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SHORT TERM *IN VIVO* STUDY OF *Oroxylum indicum* WITH THE COMBINATION OF *Catharanthus alba*, *Commiphora mukul*, AND *Cynodon dactylon* IN DLA TRANSPLANTED SWISS ALBINO MICE TO UNDERSTAND ITS ANTICANCER PROPERTY

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ABSTRACT

A comparative study of anticancer effect of *Oroxylum indicum* with the combination of *Catharanthus alba*, *Commiphora mukul* and *Cynodon dactylon* in DLA (Daltons Lymphoma Ascities tumor cell lines) transplanted Swiss albino mice (*Mus muculus)* by *in vivo* methods were done. Swiss albino male mice (32 in number) treated with DLA cell lines were used for this study. In this, the parameters like body weight, abdomen size, Cytotoxic study-Trypan blue and M.T.T Assay were included. The group III, which were treated with *O. indicum* for a period of one month, later treated with DLA celllines were showing a significant increase in the life span, WBC, R.B.C and TLC count and M.T.T. assay. While studying the results of each experiment, it was concluded that *Oroxylum indicum*, the unexplored medicinal plant was promisable in cancer treatment with high mode of activity and low cost in the contest of sky rocketting prices of drugs.

Keywords: *Oroxylum indicum*, DLA cell lines, *Catharanthus alba*, *Commiphora mukul*, *Cynodon dactylon* and M.T.T. assay.

INTRODUCTION

Researchers now days are concentrating mostly on plant derived drugs because of their high medicinal value, accuracy and lower toxicity. There is an increasing trend in the use of medicinal plants for cancer treatment. (Dinnen.R.D; Rameswar, 1997). Here comes the importance of the anti–cancerous effect of *Oroxylum indicum*, a medicinal plant, using widely in ayurvedic medicine.

Oroxylum indicum is commonly known as the 'Indian Trumpet Tree' or 'Syonaka' (Sanskrit); (Bignoniaceae family). It has been used for centuries in ayurveda against inflammation, asthma, cardiac disorders, rheumatoid arthritis and wounds (Binuttu.O.A.,1994). It is a small-medium sized, ornamental plant, with very large leaves (90 - 180 cm). The fresh root bark is soft and juicy, creamish yellow to gray in colour with much medicinal property. The barks of wood and roots are mainly used in ayurveda and have the properties for a good anticancer, antileukemic drug and can be used against skin ulceration, cough and as a purgative. 'Lapachole and β-lapachone' are the two important Naphthaquinone found in the bark and wood of plants in Bignoniaceae family. These inhibit the action of the DNA-Topoisomerase I, enzyme that unwinds the DNA and helps in the replication of the cell. The bio-chemical structure of Lapachole is (2-hydroxy-3-methyl-2-butenil)-1,4 Naphthaquinone (Mao; Nakkahara; 2002). Few other medicinal plants like, *Catharanthus alba, Commiphora mukul* and *Cynodon dactylon* is also selected for comparing the anti-cancer effect in Swiss albino mice inoculated with Daltons Lymphoma Ascites (DLA) cell line (Anesin., 1993; Amma, M. K. P., 1978, Chakrabarthi. S,1984).

Catharanthus alba is an erect, beautiful herb with a number of alkaloids mainly like 'Vincristine and Vinblastine'. It was used as a folk remedy for cancer, diabetics and have high potensive, sedative and transquilisng property. *Commiphora mukul* (Guggul) can been seen in wild arid zones. This woody shrub is highly aromatic and the pale, yellowish brown aromatic gum is used for anticancer, anti-cholesterol, anti-inflammatory uses. The major contents are E-guggul steron, and Guggul Sterol - I,I,III. *Cynodon dactylon* is a long, creeping grass with antibacterial, antifungal, anticancer, diuretic and dropsy properties. The active constituent 'Arggopyrine' is having good wound healing properties.

MATERIAL AND METHODS

Swiss albino (*Mus muculus)* male mice (32 in number, 25-30 g), where used for the studies and animals were maintained under standardized, environmental conditions (22-28°C, 60-70 % relative humidity, 12 hours dark/light cycle and water *ad libitum.* All the experiments were conducted under the guidelines of Institutional Animal Ethical Committee.

