Antitumor Activity of Mylabris cichorii Extracts Against Murine Ascites Dalton’s Lymphoma


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ABSTRACT

A blister beetle, Mylabris cichorii has been used by the traditional healers of some part of Assam, India for the treatment of cancer suspected diseases of skin. In the present study, we evaluated in vivo antitumor activity of beetle extract against murine ascites Dalton’s lymphoma (DL). The cytotoxic activity of the extract on Dalton’s lymphoma cells, thymocytes and peritoneal exudate cells (PECs) was also studied in vitro. The methanol extract of beetle’s powder was dissolved in distilled water and successively fractionated with n-hexane, chloroform and n-butanol to yield respective fractions. The tumor transplanted mice were treated daily separately with different fractions for 5 days and increase in life span (ILS) was determined. Out of these fractions, highest antitumor potential was found with the n-butanol fraction of M. cichorii at the dose of 25 mg/kg/day showing about 63% ILS. In vitro cytotoxicity assay with the trypan blue exclusion test revealed about 65% tumor cell deaths. Further, the light microscopic study showed that n-butanol fraction treatment caused more leukocyte infiltration towards the tumor cells with the appearance of membrane vacuoles and gradual disintegration of plasma membrane leading to death of the tumor cells. The results of the present study demonstrate that the n-butanol fraction of M. cichorii extract is quite effective against Dalton’s lymphoma cells both in vivo and in vitro. However, further studies for the characterization of the active principle from this fraction of beetle extract need to be carried out.

Key words: Antitumor activity, Mylabris cichorii, Dalton’s lymphoma, Cytotoxicity

INTRODUCTION

Traditional human populations have a broad natural pharmacopoeia consisting of wild plant and animal species [5,9,19]. Ingredients sourced from wild plants and animals are not only used in traditional medicines, but are also increasingly valued as raw materials in the preparation of modern medicines and herbal preparations. Animals and products derived from different organs of their bodies have constituted part of the inventory of medicinal substances used in various human cultures since ancient times [1, 10], and such uses still exist in traditional medicine.

North-Eastern region of India is comprised of rich floral and faunal diversity, and many of the animals and plants of this region are traditionally being used by the people of this region for treating different ailments and diseases [8, 10]. Our preliminary survey also revealed that the people of this region frequently use many animals and their products for the treatment of different ailments including cancer suspected cases. Mylabris cichorii, commonly known as blister beetle and locally as “Helicaptor pok” is one of them whose water extract is used by the indigenous people of Karbi Anglong and North-Cachar Hills district of Assam, India, against cancer suspected cases. The size of M. cichorii ranges from 0.5 to 2 inches, with black, brown, or gray body colors. Adults have spots or stripes of alternate yellow and black color (Figure 1). It belongs to the class Insecta, order Coleoptera, family Meloidae. M. cichorii have been reported to be one of the oldest treatments for cancer in recorded history. For more than 2000 years, blister beetles in powdered or tincture form have been used medicinally in Europe, China and elsewhere [12]. It is still employed in traditional Chinese medicine, and interest in the clinical use of cantharidin as an antitumor agent has re-emerged following the reports that cantharidin produce cytotoxic effects in a number of human tumor cell lines and primary tumor cells [11,12]. Although cantharidin showed acute toxicity in animal cells, at lower concentrations (2-5 μM), it can elicit a strong apoptotic response in many types of tumor cells, characterized by membrane blebbing, caspase activation, DNA fragmentation, activation of p53 and increased levels of both p21[waf1/cip1] and Bax [17, 18]. Based on the literature survey and primary information received from the elders and local herbal practitioners through interview, the present study was undertaken to evaluate the possible antitumor activity of M. cichorii fractions in murine ascites Dalton’s lymphoma cells.

Antimicrobial activity study

Extracts of different fractions were dissolved in phosphate buffered saline (PBS, pH 7.4) and their antimicrobial activity was determined following the method described by Ahluwalia et al. 1984 [1]. Tumor cells were transplanted intraperitoneally in 10-12 weeks old male mice and the day of tumor transplantation was taken as day zero. The tumor-transplanted animals were divided into seven groups with 10 mice in each group. Treatment with different doses (10, 25, 50, 100, 200 mg/kg) was given by intraperitoneal injection for 5 consecutive days. Tumor size was recorded by length and width of tumor. The percentage of inhibition in tumor growth was calculated using the following formula:

\[
\% \text{ yield} = \frac{\text{weight of the fraction (mg)}}{\text{weight of the whole tissue powder (mg)}} \times 100
\]

\[
\% \text{ inhibition} = \frac{\text{tumor size of control}}{\text{tumor size of treated}} \times 100
\]

\[
\% \text{ inhibition} = \frac{\text{tumor size of control}}{\text{tumor size of treated}} \times 100
\]
100 and 200 mg/kg body weight/day of M. cichorii fractions and cisplatin (2 mg/kg body weight/day) were given for five consecutive days starting from day 6 of tumor transplantation, and the host survival patterns were recorded. The group of tumor-bearing control mice received same volume of vehicle (PBS) alone. Cisplatin treated group served as positive control. The deaths of animals, if any and body weight of animals in different treatment groups were recorded daily. The antitumor efficacy was reported in percentage of average increase in life span (%ILS) calculated using the formula:

\[
\% \text{ILS} = \left( \frac{T}{C} \times 100 \right)
\]

where, T and C are the mean survival days of treated and control groups of mice respectively. The most potent fraction, i.e., n-butanol, showing highest antitumor activity was selected for further viability and light microscopical studies.

