ALCOHOLIC EXTRACT OF CURCULIGO ORCHIOIDES GAERTN. EFFECTIVELY DOWN REGULATES DLA AND EAC INDUCED TUMOUR DEVELOPMENT AND ENHANCE THE TUMORICIDAL ACTIVITY OF MICE PERITONEAL MACROPHAGES

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ABSTRACT
Curculigo orchioides Gaertn. (family: Amaryllidaceae) commonly known as Black-Musali is a small stemless herb found to be growing in Tropical and Subtropical Asia. Immunostimulatory, anti-inflammatory and antioxidant properties of the plant have been reported earlier. In the present study methanolic extract of Curculigo orchioides was studied for its antitumor activity against Daltons lymphoma ascites induced solid tumour model and Ehrlishs ascites carcinoma induced ascites model. The extract was found to be 100% toxic at a concentration of 0.8 mg/ml to Dalton’s lymphoma ascites (DLA) and Ehrlich ascites carcinoma (EAC) cells in short term in vitro cytotoxicity assay. Curculigo orchioides extract was also found to be cytotoxic towards L929 cells in culture at a concentration of 0.1 mg/ml during MTT assay. Solid tumour volume was found to be reduced by the administration of C. orchioides extract (50mg/Kg body weight) and also increased the lifespan of the ascites tumour bearing animals by 65%. Serum proinflammatory cytokine levels were also lowered by the extract in DLA induced animals. Treatment with the extract also lowered serum gamma glutamyl transpeptidase (GGT) and nitric oxide (NO) levels in ascites tumor bearing animals. C. orchioides along with the peritoneal macrophages elicited by sodium caseinate were found to be effective in increasing the lifespan of ascites tumour bearing animals. Thus C. orchioides through its immune stimulatory, anti inflammatory and cytotoxic properties found to be effective against DLA and EAC induced tumour models in mice.
Key Words: Curculigo orchioides, Daltons lymphoma ascites, Ehrlich ascites carcinoma, peritoneal macrophages, proinflammatory cytokines, gamma glutamyl transpeptidase.

INTRODUCTION
Cancer is one of the most life-threatening diseases and serious public health problems in both developed and developing countries. It is a group of diseases characterized by the dis regulate proliferation of abnormal cells that invade and disrupt surrounding tissues \[^{[1]}\]. One of the main reasons for the rapid progression of human cancers is the ability of tumor cells to escape from the immune surveillance mechanism of the body. Cancer cells may secrete immunosuppressive factors to modify the host’s immune responses. These factors can suppress immune responses, thereby impairing the inflammatory responses, chemotaxis of macrophages, and the complementary cascade. Some of these factors seem to be non-specific and lead to a generalized decline of immunity. Due to the toxic and adverse side effects of synthetic drugs as well as conventional treatments are being failed to fulfil their objectives, for these consequence herbal medicine has made a comeback to improve the fulfilment of our present and future health needs \[^{[2]}\].

The potential for treating cancer patients by immunological approaches is a promising field for oncologists and immunologists. The hallmark feature of an effective immunotherapy is its ability to stimulate lasting tumor specific immunity. Immunopharmacological agents or immunomodulators are chemicals that can influence the host response to foreign particles, which can conventionally be divided to immunosuppressive and immunostimulating drugs based on therapeutic need \[^{[3]}\]. Immunomodulators, which can be used for long period without or less side effects, are appreciable in the cancer therapy. Several medicinal herbs have shown to promote immunity in different ways. They have shown to augment specific cellular and humoral immune response \[^{[4]}\]. We have reported the immunomodulatory activity of some plants, such as Viscum album \[^{[5]}\], Tinospora cordifolia \[^{[6]}\], Withania somnifera \[^{[7]}\], etc.

Curculigo orchioides Gaertn (Hypoxidiaceae) is popularly known as black musali in India. The rhizome, as well as the tuberous roots of the plant has been extensively used in indigenous systems of medicine in India, Pakistan and China for the treatment of various diseases, including cancer, jaundice, asthma and diarthrosis wound healing \[^{[8]}\]. The juice extracted from the rhizome has also been used as a tonic to overcome impotency \[^{[9]}\]. The immunomodulatory activity of the plant was previously reported \[^{[10, 11]}\] and its methanolic extract has been shown to enhance phagocytic activity of macrophages \[^{[12]}\]. The present study
is focusing on the effect of whole plant methanolic extract of *C. orchioidees* to reduce DLA induced solid tumour development and its effect to enhance the tumouricidal activity of mice peritoneal macrophages using animal models.