Methods for inducing cancer in mice:

The Swiss Albino male mice used for the study were taken. The Dalton's Lymphoma Ascites (DLA) cell lines from a cancerous mice were taken (1x 10^6cells) in 1ml syringe, containing 0.1ml of P.B.S (pH = 7.3) and injected in to the mice intraperitonially. Tumor was developed in the abdomen of mice, after 15 days of inoculation. The cell lines were maintained by subculturing within the mice (3 x 10^6 DLA cells/ml were taken in 300ìl PBS and inoculated in the intraperitonial cavity of the mice) itself.

Dosage of medicine:

The normal mice is treated as vehicle (Group-I) and the tumor alone,(groupII) without any treatment. The third group was treated with the extract of *O. indicum* first, after one month DLA cell lines.(10ml / Kg body weight).So 0.75ml three times daily.The fourth group mice were treated with *O.indicum*, in the same day (After DLA induction), 0.75ml, three times daily. Group V was, mice with DLA, treated with mixture, 0.75 ml, three times daily. The duration of the study was 7 weeks.

Effect of medicinal plant in the life span of mice:

The life span of mice in each group were studied and reported. The survival days of animals injected with DLA (10 ^{6}cells / animal) were observed for 50 days. The percentage increase in life span (percentage ILS) was calculated using the formulae

$$\%ILS = \frac{T-C}{C} \times 100$$

Where, 'T' is the mean survival time of treated mice and 'C', the mean survival time of the control. (Expressed in days).

Experimental Design - Grouping of Animals:

S.No.	Group	Description	No of Mice
1	I	Normal Swiss Albino male mice (vehicle)	6
2	II	Swiss Albino male mice with DLA cell lines	6
3	III	Swiss Albino male mice treated with *Oroxylum indicum* extract (one month before the inoculation of DLA)	6
4	IV	Swiss Albino male mice treated with *Oroxylum indicum* extract (after the inoculation of DLA)	6
5	V	Swiss Albino male mice treated with the 'Mixture' of *C. alba*, *C. mukul* and *C. dactylon*	6

Body Weight Study:

The body weight of mice from each group recorded once in every six days and done a comparative study.

Abdomen Size:

Inflammation of abdomen was used as a sign of building up of ascitic fluid in the peritoneal cavity.

The DLA cell count:

The number of DLA cell lines in each group of animal's showing the tumor development. This was done on the 3rd, 15th, and 21st day.

***In vivo* cytotoxic study - Trypan Blue**

It is based on the principle that the viable cells exclude the dye and remain unstained, while non – viable cells takes up the dye and are stained as blue. The DLA cells from mice were taken and washed with saline. 0.1ml containing 1x10 ^{6} cells was used in *in vivo* assay. Various concentration of sample incubated at 37ÚC for three hours and stained with Trypan blue and counted in a heamocytometer. The dead cells were blue in colour and the percentage was counted (percent cytotoxicity).

$$\text{Percent Cytotoxicity} = \frac{\text{Dead cell count}}{\text{Dead cell count + Viable cell count}} \times 100$$

Cytotoxicty study by M.T.T. assay (3-(4,5- dimethyl thiazolyl), 5–diphenyl tetrazolium bromide):

It is based on the principle that the cleavage

of tetrazolium salts into a blue coloured product (Formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells are found to be proportional to the extent of formazan production by the cells used. The 5mg / ml of M.T.T reagent (Sigma) dissolved in PBS was mixed with the tumor cell and kept for 5 hours incubation, at 37ÚC in a 96 well plate. Remove the media and add DMSO (Sigma) to each well plates and kept for 5 minutes incubation and measured the absorbency at 550nm. The cleavage of tetrazolium salt M.T.T will help to study the percentage of cytotoxicity in a cancer cell by any drug.