**Collection of thymocytes, peritoneal exudates cells (PEC) and Dalton’s lymphoma (DL) cells**

The thymus glands were removed from normal mice, cut into small pieces; crushed using clean glass slides and flushed out in a petri dish with ice cold sterile PBS. The cell suspension was washed twice with PBS by centrifugation at 600 rpm for 5 min, and resuspended in ice cold PBS. Peritoneal exudate cells (PEC) were collected from the peritoneal cavity of normal mice by lavaging ice cold sterile PBS. The glass adherent cells were separated by adhering PEC over glass Petri dish at 37°C for 2 hrs in 5% CO2 humidified incubator. To analyse the comparative cytotoxicity of the extract fractions, mice were subcutaneously injected with a suspension of 25 mg/ml of n-butanol fraction (25 mg/kg/day) and cisplatin. After 3 hours of incubation, cell viability was checked by trypan blue exclusion test. The percentage yield of different fractions of M. cichorii was taken on a clean slide and a thin smear was made. The smear was air-dried, fixed in absolute methanol for 15 min and stained the following day with Leishman’s stain. The cells were then observed and studied under the microscope. The percentage of viability was calculated using the formula:

\[
\% \text{viability} = \left( \frac{\text{total viable cells of treated}}{\text{total viable cells of control}} \right) \times 100\%
\]

The concentration of extracts giving 50% inhibition of cell viability (IC50) was determined using the dose-response graph.

**Light microscopical studies**

For the light microscopical study, tumor-bearing mice were treated with n-butanol fraction (25 mg/kg/day) and cisplatin (2 mg/kg/day) for five consecutive days starting from day 6 of tumor transplantation. Animals were sacrificed by cervical dislocation after 24, 48, 72 and 96 h of treatment. The ascites tumor was collected and centrifuged at 1000 rpm for 5 min at 4°C, washed twice in PBS (0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4).

The cell pellet was resuspended in PBS (1:4, v/v) and a drop of the cell suspension was taken on a clean slide and a thin smear was made. The smear was air-dried, fixed in absolute methanol for 15 min and stained the following day with Leishman’s stain. The cells were then observed and studied under the microscope.

**Statistical analysis**

Results are expressed as Mean ± SD. Significant differences between control and different treatments were calculated using Student’s t-test. Number of replicates (N) = 5, p-values of = 0.05 were considered significant.

**RESULTS**

**Percentage yield of the extracts**

The percentage yield of different fractions of M. cichorii ranges from 0.87% to 15.2% (Figure 2). The lowest percentage yield (0.87%) was observed from the n-hexane fraction while the highest percentage yield (15.2%) was found from the methanol fraction.

**Table 1. Antitumor activity of different fractions of Mylabris cichorii and the reference drug, cisplatin, against murine ascites Dalton’s lymphoma in vivo**

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Fractions</th>
<th>Doses* (mg/kg body wt/day)</th>
<th>Survival time (days)</th>
<th>% ILSb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>Vehicle</td>
<td>20±2.4</td>
<td>-</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1.0</td>
<td>21.5±1.8</td>
<td>35.0±1.8</td>
<td>73.3</td>
</tr>
<tr>
<td>M. cichorii</td>
<td>Aqueous</td>
<td>10</td>
<td>19.5±0.8</td>
<td>-3.46</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>10</td>
<td>24±0.8</td>
<td>18.8</td>
</tr>
<tr>
<td></td>
<td>n-hexane</td>
<td>25</td>
<td>20±0.4</td>
<td>-0.99</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>10</td>
<td>17±0.2</td>
<td>-15.84</td>
</tr>
<tr>
<td></td>
<td>n-butanol</td>
<td>10</td>
<td>20±0.2</td>
<td>43.5</td>
</tr>
</tbody>
</table>

Values were the means of five replicate samples (n = 5) and all data were expressed as mean±S.D.

**Table 2. 50% inhibition of cell viability (IC50) values of different fractions of Mylabris cichorii towards Dalton’s lymphoma (DL) cells, thymocytes and macrophages in vitro determined by the trypsin blue exclusion method.**

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Incubation time (hour)</th>
<th>IC50 (µg/ml)</th>
<th>Thymocytes</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>3</td>
<td>32.2±2.1</td>
<td>59.4±3.1</td>
<td>202.4±6.2</td>
</tr>
<tr>
<td>M. cichorii</td>
<td>Aqueous</td>
<td>17.4±2.2</td>
<td>8.4±1.1</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>n-hexane</td>
<td>10.3±1.4</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>19.4±1.3</td>
<td>71.6±1.5</td>
<td>200.7±4.5</td>
</tr>
</tbody>
</table>

Results were mean±S.D. Student’s t-test, number of mice in each group, N = 10. *0.25 ml volume of the drug extracts were administered daily for 5 days. Activity criteria are passed for percentage increase in life span (%ILS) = 20%. Control animals received the same volume of extract vehicle alone.

