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In-vivo Anticancer Activity of Root and Leaf extract of *Jurinea dolomiaea* Boiss (Asteraceae) against Ehrlichs Ascites Carcinoma (EAC) Cell Line

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ABSTRACT

Cancer is a type of disease of uncontrolled growth cells. The currently available treatments are radiotherapy, chemotherapy, hormone therapy and surgery, in which all of them have side effects. Due to the adverse side effects, it is challenging to develop new drug for cancer treatment. Hence, the scientists are trying to seek the compounds from natural sources to treat cancer. Therefore, in this present investigation, root and leaf extracts of *Jurinea dolomiaea* Boiss has subjected to evaluate its anti-proliferative effect along with molecular signaling of apoptosis in Ehrlich ascites carcinoma (EAC) cell line. *Jurinea dolomiaea* root and leaf extracts exhibit a considerable scavenging activity in comparison to a standard antioxidant BHT. Moreover, root and leaf extracts were able to agglutinate 2% RBC of goat blood at minimum 12.5 μg/mL and 50.0 μg/mL concentration respectively. Cytotoxic activity was also found in root and leaf extract. In haemocytometic observation, the root and leaf extract exhibit about 62.54±2.41% and 53.96±2.34% cell growth inhibition respectively where as standard anticancer drug Bleomycin showed 79.43±1.92% growth inhibition. Morphological change under fluorescence microscope showed nuclear condensation and fragmentation which are the sign of apoptosis. Therefore, current results depict root extract of *Jurinea dolomiaea* Boiss have anti-proliferative activity against EAC cell line and can be a good source of anticancer agents to treat cancer.

1. Introduction

Cancer is considering the burning health issue and is one of the most life-threatening diseases in both developed and developing countries [1]. Cancer is occurred all around the world and can cause the death [2]. It is a group of diseases caused by loss of cell cycle control leading to abnormal and uncontrolled growth of cells [3]. Cancer development is associated with the alteration of oncogenes, tumor suppressor genes, and DNA repair genes [4]. Both external factors such as tobacco, chemicals, radiation and infectious organisms and internal factors such as inherited mutations, hormones immune conditions are responsible for such disease [5]. Cancer is a serious burden on the public health and its treatment and curing is scientifically challenging [6]. The conventional approaches of cancer treatments are chemotherapy, radiotherapy, hormone therapy and surgery. But these treatments have severe side effects [7]. The adverse effects of anticancer drugs are the reasons that the researchers to look for new and more effective drugs with least side effects [8]. Due to these limitations, scientists are in constant search of natural compounds which might be capable of curing cancer [9]. Many natural compounds such as terpenoids, phenolicacids, lignans, tannins, flavonoids, quinones, coumarins and alkaloids were discovered from plant sources that contain significant antioxidant activities and play a vital role in cancer treatment [10]. Several antioxidant compounds show anti-inflammatory, antitumor, antimutagenic and anticarcinogenic activities [11]. Natural compounds with antioxidant activity can inhibit directly the cell proliferation and stimulate the immune system [12]. In recent year, the drug industries largely depend on natural compounds as a source of medicine. Near about 60% anticancer drugs have herbal origin [13]. These are worldly accepted as a source of complementary and alternative medicine [14] in various diseases especially in cancer [15]. Due to the favorable climatic condition, Indian subcontinent is the home of wide range of plant species with medicinal properties. Ayurveda is one of the most ancient medicine systems which are developed in India with a fundamental principle [16]. This treatment system comes out on plant material and is running smoothly from the very ancient time till today. Reportedly Ayurveda gains an excellent achievement in the world medicine. So, the current focus is to identify many bioactive compounds with anticancer properties. *Jurinea dolomiaea* Boiss is a fast grown leafy plant with high medicinal values [17]. It contains fiber, ash, calcium, vitamins, thiamine, riboflavin, niacin etc. and is traditionally used as an antidote, aperients, astringent, demulcent, diuretic, febrifuge, laxative, and rubefacient [18, 19]. Its root is also used in the treatment of inflammation, atherosclerosis, stroke, heart disease, diabetes mellitus, multiple sclerosis, Parkinson's disease, Alzheimer's disease, etc. [20]. Moreover, leaves and root of the plant is used to recover male infertility [21]. Therefore, present study was designed to investigate the anticancer potential of root and leaf extracts of *Jurinea dolomiaea* Boiss.

