. The % growth inhibition was calculated by using the formula given below.

$$\% \text{ Viability} = \frac{\text{Total cells} - \text{Dead cells}}{\text{Total cells}} \times 100$$

$$\% \text{ Growth inhibition} = 100 - \% \text{ Viability}$$

Long term survival studies

These studies were performed by clonogenic assay.[12] A series of cultures seeded with $5x10^4$ cells/ml were prepared and incubated for 48hrs at 37°C. As growth medium was decanted, the cultures were exposed to test agents of different concentration (200 to 1.6µg/ml) and incubated at 37°C for a period of 72 h. Thereafter the cultures (1000 cells/ml) were washed, trypsinized and seeded in 24 well microtitre plates for clonal growth and incubated until colonies formed. The colonies were fixed with 1% crystal violet in absolute ethanol subsequently washed and dried. The individual colonies were counted (> 5 generations). The procedure was repeated thrice. The number of colonies of the treated cells is compared with the number of colonies from untreated control cells. The plating efficiency was calculated by using the formula given below.

$$\text{Plating efficiency} = \frac{\text{No. of Colonies}}{\text{No. of Cells seeded}} \times 100$$

Nuclear damage studies in cell culture

Ethidium bromide staining method was used to observe apoptosis morphological changes.[13] A series of 2.5 ml monolayer trypsinized cell cultures (50,000 Cells/well) added on cover slip was placed in 6 well microtitre plates. The cell culture wells were exposed to different drug concentrations and one well with the only maintenance medium was used as a control were incubated at 37°C, in 5% CO_2 atmosphere for overnight followed by cells, washing with PBS and fixed with 1ml of methanol (90%) at 20°C for 20 min. Consequently fixing was repeated replacing methanol by acetone for 10 sec and the plates were washed with ice cold PBS for 2-3 times. Eventually the cells were incubated with PBS containing 1% BSA and 0.1 % triton X-100 at 37°C for 30 min followed by washing with PBS 2-3 times. Finally 100µl of ethidium bromide (1µg/ml in PBS 7.4 pH) was added and incubated at 37°C for 20 min. Subsequently cover slips were washed thrice and observed under a fluorescent microscope (Hund, Germany) for nuclear changes. Apoptosis was confirmed by nuclear condensation, membrane blebbing and formation of round apoptotic bodies.

In vivo anti cancer studies

Toxicity studies

The *in vivo* anticancer studies were performed by DLA cell model in Swiss albino mice. DLA cells were used to induce tumors in mice and the effect of Orange Peel Extract was tested on them. The concentrations of orange peel flavonoids and standard methotrexate were prepared by suspending in distilled water using 0.3% sodium carboxy methyl cellulose (SCMC).[14-15]

Swiss albino mice were divided into nine Groups (6 mice/group) and all the groups were received DLA cells ($1X10^6$ cells) intra peritoneally except the Group I. The Group I and Group II were served as normal control and tumor control, respectively and received SCMC suspension orally. Group III served as positive control and treated with standard

methotrexate. Meanwhile Group IV-V received flavonoids (50-200mg/kg) intraperitoneally after the 24h of DLA cells inoculation for 10 days.

The hematological parameters such as RBC, total WBC and hemoglobin (Hb) were estimated from the 11th day of tumor induction. In addition to the above parameters, antitumor parameters such as average lifespan, % increase in the life span and bodyweight analysis were also studied.

Average life span

Average life span of all the groups was determined and noted.

Percentage increase in the life span (% ILS)

The effect of Orange Peel Extract on tumor growth was monitored by recording the mortality, daily for a period of 6 weeks and percentage increase in the life span (%ILS) was calculated.

$$\% \text{ increase in the life span} = \frac{\text{MST of the treated group} - \text{MST of the control group}}{\text{MST of the control group}}$$

Where,

$$\text{MST} = \frac{\text{Day of first death} + \text{day of last death}}{2}$$

Body weight analysis

Body weights of the experimental mice were recorded both in the treated and control groups over a 15 day period. Percentage increase in body weight was calculated using following formula.

$$\% \text{ Increase in body weight} = \frac{\text{Body weight of an animal on 15th} - \text{Body weight of an animal on 0 day}}{\text{Body weight of animal on 0 day}}$$

Thereafter haemolysate was prepared from the remaining blood and used for estimation of total antioxidant capacity by measuring catalase and lipid peroxidase (LPO) levels in haemolysate.[16]

Estimation of catalase

Catalase was estimated by the modified method of Joharapurkar.[17] To 50µl of lysate, 2ml of phosphate buffer (p^H 7.0) and 1ml of 30mm H_2O_2 were added and absorbance was measured at 240 nm using a spectrophotometer.