**Figure 1. Photograph showing Mylabris cichorii collected from Karbi Anglong and North Cachar Hill district of Assam, India.**

**Figure 2. Histogram showing the percentage yield (wt./wt.) of different fractions of Mylabris cichorii.**
Antitumor activity of the extracts

The various doses of fractions and their effects on the survivability of the hosts in different experimental groups have been shown in Table 1. In antitumor activity study, extracts/drugs showing the ILS of less than 20% were not considered to have antitumor activity. In the present study, among five different fractions of *M. cichorii* (methanol, water, n-hexane, chloroform and n-butanol fractions), n-butanol fraction showed much better antitumor activity against ascites Dalton's lymphoma with ILS of 43.5% and 63.4% at 10 and 25 mg/kg/day respectively. Cisplatin, a positive control showed 73% ILS at a dose of 2 mg/kg/day. A comparative percentage survivability of tumor-bearing hosts after treatment with the respective most potent dose of different fractions are also shown in Figure 3, where n-butanol (25 mg/kg/day) showed 100% survivability of the hosts for 33 days while cisplatin showed 100% survivability for 35 days. As compared to tumor-bearing control, significant decrease in the tumor volume and body weight was observed after treatment with n-butanol fractions of *M. cichorii*, while other fractions did not show significant changes. As compared to 20th day
of control, treatment with n-butanol fraction (25 mg/kg/day) decreased tumor volume by about 95% after 35 days of tumor transplantation, whereas, cisplatin decreased tumor volume by about 95% (Figure 4). Measurement of total body weight also showed a significant increase during tumor growth, and the data of present study showed significant decrease in body weight after treatment with n-butanol as well as cisplatin (Figure 5).

**Cell viability**

In the dose-response curve obtained from the trypan blue dye exclusion test, a significant decrease (p=0.05) in the percentage of cell viability was observed after the cells were treated with n-butanol fraction and cisplatin at 10, 20, 30 and 40 µM/g for 3 h (Figure 6, 7). n-butanol fraction showed more cytotoxic effect towards DL cells as compared to thymocytes and PECs of normal animals. The IC₅₀ values of n-butanol fraction determined by the trypan blue exclusion test were observed to be 20, 71 and 200 µg/ml in DL cells, thymocytes and PECs respectively (Table 2).

**Microscopic studies**

The light microscopic observation showed that control tumor cells were round in shape, surrounded by a very few leukocytes (Figure 8a). After treatment with n-butanol fraction (24 h), more leukocyte infiltration towards the tumor cell was noticed (Figure 8b). Leukocyte infiltration towards the tumor cell was noted to be more during 48–72 h of treatment (Figure 8c) as compared to 24 h. Treatment with n-butanol fraction for 96 h resulted in the appearance of membrane vacuoles (Figure 8d) and gradual disintegration of plasma membrane leading to lysis of the tumor cells (Figure 8e) (arrows). Cisplatin treatment also showed a very similar pattern of changes in DL cell membrane and leukocyte infiltration towards DL cells.

**DISCUSSION**

In the studies related with the assessment of antitumor activity, ascites Dalton’s lymphoma has been commonly used as an important murine experimental tumor model [15]. The result of present studies indicate that out of five different solvent extracts/fractions of *M. cichorii*, mainly n-butanol fraction showed comparatively better antitumor activity against ascites Dalton’s lymphoma in mice. Changes in tumor volume and body weight of mice in treated group showed significant decrease with longer survivability of the hosts as compared to control group (Figure 4, 5). From the data, it may be suggested that 10 and 25 mg/kg/day may cause to induce cancer cell death, while higher doses did not show significant increase in survivability of the hosts which could be due to some toxic effect of the extract [15]. Huan et al., 2006 [3] also reported that cantharidin, at a concentrations >10 µM/L showed acute toxicity in animal cells.

In conclusion, out of different fractions of *Mylabris cichorii* studied n-butanol fraction showed better antitumor activity against murine ascites Dalton’s lymphoma. n-butanol fraction also showed comparatively higher cytotoxic effect towards DL cells as compared to thymocytes and PECs of normal animals. n-butanol fraction-mediated plasma membrane disintegration and the appearance of membrane vacuoles on the tumor cells may also indicates tumor cell lysis, thereby leading to cell death. The exact mechanism(s) involved in the antitumor activity of *Mylabris cichorii* may not be clear at present. Therefore, further investigation is necessary to determine a detailed mechanism(s) involved.

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