2. Experimental Methods

2.1 Collection and Preparation of Root and Leaf Extracts

Jurinea dolomiaea Plant was collected from Hemalian region of upper Danchigam in south Kashmir about 38- 45 meters above the sea level. The plants were identified taxonomically and authenticated at the Herbarium, Department of Botany, Kashmir University. Plant's roots and leaves were washed thoroughly 2 - 3 times with running tap water and then with sterile water. About 500 g fresh root and leaf were dried at about 40 °C for 10 days to 15 days. Upon drying, the plant materials were powdered with a power grinder. About 25 g of both powdered root and leaf were dissolved in 250 mL of distilled water in separate conical flask and overnight shaking was done by a rotator. Subsequently, the samples were sonicated at medium frequency for 15 min in an ultrasonic bath and filtered with Whatman No.1 filter paper. The filtered materials were lyophilized using freeze dryer and stored at 4 °C until for further use.

2.2 Experimental Animal

Healthy Swiss Albino mice weighing 25 ± 2.0 g were collected from the animal house of Pharmacy Department, Barkatullah University, Bhopal. The mice were clustered into three groups (control, root treated, and leaf treated) containing 6 mice in each group. They were housed in plastic cage

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and maintained with standard condition (25±2 °C) with 12±1 hourdark/light cycle. Mice were fed with pellet feed containing proper nutrients.

2.3 Collection and Maintenance of Cell Line

The initial inoculums of Ehrlich ascites carcinoma (EAC) cells were kindly provided by Protein and Enzyme Laboratory, Department of Biochemistry and Molecular Biology, Barkatullah University, Bhopal. The EAC cells culture and aspiration were maintained by following the procedure of Alarm et al. [22]. In short, the EAC cells were propagated intra peritoneal biweekly by injecting cells, freshly drawn from a donor Swiss Albino mouse bearing 6-7-day-old ascites tumor. The freshly drawn fluid was diluted with normal saline (1% NaCl solution). The aspirated cells were kept in a cell culture petri dish for 1 hour at 38 °C in an air incubator. All macrophages are distinct from tumor cells, became firmly fixed to the bottom of the culture vessels [23]. Then the petri dish was slightly vortexed and the fluid was collected for EAC cells which were used in further experiments. The tumor cell numbers were adjusted to about 2×106 cells/mL by counting the cell number by haemocytometer. The viability of tumor cells was observed by trypan blue dye (0.4%) exclusion assay. Strict aseptic condition was maintained throughout the transplantation process.

2.4 Chemicals and Reagents

Hemagglutination buffer, Saline water, Sodium citrate (anticoagulant),1,1-diphenyl-2-picrylhydrazine (DPPH), Methanol, Ethanol, Butylated hydroxyl toluene (BHT), Phosphate buffer saline (PBS), Agarose, Ethidium bromide, TBE buffer, Trypan blue, Benedicts reagents, Biuret reagents, Wagner's reagents, HCl, $FeCl_3$, H_2SO_4.

2.5 Phytochemical Screening

Standard procedures were used to screen the presence of different phytochemicals from root and leaf of *J. dolomiaea* Boiss. The crude extracts of root and leaf of *J. dolomiaea* Boiss were tested for the presence of carbohydrates(Benedict's test) [24], proteins (Biuret test) [25], fats (Filter paper test) [26], glycosides (Salkowski's test) [27], alkaloids (Wagner's test) [28], tannins (Ferric chloride test) [29], phlobatannins (HCl test)[30], saponins (Frothingtest) [31], flavonoids (HCl test) [32], steroids (Salkowski's test), phenolic compounds (ferric chloride test) and phytosterols (sulfuric acid test) [33]. The qualitative results are expressed as (+) for the presence and (-) for the absence of phytochemicals.

2.6 Antioxidant Activity Assay

The DPPH scavenging activity of root and leaf extracts was determined by Brand-Williams et al. in 1995 with slight modification in micro titer plate [34]. According to this, 1 mL of methanol solution of root and leaf extracts were taken in ten (10) test tubes containing different concentrations such as 40, 80, 120, 160, and 200 μg/mL from stock solution (1 mg/mL). Subsequently, 1.5 mL DPPH (1 mg/25 mL) was added to these test tubes and the mixtures were allowed to keep at room temperature in the dark place for 30 minutes. Finally, absorbance was measured at 517 nm using spectrophotometer (GENESYS10S UV-Vis, Thermo-Scientific, USA). BHT was used as standard and methanol as negative control. The percentage of DPPH scavenging capacity was calculated according to the formula below,

$$\text{DPPH scavenging (\%)} = C–E/C \times 100\% \quad (1)$$

where, C is absorbance of the control and E is absorbance of root and leaf extracts. The 50% inhibition concentrations of the extracts, IC50 values were determined by plotting the graph of percentage DPPH scavenging activity against the different concentrations of extracts.

