Evaluation of Antitumor and Antioxidant Potential of a Polyherbal Extract on Ehrlich's Ascites Carcinoma Xenografted Mice

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Abstract: *Objective*: Indigenous herbs alone or in combination are widely used in Indian system of medicine to treat innumerable ailments since time immemorial. Many strategies has been adopted to enhance anticarcinogenic responses and to establish therapeutic benefits. Poly herbal extracts (PHE), one of the emerging trends of modern medicine, where the assorted active principles work vibrantly to produce a maximum therapeutic activity with minimal toxicity by virtue of its additive, potentative, synergistic, agonistic or antagonistic effects. Though, *Withania somnifera*, *Oroxylum indicum* and *Calotropis gigentia* are independently established as potent antineoplastic agents, their antitumor and antioxidant perspective in combination is yet to be studied. The proposed study ascertains the assorted antineoplastic and antioxidant potential of the said potent herbs in PHE.

Method: The antitumor potency of the PHE at a dose of 400 mg/kg body weight was screened on Ehrlich's ascites carcinoma (EAC) xenografted swiss albino mice. The *in-vivo* anti-oxidant activity was investigated on the basis of hepatic anti-oxidant enzymes' levels.

Result: The PHE at the aforementioned dose showed a restoring effect on altered hematological parameters ($^{***}P< 0.05$ considered to be significant), down turn in ascitic tumor volume and increase in mean survival time. A significant improvement in biochemical parameters (Enzymic antioxidants) was too observed.

Conclusion: The study epitomizes the PHE (400 mg/kg body weight) as a potent anti tumor and anti-oxidant preparation with synergistic effects on EAC bearing mice.

Keywords: Anti-oxidant activities, polyherbal extract (PHE), antitumor activity, EAC.

1. INTRODUCTION

Plant products and materials of natural origin are in use for the treatment of cancer since decades [1]. Recently, cancer treatment by conventional anticancer drugs along with the herbs has been promoted to a greater extent [2]. The use of poly herbs with diverse mechanisms of action may be effective at single target or a disease and treat it cogently with fewer adverse effects. For these therapeutic benefits, herbal extracts in combinations have been widely used and became the prime alternative against the most dreadful disease like cancer.

Withania somnifera (family Solanaceae) commonly known as Ashwagandha is usually considered as the Indian ginseng [3]. The plant parts were reported to have diverse chemical constituents which are efficacious in leukemia and pancreatic cancer cells [4, 5]. Moreover, the plant extract were found to be lethal against breast cancer cells [6, 7] and lymphoma U937 cells [8]. The plant also reported to have strong anti-oxidant activity [9].

Oroxylum indicum (family Bignoniaceae) reported to have discrete ethno-pharmacological uses [10]. Various parts of the plant ascertained to have variegated chemical constituents chiefly flavones [11, 12]. The plant proved to be significant against different cancer cell lines [13]. The plant extract was found to have antioxidant activity [14].

Calotropis gigantea belongs to family Asclepiadaceae, popularly known as milkweed is a common wasteland weed in India. The plant parts like root bark, leaves, flowers and latex possesses miscellaneous active constituents. Extensive literature survey reveals multifarious pharmacological actions of the plant, particularly cytotoxicity [15], anti-oxidant [16] and anticancer [17].

In this communication, the study rationale is rooted on the hypothesis that the combination of these three plant extracts may have additive, synergistic or

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improved anticancer and antioxidant effects on mice bearing Ehrlich's ascites carcinoma.

2. MATERIALS AND METHODS

2.1. Chemicals

5-fluorouracil (5-FU) as a standard and Tris Hcl, were obtained from Sigma-aldrich (Mumbai, India). Trichloroacetic acid, sodium pyrophosphate, ferrous sulphate, ascorbate, 1,1,3,3- tetra ethoxy propane and thiobarbituric acid were procured from finar limited (Ahmedabad, India). Pyrogallol, hydrogen peroxide, potassium dichromate, acetic acid, Ethylene diamine tetra acetic acid (EDTA), Sodium aside, were also purchased from finar limited (Ahmedabad, India). Rest all chemicals like Imodium hydrogen phosphate, 5, 5-dithio bis (2-nitro benzoic acid) (DTNB), glutathione, 1-chloral 2,4- dinitrobenzene (CDNB) were of high reagent grade and pure.

