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IN-VITRO AND IN-VIVO CYTOTOXIC EFFECT OF *SALVIA LEUCANTHA* CAV. AGAINST EAC CELL LINES

SRIDHARAN G*, BRINDHA P, RAJA R*** AND AMUTHA PRIYA R*****

***Department of Biochemistry, Srimad Andavan Arts and science college, Trichy 620005. Tamilnadu, **CARISM, SASTRA University, Thanjavur -613401, ***Department of Biochemistry, Srimad Andavan Arts and science college, Trichy 620 005. Tamilnadu. Email: brindhajana@yahoo.co.in**

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ABSTRACT

The current study aims at investigating antitumor potential of ***Salvia leucantha* Cav** (EESL) on Ehrlich Ascites Carcinoma cell lines. In-vitro cytotoxic assay such as Trypan dye exclusion and MTT assays were carried out against tested cell lines. For In-vivo studies Ehrlich Ascites Carcinoma cells at the dose level $1X10^6$cells/mouse was injected to the experimental animals. Ethanolic extract of ***Salvia leucantha* Cav** was administered at varying doses for 14 days the antitumor effect of EELN was accessed using the biochemical parameters such as Tumor volume, PCV, viable and non-viable cell counts, life span. The data obtained were in-par with the standard drug 5-Flurouracil (20mg/kg.bw). The data of the results of in-vitro and In-vivo studies suggested that EESL possesses potent antitumor activity against EAC cell lines.

Keywords: Ehrlich Ascistes Carcinoma cell Lines (EAC), Ethanolic extract of *Salvia leucantha* Cav (EESL), MTT -3-4, 5-dimethyl thiazol-2-yl 2,5 di phenyl tetrazolium bromide, Mean survival time (MST), Tumor growth response, MDA, GSH, SOD, Catalase.

INTRODUCTION

Cancer is a class of disease requires newer approach for its treatment, control and prevention. Cancer is one of the major health problems in both developed as well as developing countries [1]. Chemoprevention is a rapidly growing field of oncology which aimed to prevent the cancer growth using natural or synthetic interventions [2]. Chemotherapy using synthetic drugs can produce severe toxic side effects, which resulted in restricted usage [3].Now a day's drugs derived from plant origin has gaining momentum in treating cancer. In the armory of modern medicine, the extracts and the chemicals isolated from plants are evaluated for their anticancer efficacy against various experimental models [4]. Many chemical molecules from plants and dietary sources have been reported for possessing potential to inhibit and delay the multistage process of tumor growth [5] . The important advantages of plant based medicines are their safety, efficacy and affordability[6]. Ramnath et.al., have reported the emergence of nearly 30 anticancer drugs as an outcome of anticancer screening of plant derived products[7]. This prompted us to develop a novel anti cancerous drug from plant sources and a survey was conducted in and around Tamilnadu to select a potential plant source. Common *Lamiaceous* member botanically identified as *Salvia leucantha* Cav. was selected and its ethanol extract was subjected to In-Vitro and In-Vivo anticancer studies.

MATERIAL AND METHODS

Plant collection and extraction

The aerial parts of *Salvia leucantha Cav.* was collected from Kodi hills Tamilnadu. After proper identification and authentication[8], ethanol extract was prepared using 500gms of plant materials employing cold extraction method.

Cells

EAC cells lines obtained from recognized centre was maintained and used for experiments.

In-Vitro Cytotoxicity

The cytotoxic effect of the etahnolic extract of the test drug was evaluated against EAC cell lines using trypan blue exclusion method[9] and MTT assay procedure [10].