Estimation of lipid peroxidase

To the 0.5 ml of haemolysate add 0.5 ml of trichloroacetic acid and centrifuged. To the 1ml of supernatant add 0.25ml of 1% TBA and boiled for 60min and cooled immediately. The absorbance was measured at 532 nm using a spectrophotometer [17]

Statistical analysis

Results were expressed as mean ± SD and the significance of all the *in vivo* data was analyzed by one way analysis of variance (ANOVA) followed turkeys multiple comparison post tests. Values of $p< 0.05$ was considered as significant. Graph pad prism 5.0 software was employed to make bar diagrams.

RESULTS

The yield of Orange Peel Extract was found to be 3% w/w and total flavonoid content of Orange Peel Extract was found to be 25.48 mg quercetin equivalents per gram of extract.

In vitro anticancer studies

Short term antitumor studies

Short term anti tumor studies of Orange Peel Extract at different concentrations against DLA tumor cells were shown in Table 1. The CTC_{50} was calculated as 275µg/ml (Figure 1).

Long term toxicity studies

Orange Peel Extract were tested for long term toxicity studies and results were given in Table 2.

Nuclear damage studies

It was evident from the study that orange peel flavonoids showed apoptotic nuclear morphology changes in cancer cell lines compared to control. The photographs of evident morphological changes were shown in Figure 2.

In vivo antitumor and antioxidant studies

The *in vivo* anticancer studies of orange peel flavonoids against DLA bearing Swiss albino mice were performed at two different concentrations (50 and 200 mg/kg p.o) for 10 days using methotrexate as a standard. The results were given in Table 3, Figure 3, 4 and 5. It was also revealed from the study that the decrease in antioxidant defense mechanism was observed in Group II animals specified by elevated levels of LPO and decrease in catalase activity. The treatment with Orange Peel Extract has restored these antioxidant enzyme levels (catalase, LPO) towards normal (Table 4,

Table 1: Determination of CTC_{50} of Orange Peel Extract by tryphan blue dye exclusion assay in DLA cells.

Name of the flavonoid	Concentrations (µg/ml)	No. of viable Cells	No. of dead cells	Mean CTC_{50} (µg/ml)
Orange peels flavonoids	1000	180	320	275
	500	320	173	
	250	336	98	
	125	358	82	
	62.5	384	64	
	31.25	534	24	

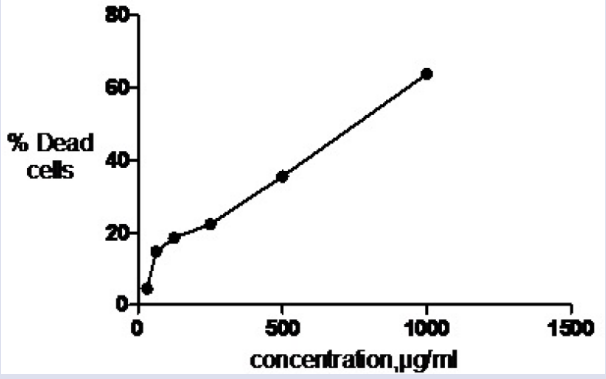

Figure 1: Determination of CTC_{50} of Orange Peel Extract by tryphan blue dye exclusion assay in DLA cells.

Figure 6). These findings also concluded that 50mg/kg bw concentration was found to be more effective than 200mg/kg bw.

DISCUSSION

Recent scientific studies have concluded that fruits rich in phenolics and flavonoids have shown significant antioxidant and anticancer activities.[18-19] Many fruits possess medicinal activities as a whole fruit, seeds, leaves and as peels. Numerous medicinal plants have been reported to have anticancer potential such as lemon,[20] orange,[21] papaya,[22] guava.[23] Among such orange peels have been utilized for long, as an active ingredient in traditional medicine. It was also reported that orange peels possess

Table 2: Determination of CTC_{50} by clonogenic assay.