2.2. Collection of Plant Materials

The roots of *W. somnifera*, stem bark of *O. indicum* and leaves of *C. gigantea* were collected from in and around Andhra Pradesh in the month of February and authenticated by Botanical Survey of India, Chennai, Tamil Nadu, India (Ref No. BSI/LS/215).

2.3. Preparation of PHE

All the plant materials were collected, washed with water and dried under shade at about 30-35ºC for one month, then pulverized to fine powder using a laboratory scale mill. The individual powder was extracted with methanol and water using a soxhlet apparatus in the ratio of 1:6 [powder (in g): solvent (in ml)]. The extract obtained were vacuum dried at 40ºC in a rotary evaporator (Buchi, Switzerland). *W. somnifera, O. Indicum* and *C. gigantea* yielded 20, 8 and 4 gm/kg, respectively. The samples were stored in a vacuum desiccator at room temperature until further use. The three extracts were mixed together in equal proportion and suspended in 5% w/v carboxy methyl cellulose for pharmacological studies.

2.4. Animals

Healthy male swiss albino mice weighing 20-25 g were obtained from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India. The animals were fed with commercial laboratory diet and had free access to water during the experiments. All the mice were reared at a temperature of 25±1 ◦C with a 12 h light/dark cycle. Experiments were carried out as per the rules of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. The whole Study was approved by the institutional animal ethical committee.

2.5. Acute Oral Toxicity Study

The overnight fasted mice were randomly divided into six groups; each group consisted of three animals. The hydroalcoholic extract was given orally at dose level of 50, 250, 500, 1000, 2000, 4000 mg/kg body weight. After administration of the extract, the animals were closely monitored during the first 2 hour for behavioral, neurological and autonomic responses and for 24 h to detect the mortality and lethality rate.

2.6. In Vivo Anti-Tumor Activity

2.6.1. Tumor Cells

Ehrlich ascites carcinoma (EAC) cells were obtained through the courtesy of Amala Cancer Research Centre, Kerala, India. EAC cells were maintained by weekly intraperitoneal (i.p) inoculation of 1×10^6 cells/mouse [18].

2.6.2. Treatment Schedule

The animals were divided into four groups, each group contained 6 mice. Whole animals were inoculated (IP) with 1×10^6 EAC cells suspended in saline except the group I (Normal-2mL/kg) which received normal saline orally for 14 days. Group II was tumor Control. Group III (treated-400mg/kg) animals received orally 400 mg PHE/kg body weight for 14 days. Group IV (5-FU treated- 20 mg/kg) animals received intra peritoneal 20mg/kg 5-FU for 14 days. After the administration of last dose of PHE followed by 18 hour fasting half of the population from each group were sacrificed for the antitumor activity, hematological and biochemical parameters. The remaining animals of each group were kept under supervision for mean survival time (MST). On 21st day of the experiment one animal from each group were photographed to observe and compare the changes in the peritoneal cavity. Antitumor activity of the PHE was determined by observation of the changes with respect to body weight, ascetics tumor volume, MST and percentage increase in life span (% ILS).

2.6.3. Tumor Volume

On the 15th day, the ascetic fluid was collected from the peritoneal cavity and measured in a graduated measuring cylinder.

2.6.4. Ascites Fluid Smearing

The ascites fluid from the peritoneal cavity of control, standard and test treated mice were smeared on glass slides and stained with giemsa dye. The slides were then observed under light microscope to check the structural changes of the EAC cells.

2.6.5. Hematological Parameters

At the end of the study followed by 18 hours fasting blood was collected by retro orbital plexus and estimated for hemoglobin (Hb), red blood cell (RBC) count, white blood cell (WBC) count, protein, packed cell volume (PVC) etc. by auto hematology analyzer (Prokan Electronics Inc, PE-6800).