MTT ASSAY

EAC were cultured in 96 well plates with growth medium RPMI (Roswell Park Memorial Institute) 1640 and 10% FCS(Fetal calf serum). Increasing concentrations of EESL was added to the cells and incubated at 37ºC for 14 hrs in CO_2 incubator with 5% CO_2. The media was replaced with a fresh growth medium along with 20 µl of 3-4, 5-dimethyl thiazol-2-yl 2,5 di phenyl tetrazolium bromide (MTT) was added to it. Again it was incubated for 4 hrs at 37ºc. After incubation purple precipitate was clearly visible under the inverted microscope then the growth medium was removed and 200ml of 0.1% 0.1N acidic isopropyl alcohol was added to the cells to dissolve the MTT, formazan crystals. Then the covered plates were kept in the dark at 18-24º overnight. The samples colur were read at 570nm. Experiments were repeated at thrice. The average was calculated, and compared with the control test samples. The percentage growth inhibition was calculated using the following formula[10]

$$\% \text{ Growth Inhibition} = \frac{\text{Control OD} - \text{Treated OD}}{\text{Control OD}} \times 100$$

Animals

7-8 weeks old body weight of 25+ 2g male Swiss albino mice were used for the studies with necessary clearance from Institutional Animal Ethical Committee

Effect of EESL on survival time

The Swiss albino mice were divided into five groups of six animals each. Treatment with EESL at various dose levels (100,200,300mg/kgbw *p.o.*) administered to group 2,3,4 respectively for 14 days after EAC inoculation. Group 5 animals were administered with 5-Flurouracil(standard drug) 20mg/kgbw for 14 days. Group1 animals served as tumor control and receivedwere administered normal saline. The percentage increase in life span was calculated as follows.

ILS (%) = [(Mean survival time of treated group/mean survival time of control group)-1] X100

MST = (Day of first death + day of last death)/2

Experimental protocol

T36 animals were divided into 6 groups of six animals.

Group I- Normal control

Group II- Ehrlich Ascites Carcinoma cell line ($1X10^6$cell/mouse)

Group III - Ehrlich Ascites Carcinoma cell line ($1X10^6$cells/mouse) treated with 100mg /kg bw of the EESL.

Group IV - Ehrlich Ascites Carcinoma cell line (1X10^{6}cells) treated with 200mg /kg bw of the EESL.

Group V - Ehrlich Ascites Carcinoma cell line (1X10^{6}cells) treated with 300mg /kg bw of the EESL.

Group VI - Ehrlich Ascites Carcinoma cell line (1X10^{6}cells) treated with 5 - Fluorouracil (5-FU) (20mg/kgbw.)(Standard Drug)

After 24 hrs tumour inoculation the test drug was administered orally for 24hrs of EAC inoculation. The plant extract administered orally for 14 days. After the experimental period animals were sacrificed by cervical decapitation, blood was collected. Liver tissue was used for the antioxidant studies. Asides fluid was collected and used for TV,TC, viable and non-viable cell counts.

Tumor volume

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and packed cell volume was determined by centrifuging at 1000rpm for 5min.

Tumor cell count

The ascitic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the number of cells in the 64 small squares was counted.

Hematological Parameters

Hematological Parameters were determined as per standard procedures [11], [12], [13].

Estimation of in vivo Antioxidants

Antioxidant potential was evaluated as per standard procedures under in-vivo condition[14], [15], [16], [17], [18].

Statistical analysis

The data obtained were analysed statistically using ANOVA.

RESULTS

Cytotoxic Effect of EESL on EAC cells (Trypan Blue method)

From the Table 1 it is evident that the death rate of Ehrlich Ascites Carcinoma cells increased with increase in concentration of ethanolic extracts of ***Salvia leucantha*** **Cav.**

Cytotoxic effect of EESL on EAC cell lines (MTT ASSAY)

The EESL was found to be more cytotoxic against Ehrlich Ascites carcinoma. The 15µg/ml concentration produced 61.31% cytotoxicity where as 1 µg/ml EESL showed 2.95% cytotoxicity (Table 2). The cytotoxicity increased with increase in the concentration of plant extract.

Effect of EESL on MST

Life span of the tumor bearing animal increased upto 5.96%,29.81% &59.17% respectively in drug treated group compared to MST of Tumor bearing animals (Table 3).

Tumor response

Elevated levels of Tumor volumes, and cell counts of tumour bearing models significantly decreased on treatment (Table 4) which further provided scientific evidences for the antitumor potentials of the test drug (EESL).