Name of the cell line	CTC_5	Concentrations (µg/ml)	No. of colonies/ well	Plating efficiency (%)
VERO	331.6	100	220	1.76
		200	150	1.2
		300	80	0.64
		Control	410	3.6
L-929	230.01	50	180	0.72
		100	120	0.480
		200	48	0.1
		Control	350	1.4

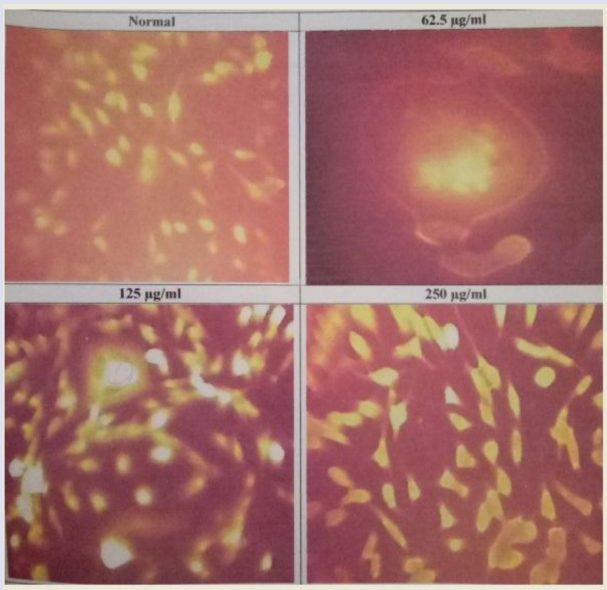

Figure 2: Induction of apoptosis in L-929 cell line by Orange Peel Extract at different concentrations.

Table 3: Effect of Orange Peel Extract on antitumor parameters of DLA bearing mice.

Parameters	Normal	DLA	DLA+ Methotrexate (3.4mg/kg)	DLA + Orange peel flavonoid (50mg /Kg)	DLA + Orange peel flavonoid (200mg /Kg)
Average life span (Days)	NA	16.50 ± 1.05	27.30 ± 0.55[d]	26.00 ± 0.51[d]	22.5 ± 0.61[d,e]
Increase in life span (%)	NA	NA	65.50	51.00	31.50
Increase in body Wt (%)	NA	33.00 ± 1.18	12.34 ± 0.85[a]	12.06 ± 0.99[a]	14.92 ± 1.10[d,e]
Total WBC (103 /mm3)	12.50 ± 0.12	23.58 ± 0.13[a,c]	15.23 ± 0.02[c]	17.45 ± 0.27[c]	21.67 ± 0.26[b,e]
RBC (1x106 /mm3)	8.83 ± 0.08	7.96 ± 0.23[a]	8.8 ± 0.08[c]	8.72 ± 0.17[c]	8.72 ± 0.19
Hb (g/dl)	11.14 ± 1.25	8.44 ± 0.24[a]	12.58 ± 0.39[d]	10.5 ± 0.46[c,f]	10.14 ± 0.46[c,f]

*The results were expressed as mean ± S.E.M. (n= 6) where a - $p<0.0001$ between normal and control, b - $p<0.05$, c- $p<0.01$, d- $p<0.0001$ between control and treated group and e-$p<0.05$ between methotrexate and flavonoids and f- $p< 0.001$.[14-15]

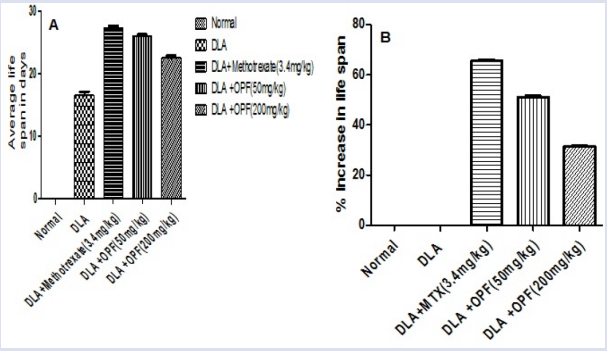

Figure 3: Effect of Orange Peel Extract on A: Average life span B: % Increase in life span of DLA bearing mice.