2.6.6. Percentage Increase in Life Span and Mean Survival Time

The effect of PHE on mean survival time was calculated based on the mortality of the mice under investigation [19].

$$Mean\ Survival\ time\ (In\ Days) = \frac{\text{Fist death} + \text{Last death}}{2}$$

$$ILS\ (\%) = \left[\frac{\text{Mean survival time of treated group}}{\text{Mean survival time of controle group}} - 1\right] \times 100$$

2.7. *In-Vivo* Anti- Oxidant Study

The antioxidant activity of PHE was carried out by using the isolated mice liver because it is well known that liver enzymes are more sensitive indicators of a distant neoplasm than blood [20]. The liver were removed, perfused in ice cold saline, blotted in tissue paper and preserved in tris buffer pH (7.4). 10% liver homogenate was prepared, centrifuged at 1200 rpm, supernatant was taken and used to estimate the antioxidant activity by assessing the biochemical parameters like: lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione S-transferase (GST) by the standard procedure followed by Sri Balasubashini *et al.* [21].

2.8. Histopathological Findings

At the end of the experiment the vital organs like liver, kidney and brain of animals were excised, fixed in 10% formalin, embedded in paraffin and 5-µm sections were stained routinely with Harry's hematoxylin and eosin (H&E). The slides were examined under light microscope for histopathological changes.

2.9. Statistical Analysis

All the experimental data were expressed as mean ± SEM (n = 6 mice per group). The data were statistically analyzed by using one-way analysis of variance (ANOVA) followed by Turkey multiple comparison test for ascitic tumor volume, haematological, meansurvival and antioxidant parameters. $P < 0.05$ was considered as the level of significance.

Table 1: Effect of the Poly Herbal Extract (PHE) on Ascites Tumor Volume, Hematological Parameters, Mean Survival Time (MST in Days) and Percentage Increase Lifespan (% ILS)

Parameters	Normal	EAC Control	PHE 400mg/kg	5-FU 20mg/kg
Ascites tumor volume (ml)	-	3.92 ± 0.057	2.50 ± 0.072***	1.85 ± 0.076***
Hemoglobin (g%)	13.46±0.20	8.83 ± 0.33	10.66± 0.40^{a,***}	11.62 ± 0.31***
RBC (10^6/mm^3)	4.55 ± 0.11	2.9 ± 0.073	3.76 ± 0.145^{b,***}	3.78 ± 0.014***
WBC (/mm^3)	8.28 ± 0.202	11.4 ± 0.180	10.1 ± 0.131***	9.15 ± 0.122***
Protein	6.48±0.10	10.35 ± 0.34	8.88 ± 0.41^{d,***}	7.89 ± 0.44^{c,***}
PCV (mm)	16.38± 0.60	24 ± 0.57	19.66 ± 0.71$^{\#\#,***}$	17.39 ± 0.61$^{\#}$
Lymphocytes (%)	42.5±1.33	52.83 ± 1.74	48.66 ±1.02	45.33 ± 1.32
Neutrophils (%)	54.66±0.49	41.66 ± 1.20	53.16 ± 0.70	54.42 ± 0.76
Monocytes (%)	0.66 ± 0.33	1.66 ± 0.21	0.62 ±0.21	0.59 ± 0.23
Mean survival time (MST in days)	-	24.16 ± 0.477	32.5 ± 0.763***	42.83 ± 0.600***
Life Span (%ILS)	-	-	34.5%	77.27%

Treatments were started 24 hours after inoculation with EAC cells and continued for the next consecutive fourteen days. N = 6 mice in each group; values are expressed as mean ± S.D

$^{***}P< 0.001$ when test and standard compared with control and test is compared with standard

$^{***}P< 0.001$ when test and standard compared with control; test and standard compared with normal and $^{a}P< 0.05$, ^{b} $P > 0.05$ test is compared with standard, $^{c}P< 0.01$ standard compared to normal, $^{d}P< 0.01$ test compared to control, $^{\#}P > 0.05$ Standard compared to normal, $^{\#\#}P< 0.01$ Test compared to standard. Data were analyzed by using one way ANOVA followed by turkey multiple comparison test.

3. RESULT

Oral administration of the PHE at a dose of 400 mg/kg body weight was significantly improved the mean survival time, life span and decreased ascites tumor volume, indifferent to the EAC control mice (Table **1**).