EESL on Hematological profiles

Hematological parameters (Table 5) of tumor animals such as levels of Hemoglobin and counts of RBC and WBC reverted back to near normal and Differential counts of Lymphocytes, Neutrophils and Monocytes, which were also altered reverted back to normal value after the test drug administration

Effect of EESL on Non-Enzymatic & Enzymatic Antioxidant Profile

From the Table 6 it is evident that the increased levels of LPO and reduced levels of Glutathione, SOD and Catalase were restored to normal level in the test drug administered animals.

Table 1: Cytotoxic effect OF EESL on EAC cells (Trypan Blue Method)

Concentration of EESL (µg/ml)	Viable cells	Viable cells(%)	Death cells	Death cells(%)
Control	125	94.7	7	5.3
25	65	77.4	19	22.6
50	85	73.2	31	26.8
100	69	65.7	36	34.3
200	60	46.5	69	53.5
500	22	19.7	90	80.3

Table 2: Cytotoxic effect of EESL on EAC cell lines (MTT Assay)

Concentration of EESL µg/ml	OD-1	OD-2	OD-3	Avg	%Cytotoxicity
control	0.305	0.312	0.297	0.305	--
1	0.295	0.299	0.294	0.296	2.95
5	0.288	0.281	0.275	0.281	7.86
7.5	0.252	0.249	0.242	0.247	19.01
10	0.181	0.175	0.172	0.176	42.29
15	0.121	0.119	0.115	0.118	61.31

IC_{50} Value = 12.23 µg/ml.

Table 3: Effect EESL on MST

Particulars	Mean survival Time	Increase in Life span (ILS) (%)
Group II	21.8±0.92	-
Group III	23.1±1.19*	5.96*
Group IV	28.3±1.92*	29.81*
Group V	34.7±0.88*	59.17*
Group VI	35.3±0.10*	61.92*

Values: ± S.E.M., n=6

*p<0.001statistically significant

Table 4: Effect of EESL on tumor growth

Group	Tumor volume(ml)	Packed cell volume (ml)	Viable cells	Non-viable cells
Group II	4.1±0.13	2.32±0.03	8.3±0.19	0.43±0.04
Group III	3.4±0.06*	1.7±0.03*	6.7±0.41*	0.66±0.07*
Group IV	2.5±0.07*	1.3±0.07*	5.1±0.63*	0.79±0.18*
Group V	1.4±0.06*	1.1±0.11*	3.7±0.17*	0.87±0.19*
Group VI	1.1±0.11*	0.6±0.14*	2.7±0.11*	0.99±0.57*

Values are mean± S.E.M., n = 6

*p<0.001statistically significant when compared to EAC control group

Viable cells: Not Stained with Trypan blue

Non-viable cells: Stained with Trypan blue

Table 5: Effect of EESL on Heamatological Parameters

Particulars	Hb content (g/dl)	RBC count	WBC count	Lymphocytes	Neutrophils	Monocytes
Group I	11.9±0.14	4.9±0.05	6.1±0.55	72±0.17	29±0.83	2.1±1.53
Group II	8.3±0.27[a]	3.6±0.14[a]	10.2±0.71[a]	31±0.31[a]	58±0.14[a]	2.7±0.57[a]
Group III	9.1±0.06[b]	3.9±0.09[b]	9.1±0.59[b]	37±1.7[b]	47±0.85[b]	2.51±0.84[b]
Group IV	10.4±0.03[b]	4.1±0.21[b]	8.4±0.31[b]	49±1.7[b]	39±0.52[b]	2.38±0.41[b]
Group V	11.2±0.01[b]	4.8±0.11[b]	7.3±0.17[b]	59±0.11[b]	31±0.15[b]	2.22±0.49[b]
Group VI	11.5±0.37[b]	4.9±0.39[b]	6.7±0.09[b]	65±1.3[b]	33±0.10[b]	2.15±0.80[b]

