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The Anticancer and Radiosensitizing Activities of EOG (Echitamidine-N-Oxide-19-o-B-D-Glucopyranoside) Isolated From The Stem Bark Extract of *Alstonia Scholaris*

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ABSTRACT

The anticancer and radiosensitizing activities of EOG (Echitamidine-N-Oxide-19-o-β-D-Glucopyranoside) isolated from the stem bark extract of *Alstonia scholaris* were studied in Swiss mice transplanted with Ehrlich ascites carcinoma (EAC) exposed to various doses of γ-radiation. Intraperitoneal administration of 0.5, 1, 2, 4, 6, 8, 10 or 12 mg/kg body weight EOG in animals transplanted with EAC tumor resulted in a dose dependent rise in tumor regression and increase in the average survival as well as mean survival time. The highest number of tumor free survivors were observed in the group receiving 10 mg/kg EOG at the end of 120 days. The administration of 0.5, 1, 2, 4, 6, 8, 10, 12 mg/kg body weight EOG in tumor bearing mice exposed to 6 Gy γ-radiation caused EOG dose dependent regression of the tumors when compared to 6 Gy alone and the maximum effect was found in animals receiving 6 mg/kg. Intraperitoneal administration of 6 mg/kg EOG into EAC mice before exposure to 0, 1, 2, 4, 6 or 8 Gy hemi body γ-radiation showed an irradiation dose dependent elevation in the tumor free survivors and a maximum number of survivor up to 120 days was observed for 6 Gy irradiation. This led to increase in the average survival and mean survival time by 31 days when compared to 6 Gy irradiation alone.

Keywords: Echitamidine-N-Oxide-Glucopyranoside(EOG), EAC, Survival studies, Radiosensitization study , Irradiation.

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INTRODUCTION

An earnest effort to search anti-cancer agents from plant sources was initiated in the 1950s, which led to the discovery and development of vinca alkaloids including vinblastine and vincristine, and isolation of cytotoxic podophyllotoxins[1-2]. Earlier National Cancer Institute (NCI) of United States to initiate an extensive plant collection program in 1960 focusing mainly on temperate regions. As a result many novel chemotypes including taxanes and camptothecins with a wide range of cytotoxic activities were discovered[3-4]. However, almost 40 years (the early 1960s to the 2012) passed before, these agents were accepted clinically for tumor treatment[4].

Radiation is an important tool to control cancer. It has been used successfully to treat a variety of tumor types, where it is delivered as an external beam alone, brachytherapy alone or a combination of both. The results of radiotherapy alone are comparable with that obtained with surgery for tumors of the oral cavity[5] oropharynx[6], supraglottic larynx[7] and glottis[8]. Radiation therapy has been found to give identical tumor control rates with less long-term sequelae[9]. Radiation therapy has been highly successful in the treatment of early stage Hodgkin's disease [10]. The radiotherapy has been often combined with surgery and/or chemotherapy to get better tumor control. The combination of conventional chemotherapy with radiation has been used in the definitive and adjuvant therapies of the many types of cancers. The randomized trials have shown that the combination treatment has been highly successful in improving survival compared to radiation alone in the treatment of locally advanced cancers of the head and neck, lung, esophagus, stomach, pancreas, and rectum[10-16].

Several agents have been used in an attempt to increase the radiosensitizing activity of tumors and use of chemotherapeutic agents including 5-fluorouracil, actinomycin D, cisplatin, gemcitabine, fludarabine, paclitaxel, doxorubicin, hydroxyurea, mitomycin C, topotecan, and vinorelbine has been found to increase the radiosensitivity of tumors [17-22]. The mechanism of radiosensitization varies among different agents. Cisplatin inhibits both SLDR and PLDR[23], whereas inhibition of repair may also explain the radiosensitizing properties of topotecan[24]. Doxorubicin increases cellular oxygen levels by inhibiting mitochondrial and tumor cell respiration [25].

The chemotherapeutic agents which inhibit the DNA synthesis and cell cycle progression like adriamycin, teniposide, etoposide, carmustine, cyclophosphomide, cisplatin, carboplatin, hydroxyurea, methotrexate, 5 flurouracil, AZT, ACV, aclacinomycin, bleomycin have been reported to enhance the radiation effects in vitro and in vivo [26-28]. Some of the natural products

like *Tinospora cordifolia* and aphanamixis polystachya have been found to enhance the effect of irradiation in HeLa cells and *invivo*[29].