**MATERIALS AND METHODS**

**Animals**

BALB/c and Swiss albino mice were taken from the breeding section, Amala Cancer Research Centre, Thrissur. The animals were kept in air-controlled room, fed with normal mice chow (Sai Feeds, Bangalore, India) and water *ad libitum*. All the animal experiments were performed according to the rules and regulations of the Animal Ethical Committee, Govt. of India.

**Cells**

L929 cells were procured from National Centre for Cell Science, Pune, India and maintained in Minimum Eagle’s Medium supplemented with 10% fetal calf serum and antibiotics. Daltons Lymphoma Ascites (DLA), Ehrlich Ascites Carcinoma (EAC) cells were procured from Adayar Cancer Institute, Chennai, India.

**Extract Preparation**

Authenticated *Curculigo orchioidees* (whole plant) was obtained from Amala Ayurvedic Centre was dried at 45°C and powdered. Fifty grams was extracted with 70% methanol, using soxhlet apparatus. Methanol was removed by evaporation using rotary evaporator, and yield was 10.2% (w/w). The extract was suspended in DMSO for *in vitro* studies. For animal experiments it was resuspended in 1% gum acacia and administered i.p. at concentrations of 50, 20 and 10 mg/kg body weight.

**Chemicals**

Minimum Eagle’s Medium (MEM) and 5′- 5′ dithiobis (2-nitrobenzoic acid) (DTNB) was purchased from Sigma-Aldrich USA. All other chemicals used were of analytical reagent grade.

**ELISA kit**

Highly specific quantitative ‘Sandwich’ ELISA kits for mouse TNF-α, IL-1β and IL-6 were purchased from Pierce Biotechnology, USA.
Toxicity analysis
Toxicity analysis of *C. orchioides* methanolic extract was performed to find out a nontoxic concentration of the extract for *in vivo* experiments in mice. LD 50 and sub-acute toxicity studies were done with different concentrations.

1) Determination of Antitumor Activities
The antitumour activities of *Curculigo orchioides* extract was determined by both *in vitro* as well as *in vivo* methods.

1.a) Determination of the *in vitro* Cytotoxic Activity of *Curculigo orchioides* to DLA and EAC cells
DLA or EAC cells were incubated with various concentrations (50, 100, 250, 500, 800 µg/ml) of *Curculigo orchioides* extract in a final volume of 1ml for 3h at 37°C. After incubation the viability of the cells were determined by the trypan blue dye exclusion method [13].

1.b) Determination of the Cytotoxicity of *Curculigo orchioides* to L929 cells in culture
Cytotoxicity of the extract of *C. orchioides* was determined using L929 cells. Cells were seeded in 96-well flat-bottom plates (5000 cells/well) and allowed to adhere for 24h at 37°C with 5% CO₂ atmosphere. Different concentrations of *C. orchioides* extract (10-100 µg/ml) were added and incubated further for 48h. Before 4h of the completion of incubation, 20 µl of MTT (5mg/ml) was added [14,15]. Percentage of dead cells was determined using an ELISA plate reader set to record absorbance at 570 nm.

1.c) Determination of the effect of *Curculigo orchioides* on solid tumor development
Solid tumour was induced by injecting DLA cells (1 x 10⁶ cells/animal) subcutaneously to the right hind limbs of five groups (8 animals/group) of Swiss albino mice. Group I was kept as untreated tumour control and Group II were treated with 10 mg/kg body weight Cyclophosphamide. Group III, IV, V was treated with ten consecutive doses of *Curculigo orchioides* extract at concentrations of 50mg/kg body weight, 20 mg/kg body weight and 10 mg/kg body weight respectively. The radii of developing tumours were measured using vernier calipers at 3 days intervals for one month and tumour volume was calculated using the formula \( V = \frac{4}{3} \pi r_1^2 r_2 \), where ‘\( r_1 \)’ and ‘\( r_2 \)’ represent the major and minor diameter, respectively [16].
1.d) Determination of the effect of *C. orchioides* on Serum pro-inflammatory cytokine levels
Blood was collected from the solid tumour bearing animals (group I, II, III and IV) on 7th day and 15th day and serum was separated to analyze the levels of proinflammatory cytokines like TNF-α, IL-1β and IL-6 using ELISA kits according to the manufacturer’s instruction.