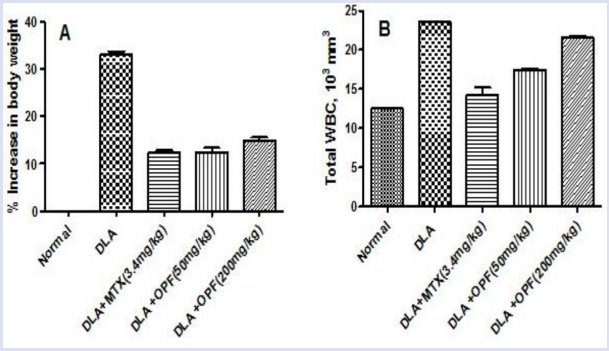

Figure 4: Effect of Orange Peel Extract on A: % Increase in body weight B: Total WBC, 10^3 mm of DLA bearing mice.

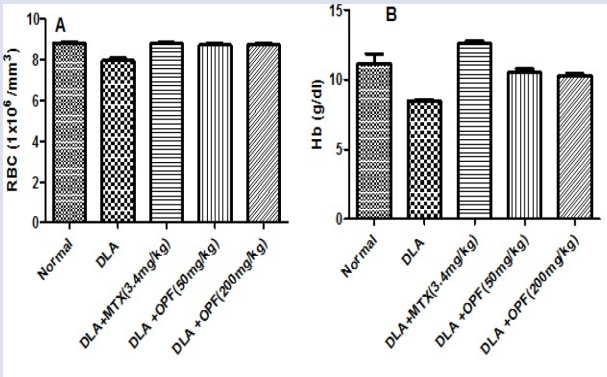

Figure 5: Effect of Orange Peel Extract on A: RBC(1x10^6 /mm^3) B: Hb (g/dl) of DLA bearing mice.

Table 4: Effect of Orange Peel Extract on antioxidant enzymes in DLA tumor bearing mice.

Group	Treatment	Lipid peroxidase (LPO), (U/g hb)	Catalase (U/g hb)
I	Normal	6.82±0.33	3.70±0.15
II	DLA control	40±1.04[a,c]	2.196±0.18[a,c]
III	Methotrexate	8.8±0.29[c]	3.69±0.043[c]
IV	Flavonoids(High Dose)	20.91±0.47[e]	2.42±0.071[e]
V	Flavonoids (Low Dose)	9.3833±0.18[c]	3.68±0.15[c]

* The results were expressed as mean ± S.E.M. (n= 6) where a - p<0.0001 between normal and control, b - p<0.01, c- p<0.0001, d- p>0.01 between treated and control groups and e-p<0.001 between methotrexate and flavonoids.[14-15]

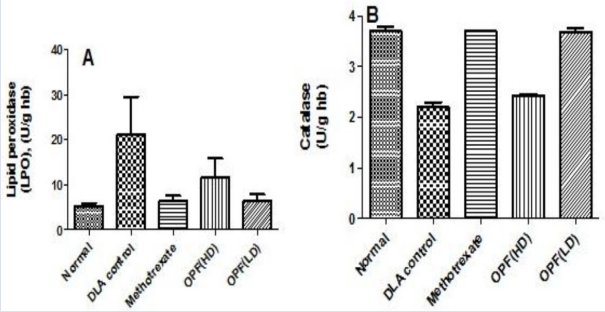

Figure 6: Effect of Orange Peel Extract on A: Lipid peroxidase B: Catalase in DLA tumor bearing mice.

significant antioxidant[24-27] antibacterial,[28] antiproliferative activity.[29] In the present study *in vitro* and *in vivo* antitumor studies of orange peel flavonoids were conducted.