Alstonia scholaris, a tree belonging to family Apocyanaceae, is a popular remedy in India for the treatment of various types of disorders in both the ayurvedic and folklore systems of medicine. The stem bark of *Alstonia scholaris* has been found to exert antineoplastic activity *in vitro* and *in vivo*[30]. It has also been reported to increase the effect of cyclophosphamide in preclinical studies in vivo. It has been reported to reduce the occurrence of tumors in chemical carcinogenesis studies[31]. Therefore, the present investigation was undertaken to study the andtineoplastic and radiosensitizing effects of active principle echitamidine-N-oxide-19-o-β-D-glucopyranoside in mice transplanted with Ehrlich ascites carcinoma and exposed to different doses of γ-radiation.

MATERIALS AND METHOD

Isolation of EOG from *Alstonia Scholaris*

The EOG was isolated from the chloroform fraction of 85% ethanol extract of *Alstonia scholaris* R. Br., (Family Apocyanaceae). Briefly, the powdered dried bark was extracted with 85% ethanol at room temperature and evaporated to dryness in vaccuo. The extract was suspended in distilled water and further extracted with hexane, chloroform, ethyl acetate and n-butanol in succession. The solvent was evaporated from chloroform fraction partitioned between 3% HCl and ethanol, the aqueous layer basified to pH 10 by addition of NaOH and extracted once again with $CHCl_3$ so as to give a crude alkaloidal fraction. The alkaloidal fraction was subjected to column chromatography, HPTLC, and HPLC so as to isolate the indole alkaloids. The glycosidic indole alkaloid obtained was identified as echitamidine-N-oxide-19-o-β-D-glucopyranoside (EOG) by UV, IR, NMR and MASS spectroscopy[32].

Animal Care and Handling

The animal care and handling were done according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Ten to twelve weeks old female Swiss albino mice weighing 30 to 36 g were selected from an inbred colony maintained under the controlled conditions of temperature (23 ± 2°C), humidity (50±5%) and light (12 h of light and dark cycle, respectively). The animals had free access to sterile food and water. Usually four animals were housed in a polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment. The

study was approved by the Institutional Animal Ethical Committee (IAEC) of the Kasturba Medical College, Manipal, India.

Tumor Model

Ehrlich ascites carcinoma (EAC) procured from the Cancer Research Institute (ACTREC), Mumbai, India, was maintained and propagated by serial intraperitoneal transplantation of EAC cells in an aseptic environment. The each swiss mouse was tumorized by injecting 10^6 EAC cells intraperitoneally in an aseptic condition and the day of tumor inoculation was considered as day zero. All the experiments on tumor bearing mice were conducted 24 h after the EAC transplantation and this day was considered as day one.

Dissolution of Drug and Mode Of Administration

The EOG was dissolved freshly in 0.5% CMC (Carboxy Methyl Cellulose) with constant trituration in a mortar, before use. The CMC, EOG or cyclophosphamide was administered intraperitoneally in the tumor bearing mice.

ANTINEOPLASTIC ACTIVITY OF EOG

A separate experiment was conducted to evaluate the optimum dose of EOG for its antineoplastic action using the standard protocol recommended by National Service Center of Cancer Chemotherapy, USA[33]. One day after tumor inoculation, the animals were divided into the following groups of ten each.

CMC group:

The animals of this group received 0.3 to 0.36 ml of 0.5% CMC daily once consecutively for 9 days.

***EOG group*:**

This group of animals was administered with 0.5, 1, 2, 4, 6, 8, 10 or 12, mg/kg body weight EOG once daily for 9 consecutive days.

Acute Toxicity:

The acute toxicity of EOG was determined [34-35] according to OECD guidelines 420-425, by administering a group of 10 animals with 0.5, 1, 2, 4, 6, 8, 10, 12, 25 mg/kg body weight of EOG intraperitoneally. The animals were monitored daily up to 14 days for symptoms.