1.e) Determination of the effect of *C. orchioides* on Serum Gamma Glutamyl Transpeptidase (GGT) and Nitric Oxide (NO) levels
Three groups (6 animals/group) of Swiss albino mice were induced ascites tumor by injecting EAC cells (1 x 10^6 cells/animal) to the peritoneal cavity. Group I: EAC alone (Control), Group II: EAC + *C. orchioides* treated (50mg/kg body weight) and Group III: EAC + *C. orchioides* treated (20mg/kg body weight) for 10 consecutive days. Blood was collected at different time points (5, 10 and 15th day), and the serum was used for the estimation of GGT [17] and NO [18].

2) Determination of the effect of *C. orchioides* on the tumoricidal activity of mice peritoneal macrophages

2.a) Effect of *C. orchioides* activated macrophages on ascites tumour development
Male Balb/c mice (6 groups, 8 animals/group) were used for this experiment. Group I animals were kept as untreated control, group II animals were treated with 200µl of 5% sodium caseinate to elicit peritoneal macrophages. Group III and IV received *C. orchioides* extract alone (50 and 20 mg/kg Body weight respectively) and group V animals received sodium caseinate along with 50 mg/kg body weight extract where as VI animals received sodium caseinate and *C. orchioides* extract at a dose of 20 mg/kg body weight. EAC cells were administered to all animals (1 x 10^6 cells/animal, ip) 24 hours after the macrophage elicitation. The death pattern of animals due to tumour burden was noted and the percentage of increase in lifespan was calculated using the formula ((T-C)/C) x 100, where ‘T’ and ‘C’ represent the number of days that treated and control animals survived, respectively.

2.b) Effect of *C. orchioides* activated macrophages on developed tumours
Ascites tumour was induced to Balb/c mice (6 groups, 8 animals/group) using EAC cells (1 x 10^6 cells/animal) intraperitoneally on 7th day of tumour inoculation. Group II animals received sodium caseinate and group III and IV animals received extract alone (50 and 20
mg/kg B.wt respectively). Group V and VI animals received sodium caseinate and *C. orchiodes* extract (50 and 20 mg/kg B. wt respectively).

**Statistical analysis**

Values are expressed as mean ±S.D. The statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett’s test. p-values less than 0.05 was considered to be significant.

**RESULTS**

**Toxicity analysis**

LD 50 studies showed that the extract has no lethal effects up to 5g/kg body weight. Acute toxicity studies showed that the *C. orchiodes* extract is non toxic up to 200mg/kg body weight (Data not shown).

1.a) **Cytotoxicity of *Curculigo orchiodes* towards DLA and EAC Cells**

Methanolic extract of *Curculigo orchiodes* was found to be 100% toxic at a concentration of 800 µg/ml to DLA and EAC cells with dose dependant increase in cytotoxicity (Table: 1).

**Table 1: Cytotoxicity of *Curculigo orchiodes* towards DLA and EAC Cells**

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>% Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DLA</td>
</tr>
<tr>
<td>50</td>
<td>14.5</td>
</tr>
<tr>
<td>100</td>
<td>23.5</td>
</tr>
<tr>
<td>500</td>
<td>62</td>
</tr>
<tr>
<td>800</td>
<td>100</td>
</tr>
</tbody>
</table>

DLA or EAC (10^6 cells) were incubated with different concentrations (0.05-0.8 mg/ml) of extract. Percentage of dead cells was determined by trypan blue exclusion method.

1.b) **Cytotoxicity of *Curculigo orchiodes* towards L929 cells in culture**

Extract of *C. orchiodes* was found to be cytotoxic towards L929 cells in culture. Methanolic extract of *C.orchiodes* was found to be 100% toxic at a concentration of 0.1mg/ml (Table 2).

**Table 2: Cytotoxicity of *Curculigo orchiodes* extract to L929 cells in Culture**

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>% toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>22%</td>
</tr>
<tr>
<td>50</td>
<td>54%</td>
</tr>
<tr>
<td>100</td>
<td>100%</td>
</tr>
</tbody>
</table>
1.c) Effect of *Curculigo orchioides* on Solid Tumor Development

There was a significant reduction of tumour volume in *Curculigo orchioides* treated animals when compared to the untreated tumour control animals (Fig. 1). The tumour reducing capability of the extract at a concentration of 50mg/kg body weight was found almost similar to that when administered with Cyclophosphamide (10mg/kg body weight).

![Fig. 1: Effect of *Curculigo orchioides* on Solid Tumor Development](image-url)

1.d) Effect of *Curculigo orchioides* on Serum proinflammatory cytokine levels

Serum proinflammatory cytokine levels were elevated due to tumour development; IL-1β level was 44.69 ± 3.52 for tumour control group on 15th day and it was significantly reduced to 32.28 ± 2.24 by the administration of *C. orchioides* (50mg/kg b.wt). Serum TNF- α level was also reduced to 198.62 ± 12.28 from the increased level of 286.48 ± 8.22 by the extract and the Extract reduced IL-6 levels also when compared to that of the tumour control animals. (Table 3).