Values are mean ±S.E.M. n=6

[a] p<0.001 statistically significant when compared with Group I

[b] p<0.01 statistically significant when compared with Group II

Table 6: Effect of EESL on Enzymatic and Non-Enzymatic Antioxidants

Particulars	LPO	Glutathione	SOD	Catalase
Group I	1.13±0.21	3.31±0.09	4.49±0.35	31.6±0.09
Group II	4.26±0.23*	0.84±0.47*	1.23±0.76*	14.3±0.04*
Group III	3.57±0.31**	1.24±0.56**	2.69±0.89**	19.2±0.07**
Group IV	2.56±0.43**	2.32±0.43**	3.32±0.43**	23.7±0.41**
Group V	1.71±0.09**	2.97±0.22**	4.13±0.22**	27.4±0.17**
Group VI	1.37±0.01**	3.11±0.27**	4.22±0.61**	29.3±0.09**

Values are mean ± S.E.M. n=6

[*] p<0.001 statistically significant when compared with Group I

[**] p<0.01 statistically significant when compared with Group II

DISCUSSION

Cancer is often associated with increased risk of death and the toxic side effects caused by the modern medicine, many cancer patients seek alternative and complementary methods of treatment such as usage of phytomedicine [19]. Now a days researcher are focusing their research towards the development of an eco friendly anti caner drug from plant sources, which resulted in newer chemotherapeutic agents such as paclitaxel, vincristine, podophyllotoxin and camptothecin existing in clinical trials. In the present study a common plant *Salvia leucantha* Cav. was selected based on Literature review its Ethanolic extract was screened for its anticancer potential against Ehrlich Ascites Carcinoma cell lines employing In-vitro & In-Vivo methods.

Plant extracts under study (EESL) was screened for their Cytotoxic potentials against Ehrlich Ascites Carcinoma cell lines. It is observed that 500 µg/ml EESL showed 80.30% cytotoxicity (Table 1).

Cytotoxic activity of the test drug was also assessed through MTT assay. 24hrs treatment with the test drugs showed inhibition of EAC cells. EESL showed 61.31% of cytotoxicity with IC_{50} value 12.23 µg/ml (Table 2) The death of the cells caused by the test drug under study might be due to the loss of mitochondria which is one of the hallmarks of the apoptosis pathway[20].

The merit of the anticancer drug can be judged by accessing the increase in life span of cancer animals [21]. It was noticed EESL increased tumor bearing animal's life span by about 5.96%, 29.81% &59.17% and is dose dependent. Tumor bearing mice possessed increased ascites fluid and cancer cell counts. Ascites fluid provides the essential nutrients for the growth of cancer cells hence increase in fluid volume directly correlated with tumor growth. Treatment with EESL at various dose levels decreased the Ascites fluid volume as well as peritoneal cell counts.

The major complication cancer chemotherapy is reduction in RBC count and myelosupression. The decreased in RBC and Hemoglobin content of tumor bearing animals are may be due to iron deficiency or break down of RBC [22]. Treatment with EESL normalizes the abnormalities found in the hematological profiles. This clearly depicts the hemopoietic protective role of EESL.

Malondialdehyde (MDA) is formed during oxidative degeneration membrane lipids by free radicals which is accepted as a marker of lipid peroxidation[23]. A higher level of MDA was reported in various Cancer tissues. [24] The elevated levels of MDA in cancerous tissue were brought back to normal after the treatment with the test drug in a dose dependent manner.

Glutathione is a potent inhibitor of neoplastic process [25]. Free radical scavenging potential of the Herbal drug under study increased the level of glutathione which in turn facilitated the inhibition of neoplastic process and contributed towards the increase of life span by inhibiting the tumor growth.

Activity of SOD and CAT increased and helped in scavenging superoxide and hydrogen peroxide[26].

The data of the results were in par with standard drug 5-fluorouracil (20mg/kg bw). It is observed that 300mg/Kg.bw. is the most

effective dose. Present finding clearly depicted the anti tumor and antioxidant potentials of the ethanolic extract of *Salvia leucantha* Cav. (EESL)

CONCLUSION

Thus the data of the results of the present work clearly depicted that EESL possess potent anticancer activity against Ehrlich Ascites Carcinoma cell lines, probably by increasing the life span, protecting hemopoietic system and through antioxidant mechanism.

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