Total White Cell Count -Trucks Fluid Method:

Blood was collected from the mice tail vein and used it for total white cell count, (Samuel, 1988)

Differential white cell count:

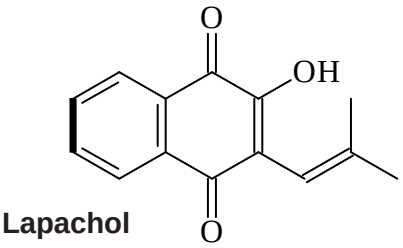
The tail vein blood of mice from each group were collected and stained with Leishman's stain and counted. (Samuel, 1986)

Total Lymphocyte Count:

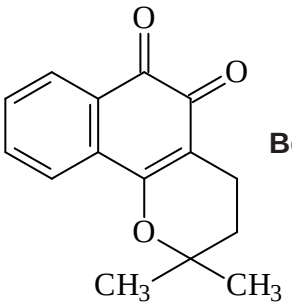
The tail vein blood was carefully layered on the Ficol Histopaque solution (Sigma) and cells were washed four-five times with Hanks Balanced Salt Solution (HBSS)(Sigma), counted in a heamocytometer and the viability checked with Trypan Blue Dye exclusion test.

Estimation of Hemoglobin:

Blood from mice from each where dissolved in Drabkins Diluting solution and mixed thoroughly. The readings were taken at 540nm, after 10minutes, in a photoelectric calorimeter. (Samuel, 1989).



Lapachol



Beta-lapachol

Chemical structure of Lapachol(2-hydroxy-3-(3methyl-2 butenil)-1,4 Naphthaquinone and Beta lapachone.

Table - 1: Body weight of swiss albino mice with and wihout dla, treated with *Oroxylum indcum* and 'mixture' of medicinal plants

Days after tumor challenging	Body weight 0	7	14	21	28
Normal mice (Vehicle)	28.2 ± 2.4 N = 6	28.3 ± 2.5 N = 6	28.4 ± 2.8 N = 6	28.6 ± 2.7 N = 6	28.6 ± 2.4 N = 6
Tumor (control)	28.20 ± 2.3 N = 6	28.1 ± 2.4 N = 6	28.4 ± 1.9 N = 6	28.6 ± 2.7 N = 6	28.6 ± 2.7 N = 6
O.indicum (before)+DLA	27.2 ± 1.4 N = 6	27.4 ± 1.1 N = 6	27.8 ± 0.9* N = 6	29.2 ± 2.6** N = 6	33.0 ± 3.4 N = 6
O.indicum (after)+DLA	27.2 ± 1.6 N = 6	28.2 ± 1.2 N = 6	27.4 ± 0.8 N = 6	31.8 ± 0.8 N = 6	31.3 ± 3. 5 N = 6
Mixture	27.3 ± 2.4 N = 6	28.3 ± 1.3 N = 6	28.6 ± 1.4 N = 6	29.4 ± 2.3 N = 6	33.3 ± 3.2 N = 6

N = No. (surviving) animals: values are meant standard deviation. Values meant with •are significantly different from control of values **P<0.01,*P<0.05, students "t" test.

Table - 2 : Life span of swiss albino mice with and wihout dla, treated with *Oroxylum indcum* and 'mixture' of medicinal plants

Groups	Mean Survival	Percentage of increase in Life Span
Normal mice	No change (Life span minimum - 2 years)	No change
O. indicum (before)+DLA	27 +2.16*	44
O. indicum (after)+DLA	25 + 1.75	37.3
Mixtur + DLA	32 .83 +2.4*	38.8
Control (DLA alone)	18 + 0.81	_

Values are meant standard deviation. Values meant with * are significantly different from control of values P<0.001,P<0.05, students 't' test, the drug was administered as a dose of 10ml / Kg of body weight.