![Table 3: Effect of *Curculigo orchioides* on Serum proinflammatory cytokine levels](table-url)
1.e) Effect of *Curculigo orchioides* on Serum Gamma Glutamyl Transpeptidase (GGT) and Nitric Oxide (NO) levels

The effect of *C. orchioides* on the serum GGT and NO is presented in Table 4. On the 15th day after tumor challenge, elevated level of GGT in the serum of control tumor bearing animals as compared to normal animals was significantly (p<0.01) reduced to 68.65 ± 2.47 nmol p-nitroaniline/ml serum after the administration of *C. orchioides*. The extract also reduced serum NO levels when compared to the tumour control animals.

**Table 4 Effect of *Curculigo orchioides* on Serum Gamma Glutamyl Transpeptidase (GGT) and Nitric Oxide (NO) levels**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GGT (nmol p-nitroaniline/ml)</th>
<th>NO (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Normal</td>
<td>27.72±2.36</td>
<td>23.64 ± 1.26</td>
</tr>
<tr>
<td>Tumour control</td>
<td>56.68±2.62</td>
<td>136.44±6.92</td>
</tr>
<tr>
<td><em>C. orchioides</em> (50 mg/kg b.wt)</td>
<td>32.34±1.24</td>
<td>48.65±2.32</td>
</tr>
<tr>
<td><em>C. orchioides</em> (20 mg/kg b.wt)</td>
<td>38.63±1.18</td>
<td>58.62±2.08**</td>
</tr>
</tbody>
</table>

The serum was collected from tail vein on 5, 10 and 15th day after tumour and assayed for biochemical parameters. Values are mean ± S.D. **p<0.01**

2.a) Effect of *C. orchioides* activated macrophages on ascites tumour development

Lifespan of tumour bearing animals was found to increase by the prophylactic administration of the extract along with elicitation of macrophages to 95%. Non treated control animals were survived up to 20 ± 1.31 days where as in the case of the sodium caseinate alone treated group the average date of survival was 23 ± 1.55.

**Table 5: Effect of *C. orchioides* activated macrophages on ascites tumour development**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of days survived</th>
<th>% Increase in lifespan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour alone</td>
<td>20 ± 1.31</td>
<td>----</td>
</tr>
<tr>
<td>Tumour + sodium caseinate</td>
<td>23 ± 1.55*</td>
<td>15</td>
</tr>
<tr>
<td>Tumour + <em>C. orchioides</em> (50 mg/kg b.wt)</td>
<td>33 ± 2.50**</td>
<td>65</td>
</tr>
<tr>
<td>Tumour + <em>C. orchioides</em> (20 mg/kg b.wt)</td>
<td>26 ± 1.16**</td>
<td>30</td>
</tr>
<tr>
<td>Tumour + sodium caseinate + <em>C. orchioides</em> (50 mg/kg b.wt)</td>
<td>49 ± 2.13**</td>
<td>95</td>
</tr>
<tr>
<td>Tumour + sodium caseinate + <em>C. orchioides</em> (20 mg/kg b.wt)</td>
<td>34 ± 1.69**</td>
<td>70</td>
</tr>
</tbody>
</table>

*Values are mean ± S.D. **p<0.01*
2.b) Effect of *C. orchioides* activated macrophages on developed tumours

Table 6 illustrate the increase in lifespan when the extract was administered along with sodium caseinate 6 days after the tumour induction. Life expectancy was enhanced to an average of 35 days for *C. orchioides* (50mg/kg b.wt) + sodium caseinate, where as the average number of days survived by the ascites tumour bearing animals were 19 and 21 for tumour control and tumour + sodium caseinate alone groups respectively.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of days survived</th>
<th>% Increase in lifespan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour alone</td>
<td>19 ± 2.00</td>
<td></td>
</tr>
<tr>
<td>Tumour + sodium caseinate</td>
<td>21 ± 1.49</td>
<td>10.5</td>
</tr>
<tr>
<td>Tumour + <em>C. orchioides</em> (50 mg/kg b.wt)</td>
<td>26 ± 2.00**</td>
<td>36.8</td>
</tr>
<tr>
<td>Tumour + <em>C. orchioides</em> (20 mg/kg b.wt)</td>
<td>23 ± 1.35**</td>
<td>21.2</td>
</tr>
<tr>
<td>Tumour + sodium caseinate + <em>C. orchioides</em> (50 mg/kg b.wt)</td>
<td>35 ± 1.85**</td>
<td>84.0</td>
</tr>
<tr>
<td>Tumour + sodium caseinate + <em>C. orchioides</em> (20 mg/kg b.wt)</td>
<td>28 ± 1.92**</td>
<td>47.4</td>
</tr>
</tbody>
</table>