RADIOSENSITIZING ACTIVITY OF EOG

Selection of Optimum EOG Dose for Radiosensitization

The optimum dose for radiosensitizing activity of EOG was determined by administering various doses of EOG to the tumor bearing mice before irradiation, where the animals were divided into two groups

CMC + irradiation:

The animals of this group received 0.3 to 0.36 ml CMC 1 h before exposure to 0 or 6 Gy γ-radiation and then once daily for another nine consecutive days after irradiation.

EOG + irradiation:

The animals of this group were injected with 0.5, 1, 2, 4, 6, 8, 10 or 12 mg/kg b wt of EOG 1 h before exposure to 6 Gy γ-radiation and then once daily for another nine consecutive days after irradiation.

Since a maximum tumor free survival was observed for the EAC mice receiving 6 mg/kg b wt of EOG in conjunction with 6 Gy γ-radiation when compared with the other doses of EOG, further experiments were carried out with this dose.

RADIOSENSITIZATION BY EOG

A separate experiment was conducted to study the effect of optimum dose of EOG on the radiation induced regression in the EAC tumor, where all the conditions were exactly similar to the above, except that the EOG+ irradiation group received 6 mg/kg EOG before exposure to 0, 1, 2, 4, 6 or 8 Gy

Irradiation

Prostate, immobilized (achieved by inserting cotton plugs in the restrainer) and unanaesthetized tumerised animals of all irradiation experiments were restrained in a specially designed well-ventilated perspex box and the lower half of the animal body, below rib cage (hemi-body) was exposed to different doses of gamma radiation. A batch of ten animals were irradiated at a dose rate of 2.0 Gy/min at a source to animal distance (midpoint) of 78.9 cm using ^{60}Co γ-radiation from a Tele Cobalt Therapy source (Theratron, Atomic Energy Agency, Ontario, Canada).

The animals of all experiments except acute toxicity studies were monitored regularly for body weight changes, signs of toxicity and mortality. The weight of animals was recorded every third day up to 30 days after tumor inoculation in all the groups. A 33% of drug related deaths or a weight loss of 5 g per mouse was considered as an index of toxicity. The animal survival was monitored daily up to 120 days, since the survival of animals up to 120 days is roughly equivalent to 5 years survival in man (Nias, 1990). The tumor response was assessed on the basis of median survival time (MST) and the average survival time (AST). The MST and AST were calculated from the animals dying within 120 days and those surviving 120 days were excluded from it (Geran *et al*., 1972). The increase in mean life span (% IMLS) and the increase in average life span (% IMLS) were calculated using the following formulae:

$$MST = \frac{\text{First death + last death in the group}}{2}$$

$$AST = \frac{\text{Sum of animal death on different days}}{\text{Number of animals}}$$

$$IMLS = \frac{\text{MST of treated mice - MST of control}}{\text{MST of control}} \times 100$$

$$IALS = \frac{\text{AST of treated mice - AST of control}}{\text{AST of control}} \times 100$$

Statistical Analysis

The statistical significance between the treatments for survival studies was determined using the "Z" test, while the student's 't' test was used for biochemical studies. Appropriate post -hoc tests were used for multiple comparisons. The data were confirmed by repetition of the experiments. Test of homogeneity was applied to determine any statistical differences between the repeat experiments. Solo 4 (BMDP Statistical Software Inc., Los Angeles, CA) was used for data analyses. All the data are expressed as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

The results after the treatment with EOG or EOG and radiation were expressed as tumor free survival in the Tables 1-2 and Figures 1-2.

Acute toxicity:

Administration of various doses of EOG did not showed any neurological and behavioural toxic symptoms and signs up to 12mg/kg b. weight. However a further increase in EOG dose to 25 mg/kg induced toxic symptoms and mortality.