2.7 Hemagglutination Assay

The hemagglutination assay of crude root and leaf extracts of *J. dolomiaea* were performed in 96-well micro-titer U-bottomed plates described by Hasan I. et al., [35]. According to this process, goat blood was collected in test tubes containing sodium citrate (anticoagulant reagent) and washed for 20 times with PBS (phosphate buffer saline) to prepare 2% (w/v) red blood cells. This 2% RBC was prepared followed by a standard process described by Rashel Kabiretal [36]. In short, the goat blood was collected from slaughtered house in sodium citrate and centrifuged at 3000 rpm for 5 minutes at 4 °C to separate the blood cells from plasma. Then, the cell was washed for three times with PBS (phosphate buffer saline). After that, 20 mg of RBC pellet was taken and dissolved in 1 mL of hemagglutination buffer. Subsequently, 50 μL of hemagglutination buffer was taken in each well of micro-titer plate. Instantly, 50 μL of root and leaf extracts were added to the first well and serially diluted. Finally, 50 μL of 2% red blood cells were added to each well of the titer plate after 30 minutes of shaking; the agglutinating activity was measured.

2.8 Cytotoxicity Assay

It was done by using brine shrimp (*Artemia salina*) nauplii hatched after 48 hours in saline water (1% NaCl). In the experiment, ten (10) different concentrations (25, 50, 75, 100, 125, 150, 175, 200, 225 and 250 μg/mL) from prepared stock solution (1 mg/mL) were taken in different ten (10) test tubes and made the total volume up to10 mL by adding 1% saline. After that, 20 pieces of hatched shrimps were taken in each test tube and kept at room temperature for observation. The live and dead shrimps were counted after 24 hours and the IC50 value was calculated.

2.9 Evaluation of Weight Loss

To evaluate the loss of weight, after inoculation of carcinoma cells, during treatment period weight of each mouse was measured by electric balance for six consecutive days. Finally, the rate of weight loss was calculated by using the average value for both control and treated mice as:

$$\text{The rate of weight loss} = \frac{\text{1st day weight} - \text{Last day weight}}{\text{Total day of treatment}} \text{ mg/day} \quad (2)$$

2.10 Cell Growth Inhibition

The cancer cell growth inhibition was conducted by a process described by Sur P. et al., [37]. To determine the cell growth inhibition, four groups (control, Bleomycin, root and leaf) of Swiss Albino mice (6 in each group) were used where 1.72 × 106 EAC cells per ml were inoculated in every mouse of each group. After 24 hours, 5.00 mg/kg/body weight root and leaf extract were treated for therapeutic evaluation and continued for six days. Mice in each group were sacrificed on day seven when the total intra-peritoneal EAC cells were isolated and diluted in normal saline (1% NaCl). First, viable cells were counted by haemocytometer using trypan blue stain as:

$$\text{Cells/mL} = \frac{\text{average count per square} \times \text{Dilution factor}}{\text{Depths of fluid under cover slip} \times \text{Area counted}} \quad (3)$$

Percentage of cell growth inhibition was calculated by comparing the total number of viable cells in the treated groups with control group as:

$$\text{Percentage of cell growth inhibition (\%)} = \frac{1 - Tw}{CW} \times 100\% \quad (4)$$

where Tw is mean of number of EAC cells of the treated group and Cw is mean of number of EAC cells of the control group.

2.11 Study on Apoptosis

These cells were identified through the morphological alteration during microscopic observation either by light or by fluorescence microscope [38]. In brief, EAC cells were collected from both control and treated mice after 6 days of treatment and the cells were washed for 2-3 times with phosphate buffer saline (PBS). Subsequently, the cells were stained with 1mg/1.6 mL of Hoechst-33342 solution (20 mM) dye at 37 °C for 20 min. Finally, the cells were washed with phosphate buffer saline (PBS) and resuspended in PBS for observation of morphological changes under fluorescence microscope.

3. Results and Discussion

3.1 Phytochemical Screening

The qualitative phytochemical constituents of root and leaf extract of *J. dolomiaea* are shown in Table 1. Analysis showed the presence of alkaloids, saponins, tannins, flavonoids, glycosides, steroids, phenolic compounds, and phytosterols. Proteins, carbohydrates, and fats were found in root in higher quantity than that of leaf. Glycosides, tannin, and phenolic compounds were present in higher amount in leaf more compared to root. The sign "+" and "–" represent the presence and absence of the particular compounds.