Table - 3 : WBC, heamoglobin and differential count in life span of swiss albino Mice with and wihout DLA, treated with oroxylum indcum and 'mixture' of medicinal plants

Treatement in mice	WBC count / mm^3	Hb Content (g%)	Lymphocytes	Neutrophils	Eosinophils
Normal mice	6,850	13.6	60.3	33	3.5
DLA - Control	1,23430.7	12.3	33.1	60.2	3.9
O.indicum (before) + DLA	13,124.7	14.7	42	53	3.8
O.indicum (after) + DLA	12,624.6	14.2	33	60	3.8
Mixture + DLA	12,421.0	13.8	37	58	3.8

RESULTS AND DISCUSSION

***In vitro* cytotoxiciy study by trypan blue exclution method**

The study of *O. indicum* and mixtures shows 86% and 68% of cytotoxicty respectively. The study shows, 86% of cytotoxicity is very good and this plant can be use for the further studies and drug development

Oroxylum indicum, the unexplored medicinal plant with antimalarial, antibacterial, and antiviral properties is widely using in Ayurveda. World wide quite few number of researches are goining on this plant. Lapachole and β-lapachone are the two important naphhaquinone, which are mainly fighting against most type of cancers. The mode of action is, it is going and binding on the Topoisomerase -I enzyme, which is helping the unwinding of DNA at the time of replication and thus stopping its action and preventing the further proliferation of the cancer cells. In future a number of research is going to take place in the same plant, which will prove this plant as a promising one.

The average body weight of the animals treated with *O. indicum* and 'Mixture' of medicinal plants were found to be less than that of 'control', on 21st day after tumor challenge. The increase in body weight was due to the tumor load in the intraperitonial cavity. The body weights in 'mixture' treated mice were not significantly different from control.

M.T.T Aassay – *in vitro* cytotoxic study (*O. indicum* against DLA cell lines)

Sample	Concentration /reading (µg / ml)	%Growth inhibition M.T.T.ASSAY	CTC_{50}
1	480	91.81	51.74%
	240	73.18	
	120	64.27	
	60	46.12	
	30	35.08	

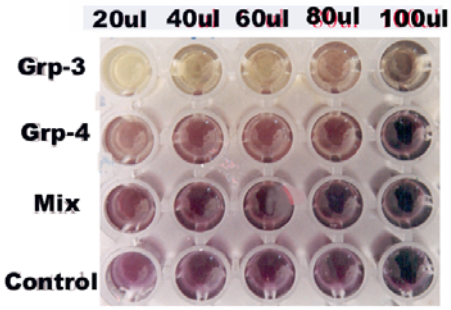


Fig.-1 : 96 Well- plate showing MTT assay of DLA cancer cells. The colour change from light grey to dark grey showing the percentage of cytotoxicity.
Well no 1 (Group 3, 20µl) is showing the least and last well (control, 100 ìl) is showing the highest cytotoxicity.

The lifespan of ascities tumor bearing mice treated with *O. indicum* and 'mixture', were found to be significantly increasing. Control animals survived only 18 days after tumor induction, while treated animals survived for 25 and 27 days, with an increased life span of 37% and 44% respectively. The group-III animals given treatment before cancer induction were showing a 58% increase in life span than that of tumor bearing animals.

The treatment is found to be effective in terms of W.B.C count as the values are near to the normal WBC count and a tremendous decrease is seen from that of group-II (DLA alone). The hemoglobin count of group-III shows a maximum result of 14.7% while the least value of 12.3% is shown in the control. In case of differential count, a gradual decrease in lymphocytes were observed GrpI>GrpIII>GrpV>GrpII>GrpIV. The neutrophils count of DLA transplanted mice increased from normal mice to the control mice and there where no much differences in the eosinophil number in all groups of animals.

The cleavage of tetrazolium salts M.T.T into a blue coloured product (Formazan) by mitochondrial enzyme succinate dehydrogenase will help to study the percentage of cytotoxicity in a cancer cell by any drug. In M.T.T assay the highest concentration of 480ìg / ml shows the highest percentage of growth inhibition (91.81%) and the CTC_{50} value (51.74%). The study also shows *O.indicum* and mixtures gave 86% and 68% of cytotoxicity in cancerous cells respectively. Lapachole and beta – lapachone are the two important naphhaquinone, in this plant, which are mainly fighting against most type of cancers. The mode of action is, it is going and binding on the Topoisomerase –I enzyme, which is helping the unwinding of DNA at the time of replication and thus stopping its action and preventing the further proliferation of the cancer cells. This result shows that the plant drug is highly toxic to DLA cell line and can be used as a good anti-cancer herbal product in future and more research studies are highly inviting.

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