The ascetic fluid smeared from the peritoneal cavity of the control, standard and test treated mice shown diversified structural changes (Figure **1**). The smear from the control group confirmed viable EAC cell clumps. However standard and test treated groups corroborate membrane bleeding, cell wall destruction, vacuolated cytoplasm and complete degradation of the cells.

The hematological parameters of the PHE treated mice merely reached to the normal values (Table **1**). The number of RBC count and hemoglobin count increased as well, but the WBC and lymphocyte count decreased as compared with the control group.

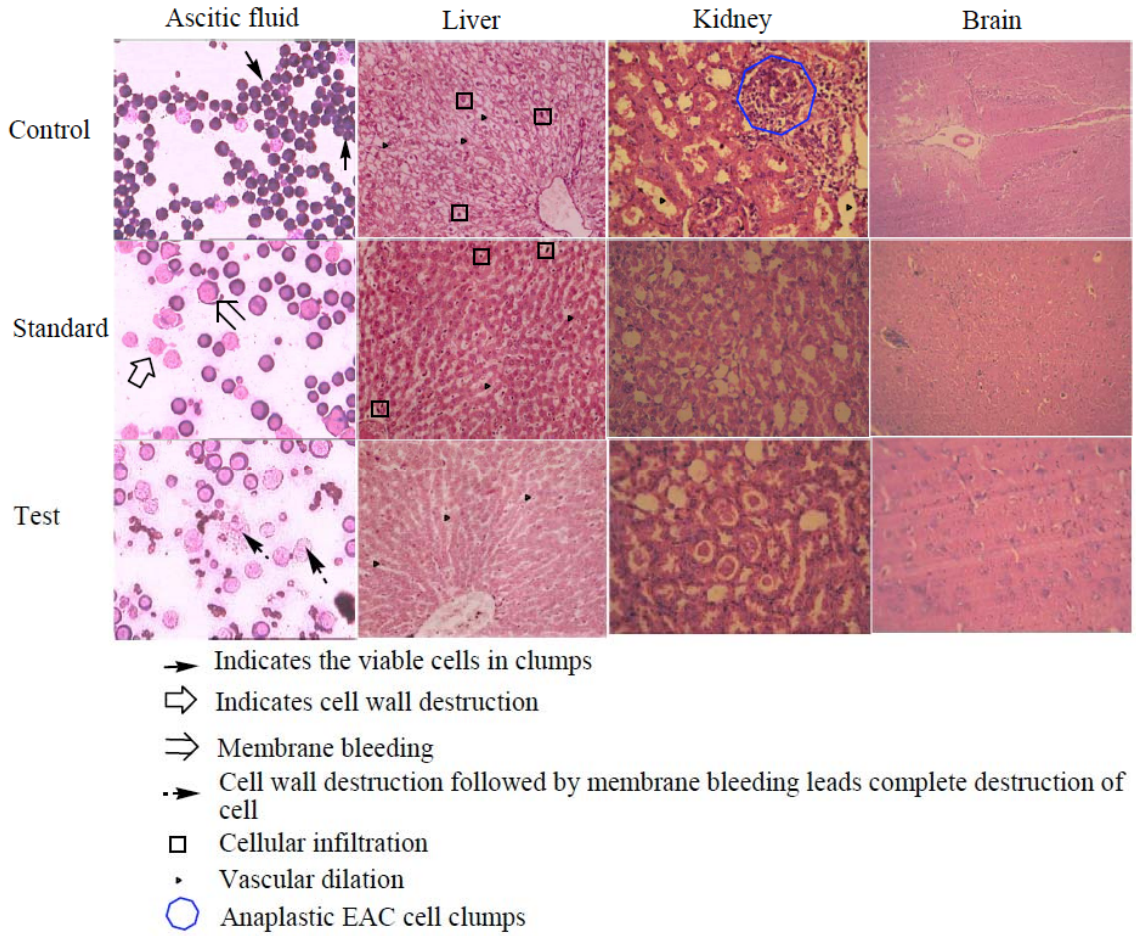


Figure 1: Ascetic fluid smear & histopathology.