Antineoplastic Activity

Mice transplanted with Ehrlich Ascites carcinoma (EAC) did not show tumor regression immediately and throughout the experimental period. Administration of EOG increased tumor free survivors in a dose dependent manner indicated by a constant decline in the weight gain due to tumor cell multiplication as compared to CMC treatment. This was reflected by the increased life span in the form of MST and AST depending on the dose of EOG. The greatest number of tumor free survivors was observed in the group of animals receiving 10 mg/kg EOG and

declined thereafter, indicating that this was the most effective dose as far as the tumor regression is concerned (Table 1). This increase in MST and AST was accompanied by an increase in the IMLS and IALS (Table 1 and Fig. 1) The MST and AST for CMC group was found to be approximately 18 days whereas the animals treated with 6, 8 and 10 mg/kg b wt. EOG showed MST of 34, 43 and 52 and AST of 35, 44 and 53 days, respectively. Likewise, there was an increase in the IMLS up to 84, 134 and 182 and IALS up to 90, 141 and 188 in animals treated with 6, 8 and 10 mg/kg b.wt of EOG, respectively, when compared with CMC treatment (Table 1, Fig 1). Administration of 8 and 10 mg/kg EOG was found to be more effective when compared to all other doses where 100% healthy survivors could be observed at the end of 30 days. The 10 mg/kg EOG proved to be the best dose as there were 50% and 33% survivors at the end of 60 and 120 days, respectively (Table 1). A further increase in EOG dose up to 12 mg/kg resulted in a decline in the survival as no survivors could be observed even at the end of 30 days and was accompanied by toxic manifestations in the form of ruffling of hairs, sluggishness. Therefore, 10 mg/kg EOG was considered as an optimum dose, as it resulted in the highest number of long-term survivors and also did not induce any toxic effects in the form of debility, loss of body weight and death. Therefore further studies were carried out using 10 mg/kg b.wt EOG.

Radiosensitizing Activity of EOG

Exposure of tumor bearing mice to 6 Gy alone marginally increased the survival when compared to CMC treatment alone. However, administration of 0, 1, 2, 4, 6, 8, 10 or 12 mg/kg b. wt EOG before exposure to 6 Gy hemi-body radiation, arrested the tumor weight gain when compared to EOG or CMC treatment alone. The combination treatment also reduced the effective dose of EOG, where 6 mg/kg proved to be the best dose as, indicated by increase in MST and AST by approximately 18 days, respectively over EOG treatment alone (Table 1). Irradiation of EAC mice with different doses of γ-irradiation showed irradiation dose dependent regression of tumor up to 6 Gy as evidenced by an increase in the AST and MST for all exposure doses. The combination of 6 mg/kg with various doses of irradiation increased the tumor free survivors in radiation dose dependent manner up to 60 days and declined thereafter.

The increase in tumor free survivors was reflected as increased MST and AST in the EOG + irradiation group when compared to CMC + irradiation group. The combination treatment resulted in a rise in MST up to 40, 46, 52 & 45 days in the animals exposed to 2, 4, 6 and 8 Gy group when compared to concurrent CMC + irradiation group (21, 24, 22 and 23 days). A similar effect was discernible in AST in the combination group. It is clear from Table 2 that the MST

and AST increased in a dose dependent manner up to 6 Gy and declined thereafter. The IMLS and IALS also accrued in a radiation dose dependent manner in both CMC + irradiation and EOG + irradiation groups, except that IMLS and IALS were many folds greater in the latter group when compared to the former group (Table 2). The greatest effect of combination treatment was discernible for 6 Gy irradiation where 33% animals did survive at the end of 120 days. However, this effect was absent for other exposure doses in the EOG + irradiation group (Table 2).

Table 1 effect of various doses of echitamidine-n-oxide-19-0-β-d-glucopyranoside alone or in combination with 6 Gy of γ-irradiation on the survival of tumor bearing mice.