3.2 Antioxidant Activity

The antioxidant activity was evaluated by using DPPH free radical scavenging assay. DPPH free radical scavenging activity of root and leaf of *J. dolomiaea* along with standard BHT is shown in Fig. 1. The IC50 value of root and leaf of *J. dolomiaea* and standard BHT were calculated as 133.41

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µg/mL,140.23 µg/mL and 81.98 µg/mL respectively. The root extract showed the better DPPH free radicals scavenging activity than leaf extract when compared with standard BHT as shown in Fig. 2.

Table 1 Phytochemical screening of Root and Leaf extracts of *J. dolomiaea* Boiss

S. No.	Name of Compounds	Root	Leaf
01	Carbohydrates	+	+
02	Proteins	+	+
03	Fats	+	+
04	Glycosides	+	+
05	Alkaloids	+	+
06	Tannins	+	+
07	Phlobatannins	-	-
08	Flavonoids	+	+
09	Steroids	+	+
10	Saponins	+	+
11	Phenolic compounds	+	+
12	Phytosterols	+	+

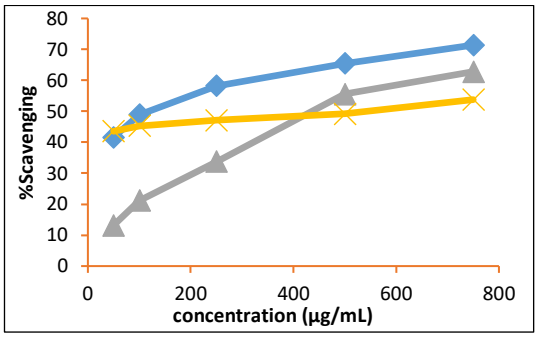

Fig. 1 The antioxidant activity assay of root and leaf extracts of *J. dolomiaea* along with standard BHT. (a)Scavenging activity of leaf and seed extracts along with standard BHT.

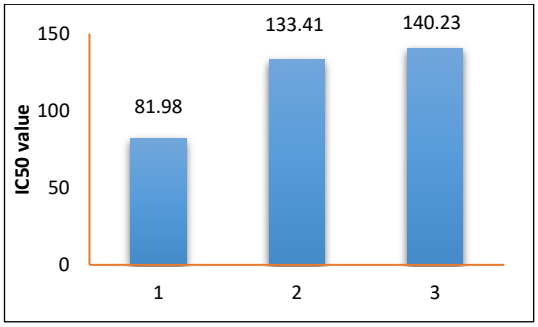

Fig. 2 Comparison of IC50 values of root and leaf extracts with standard BHT

3.3 Hemagglutination Activity

It was carried out to find the presence of lectin protein which has potent anticancer and growth inhibition activity. The hemagglutination activity of these extracts of *J. dolomiaea* is shown in Fig. 3. Both the extracts show the concentration dependent hemagglutination activity on goat blood. In the present study, the root extract is able to agglutinate 2.0% blood at minimum 12.5 µg/mL concentration whereas the minimum concentration of leaf extract was 50.0µg/ml to agglutinate goat blood. Therefore, the root extract is required in higher concentration than leaf extract to agglutinate goat blood which indicates that root extract contains more lectin protein than leaf extract.

Fig. 3 Hemagglutination activity of root and leaf of *J. dolomiaea* Root extracts showed better hemagglutination activity on goat blood when compared to control (without extract)

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3.4 Cytotoxicity Test

In cytotoxicity both root and leaf extract of *J. dolomiaea* showed excellent activity which indicates that they are biologically potent [39]. The 50% lethal concentration (LC50) values of each test sample were calculated from the corresponding regression equation and the LC50 values of root and leaf extracts were 155.84±6.69 µg/mL and 173.95±6.31 µg/mL respectively, that is shown in Fig. 4. The results evident that root and leaf extract of *J. dolomiaea* exhibit notable toxic effects against brine shrimp nauplii. Root extract showed the better cytotoxic activity than leaf extract (Fig. 5).