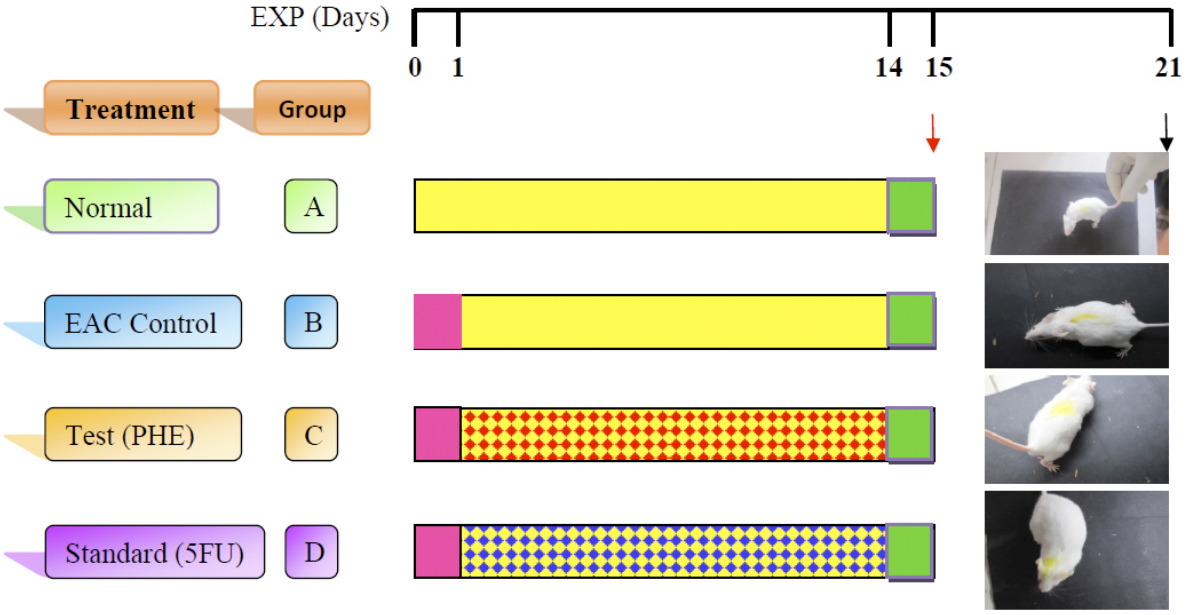
Table 2: Effect of the Poly Herbal Extract (PHE) on Activities of Antioxidant Enzymes in Liver of EAC-Bearing Mice

Parameters	Normal	EAC Control	PHE 400mg/kg
SOD	39.11 ± 0.695	19.30 ± 0.531	29.53 ± 0.551[a,b]
Catlase	0.483 ± 0.005	0.183 ± 0.005	0.23 ± 0.004
LPO	7.93 ± 0.076	24.19 ± 0.33	19.68 ± 0.43[a,b]
GPX	16.54 ± 0.286	45.18 ± 0.448	28.49 ± 0.388[a,b]
GST	0.181 ± 0.003	0.289 ± 0.003	0.208 ± 0.006

Notes: n=6; results are expressed as mean±S.D; [a]P<0.001 compared to normal group; [b]P<0.001 compared to control group; Data were analyzed using one way ANOVA followed by turkey multiple comparison test. LPO=µ moles of MDA/min/mg protein; GPX =µ moles of GSH oxidized/min/mg protein; GST=µ moles of CDNB conjugation formed /min/mg protein; SOD=Units/min/mg protein; CAT=µ Moles of H_2O_2 consumed/min/mg protein.

It has been well established that tumor-bearing animals can experience a systemic alteration of antioxidant enzymes in organs far from the tumor like liver. In this study, there was a significant downturn in the activities of (SOD) and (CAT), at the same time increased level of (LPO), GPX and GST were contemplated in the control group. However, the administration of PHE at a dose of 400mg/kg body wt restored the biochemical parameters (enzymic antioxidants) near to the normal (Table **2**) there by reducing the oxidative stress on liver.