EOG (mg/kg)	Treatment															
	Sham-irradiation								EOG+irradiation							
	Survival (days)				Percent Survivors (days)				Survival (days)				Percent Survivors (days)			
	MST	IMLS	AST	IALS	30	60	90	120	MST	IMLS	AST	IALS	30	60	90	120
CMC	18.5	-	18.4	-	0	0	0	0	21.7	17.3	22.3	21.2	0	0	0	0
2	20	8.1	19.9	8.2	0	0	0	0	23.6	27.6	23.4	27.2	0	0	0	0
4	27.5	48.64	26.8	45.65	0	0	0	0	37.6	103.2	37.1	101.6	70[a]	30[a]	0	0
6	34.5	84.86	35.1	90.76	66.66[a]	0	0	0	52.5	183.8	53.2	189.1	100[a]	58.3[a]	33.3[b]	33.3[b]
8	43.4	134.59	44.5	141.84	100[a]	38.4[a]	16.6[a]	16.66[a]	46.7	152.4	46.1	150.5	90[a]	50[a]	10[a]	0
10	52.3	182.7	53.1	188.5	100[a]	50[a]	33.3[a]	33.33[a]	39.5	113.5	39.1	112.5	66.66[a]	10[a]	0	0
12	28.4	53.51	28.6	55.43	0	0	0	0	34.6	87.0	34.2	85.9	40[a]	0	0	0

a= $p < 0.05$; b = $p < 0.0001$; (when compared with SPS), SPS= Sterile Physiological Saline or concurrent Sham-irradiation group

CMC= carboxy methyl cellulose, EOG=Echitamidine-N-Oxide-19-0-β-D-Glucopyranoside

Table 2 Effect of 6 MG/KG of echitamidine-N-oxide-19-0-B-D-glucopyranoside in combination with different doses of γ-irradiation on the survival of Tumor Bearing Mice.

Exposure Dose (Gy)	Treatment															
	CMC + irradiation								EOG + irradiation							
	Survival (days)				Percent Survivors (days)				Survival (days)				Percent Survivors (days)			
	MST	IMLS	AST	IALS	30	60	90	120	MST	IMLS	AST	IALS	30	60	90	120
0	18.5		18.4		0	0	0	0	34.5	86.5	35.1	90.76	40[a]	10[a]	0	0
2	20.6	11.35	21.2	15.21	0	0	0	0	39.6	114.78	40.1	117.9	90[a]	30[a]	0	0
4	21.4	15.67	21.8	18.47	0	0	0	0	46.3	150.3	45.9	149.4	100[a]	50[a]	0	0
6	21.7	17.29	22.3	21.19	0	0	0	0	52.5	183.8	53.2	190.2	100[a]	58.3[a]	33.3	33.3[a]
8	22.7	22.7	23.4	27.17	0	0	0	0	44.3	139.5	44.8	143.4	95[a]	45[a]	0	0

a = $p < 0.0001$; b= $p < 0.05$ when compared with SPS + irradiation treated group