The results from *in vitro* studies concealed that flavonoids showed moderate cytotoxicity and acted specially on cancer cell culture (L929) compared to the normal cell culture (vero). The short term toxicity studies against DLA culture by tryphan blue dye exclusion assay revealed moderate toxicity of Orange Peel Extract. Long term studies were carried out to demonstrate survival of cells rather than cell toxicity which is significant for cancer treatment. This is used to evaluate the regenerative capacity, which is measured by plating efficiency and studies also revealed that plating efficiency was found to be less for cancer cell line than normal cell line. This shows that Orange Peel Extract showed a higher regenerative capacity in normal cell culture compared to cancer cell culture which demonstrates its specificity towards cancer cells. Nuclear cell damage studies by ethidium bromide revealed that orange peel flavonoids treated cells showed nuclear morphological changes similar to that of apoptotic cell morphology.

The results from *in vivo* anticancer studies confirmed the *in vitro* anticancer activity of orange peel flavonoids. They have shown the prolongation of life span of Group IV and V animals which being the reliability criteria for judging the value of an anticancer drug. The Group IV and V animals treated with orange peel flavonoids have shown a significant reduction in body weight indicating the potent anticancer properties of Orange Peel Extract. This study also explored that low dose of Orange Peel Extract showed better anti cancer activity than its high dose counterparts. The two dose levels selected (50mg/kg and bw and 200mg/kg bw) were in the proportion of 1:4. The highest dose (200mg/kg bw) might be toxic to animals, hence there is a decrease in average life span and other parameters. The haematological parameters such as Hb content, WBC and RBC count were increased in Group IV and V animals compared to normal clearly indicating that Orange Peel Extract possesses protective action on haemopoetic system. It was also evident from the study that the treatment with Orange Peel Extract has shown significant restoration of catalase and LPO levels towards normal, which might be responsible for the increase in the life span of treated animals.

CONCLUSION

In the present study the orange peel flavonoids showed promising anticancer activity which was evident from the *in vitro, in vivo* studies conducted. It has increased the average life span of treated animals and restored the antioxidant enzyme levels (catalase, LPO), biochemical and haematological parameters to normal which was comparable to that of standard methotrexate. This has paved a way to use fruit peels of herbal medicine as a source of bioactive constituents for the treatment of diseases.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

H: Hrs; **Min:** Minute.

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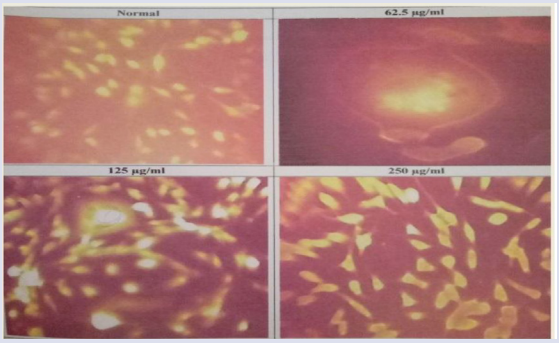
GRAPHICAL ABSTRACT



SUMMARY

Cancer is one of the leading life threatening disease and 2nd leading cause of death world wise. As current treatments for cancer are suffering from shortfalls, alternative therapy need to be searched for successful treatment with less or no side effects. Apart from their cultural and spiritual points, herbal medicine has been used for the treatment of cancer since ancient days. Orange peels are being used as food, drug and cosmetic products. In the present work, the flavonoid fraction of orange peels have evaluated for *in vivo* and *in vivo* anti tumor activity.

ABOUT AUTHORS

Mrs. Godishala Shirisha, M.pharmacy, Department of Pharmaceutical Biotechnology, JSS college of Pharmacy, Ootacamund, Tamilnadu, India.

Dr. Kiranmai Mandava, Professor, Department of Pharmaceutical Chemistry, Bharat Institute of Technology, Mangalpally, R.R. District, Telangana, India.

Mrs. B. Uma Rajeswari Batchu, Assistant Professor, Department of Pharmaceutical Chemistry, Bharat Institute of Technology, Mangalpally, R.R. District, Telangana, India.

Dr. Kesav Rao Tammana, Professor, Department of Pharmaceutical Chemistry, Bharat Institute of Technology, Mangalpally, R.R. District, Telangana, India.

Mrs. Vijaya Laxmi Turpu, Assistant Professor, Department of Pharmaceutical Chemistry, Bharat Institute of Technology, Mangalpally, R.R. District, Telangana, India.

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