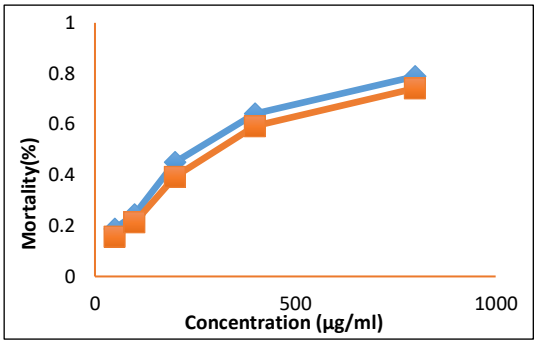

Fig. 4 Cytotoxic activity of root and leaf extract of *J. dolomiaea* Boiss

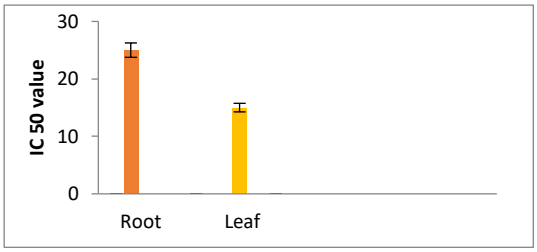

Fig. 5 Comparison of LC50 values of Root and Leaf extracts

3.5 Evaluation of Weight Loss

The unexpected weight loss is a common symptom of cancer. The experimental mice lose their weight gradually after inoculation of cancer cells. The rate of weight loss of control, root and leaf treated mice was evaluated. The rate of weight loss of root treated mice was less than that of leaf treated mice whereas control mice showed the highest rate of weight loss and is represented in Fig. 6.

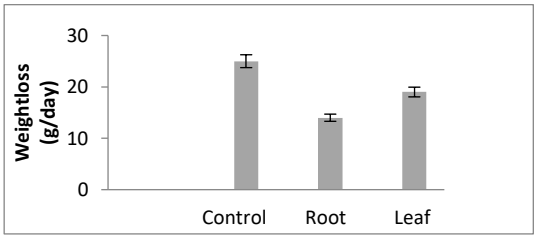

Fig. 6 Evaluation of weight loss of root and leaf extract in Swiss Albino mice

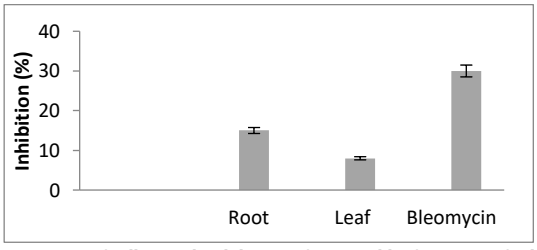

Fig. 7 Comparison of cell growth inhibition of root and leaf extracts of *J.dolomiaea* along with standard (Bleomycin)

3.6 Cell Growth Inhibition

It is widely used to determine the number of cells in a collective sample by using a hemocytometer which can easily separate the live cells from dead cell by using trypan blue dye exclusion [40]. The cell growth inhibitions of 5.00 mg/kg/day root and leaf extracts of *J. dolomiaea* are shown in Fig. 7. The percentage of cell growth inhibition of root and leaf extracts were calculated as 62.54±2.41%and 53.96±2.34 %, respectively, whereas inhibition by standard (Bleomycin) was 79.43±1.92%.

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Hemocytometer counting of EAC cells using trypan blue showed that viability of EAC cells decreases as compared to control. According to the data presented in Fig. 7, root extract shows higher growth inhibition than leaf extract.

3.7 Study on Apoptosis

Morphological changes of EAC cells collected from both control and treated mice were investigated under fluorescence and optical microscope was marked by arrow which is shown in Fig. 8. Under fluorescence microscope, round, regular and normal shaped nucleus were observed from control mice whereas irregular, fragmented and condensed nucleus were found in treated cells. Under optical microscope regular and normal shaped cells were seen in control mice and shrinkage and segmented cells were appeared in treated mice.

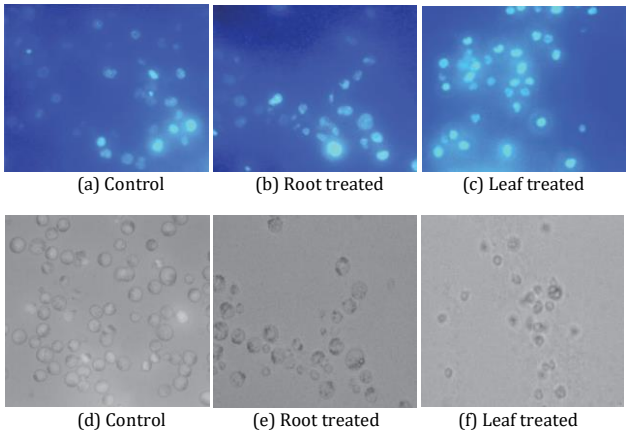

Fig. 8 Fluorescence and optical microscopic observation of EAC cells

4. Conclusion

Cancer is a devastating fatal disease all over the world and scientists are still trying to find out an effective way to combat this disease. This study showed that *J. dolomiaea* Boiss plant is a natural source of different bioactive phyto-nutrients having significant antioxidant, cytotoxic, hemagglutination, and growth inhibition activity against cancer cells. The current investigation provides solid evidence that the root and leaf extracts of *J. dolomiaea* contain significant anti-proliferative features with well-known massive health benefits.

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