CMC=carboxy methyl cellulose, EOG=Echitamidine-N-Oxide-19-0-β-D-Glucopyranoside

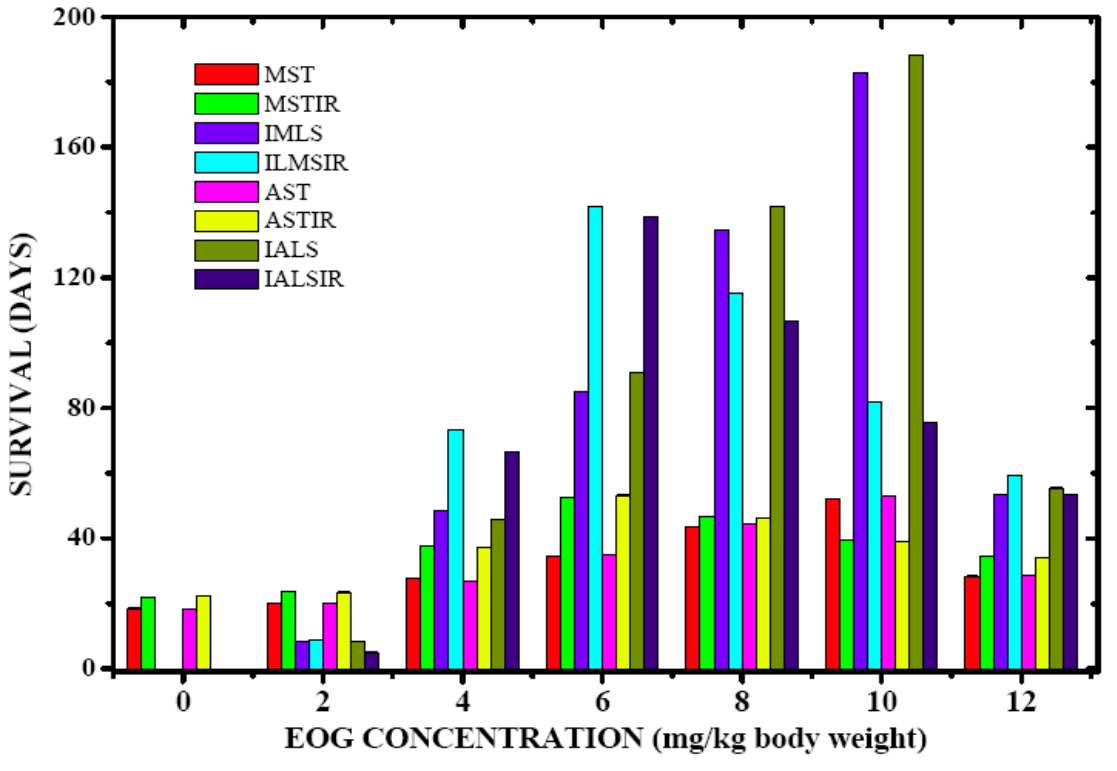

Figure 1 Effect of Various Doses of Echitamidine-N-oxide-19-0-B-D-Glucopyranoside in combination with 6 GY of γ-irradiation on the survival of Tumor Bearing Mice.

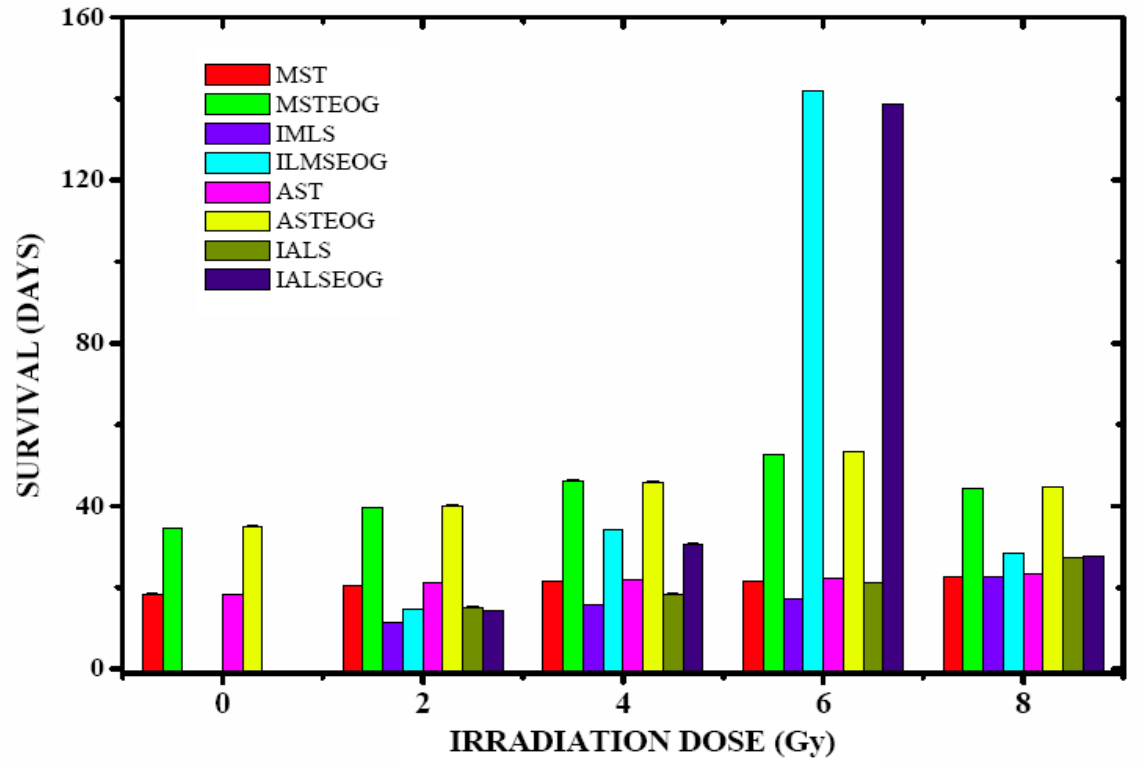

Figure 2 Effect of 6 MG/KG OF echitamidine-N-oxide-19-0-B-D-glucopyranoside IN combination with different doses of Γ-irradiation on the survival of tumor bearing mice.