In addition to the antitumor and antioxidant activities observed for the PHE, the histopathological examinations (Figure **1**) of the drug treated and control group mice were investigated to explore more details of the mechanism of this extract and find out toxicity if any. The liver histopathology of control showed cellular infiltration of the anaplastic carcinoma cells. In case of standard and test, very fewer cellular infiltrations were found as compared to control, but the standard showed congestion and mild central vein dilation, which were subsided in the test treated group suggesting less toxicity. The histopathology of kidney tissues showed



Basal diet; drinking water, ad libtum

A single intra-peritoneal (I.P) injection of 1 $\times 10^6$ EAC (Ehrlich Ascites Carcinom a) cells on 0^{th} day of the experiment

PHE (Poly Herbal Extract) 400m g/kg body we ight P.O (per or al) daily from 1^{st} to 14^{th} day of the experiment

Intra-peritoneal (I.P) injec tion of 5-FU (5-Fluoro Uraci l) 20mg/kg body weight daily from 1^{st} to 14^{th} day of the experiment

18 hrs fasting prior to sacrification

Mice (half of the population from each group) sacrificed on 15^{th} day of the experiment

Photographs taken on 21^{st} day of the experim ent showing changes in the size of the peritoneal cavity of various groups

Figure 2: Colored flow chat of treatment schedule and the mice bearing EAC cells.

cellular infiltration in control, simultaneously little sign of nephrotoxicity in the standard group was observed due to cellular and glomerular infiltration. The PHE treated group also showed mild glomerular infiltration but significantly less than control and standard treated groups. There was an absence of tubular necrosis, casts and glomerular congestion. The brain histopathology illustrates neuronal degeneration with purkinje and glial cells necrosis. The standard and test treated groups assured momentous retrieval of structural changes. However, no evidence of neurotoxicity was found in test treated group, which proves PHE (400mg/kg) as significant therapy in the treatment of EAC bearing mice.

On the 21st day of the experiment, one mouse from each group was photographed and examined. Reduction in the tumor burden was observed with decreased swelling of abdominal cavity in standard and test treated mouse as compared to control (Figure **2**).

4. DISCUSSION

In the proposed study, the anti-tumor and anti oxidant potential of the PHE were well established on Ehrlich ascitic tumor bearing mice. There was an induction of local inflammatory reaction, with increasing vascular permeability, resulting in an intense edema, cellular infiltration and progressive ascitic fluid with the Ehrlich ascitic tumor implantation [22]. A rapid growth in ascetic fluid is quite obvious to cater the nutritional requirement of the tumor cells as the ascetic fluid is a suitable dietary source for the tumor [23]. The result of the proposed study portraits a significant reduction in the ascetic tumor volume by the treatment of PHE at 400 mg/kg body weight with no adverse effects, which was well supported by our histopathological findings. The hematological reports, increase in the mean survival time and percentage increase in life span of the test treated group is momentous. Cell proliferation in cancer is inversely proportional to lipid peroxidation. An increased level of lipid peroxidation in the liver of tumor bearing animal could lead to decrease in GPX and GST activity. The latter two are biotransformation enzymes, which detoxify carcinogens, xenobiotics and free radicals, ultimately protecting the cells and organs from oxidative stress. SOD and CAT are the primary anti oxidant enzymes as they are engrossed in direct elimination of active oxygen species [24]. Depression of these two enzymes within the liver of EAC bearing mice is a common phenomenon. Our results affirmed an excellent restoration of all biochemical parameters (Enzymic antioxidants) there by reducing the oxidative stress in EAC bearing subject. In this study, all the data superseded the previous reports when these three plants were screened individually on various cancer models. Hence, it could be concluded that the PHE of *Withania somnifera, Oroxylum indicum* and *Calotropis gigentia* justifies the objective of the study. The improved anticancer and antioxidant potency of the PHE is believed to be due to synergistic effect. Out of these three plants, Calotropis gigentia is known to be a toxic plant; however, in this combined extract no such toxicity or adverse effect was observed. Therefore, the PHE is proven to be a potent anticancer and antioxidant preparation effective on Ehrlich's ascites carcinoma xenografted mice.

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