*Values are mean ± S.D.** p<0.01.*

**DISCUSSION**

Cancer is a class of diseases characterized by out-of-control cell growth. Normal cells in the body follow an orderly path of growth, division, and death. Cancer cells display the traits of un-controlled growth, invasion sometimes metastasis. Cancers are caused by abnormalities in the genetic material and also in their patterns of gene expression \[19\]. It is the second largest cause of death in the world.

Anticancer, or antineoplastic, drugs are used to treat malignancies, or cancerous growths. Drug therapy may be used alone, or in combination with other treatments such as surgery or radiation therapy. Most commonly, chemotherapy acts by killing cells that divide rapidly, one of the main properties of most cancer cells. Chemotherapy drugs, are often feared because of a patient's concern about toxic effects. Their role is to slow and hopefully halt the growth and spread of a cancer. A major complication of chemotherapy is its toxicity to normal cells, which is due to the inability of drug to differentiate between normal cells and malignant cells. This often impacts the efficacy of the treatment and even makes it impossible to cure the patients. One of the requisites of cancer chemo preventive agent is elimination of damaged or malignant cell through cell cycle inhibition or induction of apoptosis with less or no toxicity to normal cells \[20\]. The use of herbal medicine or dietary agents is being increasingly utilized...
as an effective way for the management of many cancer treatments [21]. The greatest recent impact of plant derived drugs is observed in the area of antitumour research, where compounds such as taxol, vinblastine, vincristine and camptothecin have dramatically improved the effectiveness of chemotherapy against some of the dreaded cancers [22]. Hence there is great potential for the development of anticancer drugs from the essentially untapped reservoir of the plant kingdom.

The results of present investigation reveal that Curculigo orchioides with wide spectrum of medicinal properties have cytotoxicity against DLA and EAC cells lines. Cytotoxicity is one of the chemotherapeutic targets of antitumor activity [23]. Most of the clinically used antitumor agents possess significant cytotoxic activity in cell culture systems. The cytotoxic activity of Curculigo orchioides against DLA and EAC cell lines partially explains its significant anti tumor activity against solid and ascites tumor. The antitumor activity was evaluated in solid tumor model. Methanolic extract of Curculigo orchioides reduced the tumor burden effectively.

Administration of C. orchioides reduced the serum gamma glutamyl transpeptidase (GGT), an enzyme that catalyzes the transfer of gamma glutamyl moieties from glutathione to other aminoacids and dipeptides [24]. Moreover, administration of C. orchioides was found to inhibit nitric oxide production in tumour cells. Ehrlich ascites tumor is a rapidly growing carcinoma with very aggressive behaviour [25]. It is able to grow in almost all strains of mice. The Ehrlich ascites tumor implantation induces a local inflammatory reaction, with increasing vascular permeability, which results in an intense oedema formation, cellular migration, and a progressive ascetic fluid formation [26]. Administration of C. orchioides extract found to increase the lifespan of the ascites tumour bearing animals. Mechanism of antitumour activity of C. orchioides extract has to be evaluated.

Macrophages respond to tumour cells in a complex manner; they can either promote the destruction of tumour or may enhance the tumour growth [27]. When macrophages are stimulated with some chemicals they shows cytolytic activity against a wide array of neoplastic cells [28]. Normal macrophages which do not exhibit cytolytic activity readily acquire such capacity by incubating with a variety of microbes or components of their cell walls and/or interactions with lymphokines. The exact mechanism of their tumour cell killing remains largely unknown, but several biologically active molecules by macrophages like IL-1, TNF, IFN-γ, GM-CSF etc are considered to be involved in it. C. orchioides extract has
found to stimulate the macrophages in tumour bearing mice and they also reduces the tumour burden and delayed the death due to tumour development.

CONCLUSION

*C. orchioides* was found to be cytotoxic to tumour cells and they also reduced the tumour development in solid tumour models. Extract has been proved earlier to exhibit immunomodulatory\textsuperscript{10} and anti-inflammatory activities. From the present study it was also observed that *C. orchioides* can also enhance the tumour killing capability of mice peritoneal macrophages.

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REFERENCE