Treatment failure due to chemotherapy or radiotherapy or chemoradiotherapy has been attributed to increased normal tissue damage, systemic toxicity, distant metastasis apart from the induction of treatment related second malignancies after administration of these therapies in the cancer patients. In several instances the acute toxicity is manifested as life threatening changes in the vital parameters in central nervous system, heart and other normal tissues, which also results in the failure of radiotherapy of malignant lesions by certain adjuvants[36]. Therefore, it is imperative to search for newer agents, which do not exhibit systemic toxicity and kill the neoplastic cells effectively. An attempt has been made to study the effect of echitamidine-N-oxide-19-o-β-D-glucopyranoside either alone in combination with radiation in the mice transplanted with Ehrlich ascites carcinoma.

EOG treatment alone increased the median and average survival of tumor bearing mice with increasing dose of EOG and the maximum number of long term survivors was observed for 6 mg/kg. The studies on the use of EOG as a novel anticancer and radiosensitizing agent are not available. Where as, echitamine chloride extracted from the alcoholic fraction of Alstonia stem bark has been reported to increase the median and long term survival of the tumor bearing mice in our earlier study[37]. Other indole alkaloids including vincristine, vinblastine, have been also reported to have the effect similar to that observed in the present study[38-39]. Likewise, the polyphenolic extract of leaves of *Ichnocarpus frutescens* has shown antitumor activity against murine Ehrlich ascites carcinoma (EAC) model[40]. The methanol extract of *Hypericum hookerianum* Wight and Arnott stem (MEHH) exhibited potent in vivo antitumor properties against Ehrlich ascites carcinoma (EAC) tumor bearing mice[41]. An extract of *Ruta graveolens* has been found to be cytotoxic Dalton's lymphoma ascites (DLA), Ehrlich ascites carcinoma[42].

Natural compound flavopiridol extracted from the the stem bark of *Dysoxylum binectariferum,* has been reported to inhibit tumor growth *in vivo* and *in vitro*[43-45]. The combination of radiation and gemcitabin has been reported increased survival in man[46]. Flavopiridol has also been reported to increase the effect of radiation in esophageal carcinoma cells[47]. Administration of temozolomide to mice bearing U251 tumor xenografts showed a greater than additive increase in radiation-induced tumor regression[48]. The glycoside ouabain has been also reported to enhance radiosensitivity in various cell lines *in vitro* [49-50].

Once the cytotoxic and radiosensitizing activity of EOG was established it was decided to probe whether it induced damage to cellular genome? The DNA damaging effect of EOG was elucidated in Ehrlich ascites tumor bearing mice, where the EAC cells were subjected to single

cell gel electrophoresis or comet assay, since it has been regarded as rapid, simple and sensitive technique for estimating DNA damage[51]

EOG treatment may have inhibited the activation of topoisomerases and polymerase enzymes resulting in damaged DNA as a result there has been formation of DNA strand breaks and subsequently apoptosis and cell death. This contention is supported by the presence of glucopyranoside ring in EOG similar to VM-26 or teniposide which is a known topoisomerase inhibitor. EOG may have inhibited transactivation of NF-κB, initiating a cascade of events leading to apoptotic cell death. It may have also activated cytochrome-c release, stimulated transactivation of p53, induced PARP cleavage and activated caspases which may have played a key role in inducing apoptosis and subsequently cell death. The apoptosis induced in the present investigation may have been due to operation of by extrinsic (Fas related) or intrinsic mechanisms involving several players.

CONCLUSION

To conclude EOG an indole alkaloid has been isolated from the chloroform fraction of 85% ethanol extract of *Alstonia scholaris* and its administration in the tumorized mice increased the life span of mice and the optimum dose was found to be 6 mg/kg. The combination of EOG and irradiation was found to be superior over EOG treatment alone as a more effective tumor regression was observed in the former than the latter. Apart from these, there may be unknown molecular events that may have elicited cytotoxic effects in the tumor cells and need further investigation.

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