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EVALUATION OF *IN VITRO* AND *IN VIVO* ANTICANCER ACTIVITY OF THE EXTRACTS OF *KEDROSTIS ROSTRATA*

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ABSTRACT

The present study was designed with the object of exploring the anticancer property of the extracts of aerial parts of *Kedrostis rostrata* by *in vitro* and *in vivo* methods. Initially, the petroleum ether, chloroform, ethyl acetate and methanol extracts were screened *in vitro* by MTT assay using MCF7 and SKMEL cell lines. The methanol extract showed a significant activity in the *in vitro* evaluation. Based on the results of *in vitro* evaluation, the methanol extract was selected for the *in vivo* evaluation on Swiss albino mice with tumour induced by Ehrlich ascites carcinoma cells. Standard drug 5-fluorouracil (20mg.kg-1) was used for the comparative evaluation. Initially, the methanol extract was subjected to toxicity evaluation. The results showed that the ED_{50} of the extract was 200mg.kg^{-1}. In the *in vivo* evaluation, two doses of extracts, 200 and 400mg.kg^{-1} were subjected to screening. Various aspects viz., clinical, hematological and biochemical parameters of the experimental animals were analyzed in the *in vivo* evaluation. The results showed that the animals treated with the methanol extract showed significant reduction in body weight comparing with tumor control animals. In case of average life span, the tumor control animals showed the life span of 47% whereas, animals treated with the methanol extract at the dose of 200 and 400mg.kg^{-1} showed the life span of 73% and 78% respectively. The reduction in the viable tumor cell count and changes in hematological and biochemical parameters also support the antitumor nature of the methanol extract of *K. rostrata*. From these studies, it is clear that further works needed to be done in the future for the development of clinically useful chemotherapeutic agents.

KEYWORDS: *Kedrostis rostrata* extracts, MTT assay, Swiss albino mice, Ehrlich ascites carcinoma cells.

INTRODUCTION

Since prehistoric period, plants has been used as the source of medicines for the treatment of ailments.[1-3] India's oldest, richest and most diverse cultural traditions associated with the use of medicinal plants is a well-known one.[4] Traditionally used plants for medicinal purposes contain wide range of active chemical constituents. Such constituents are termed as phytochemicals that includes alkaloids, glycosides, saponins, resins, oleoresins, sesquiterpene, lactones and oils, both essential and fixed.[1,5] Nowadays, the traditional herbal medicines getting significant consideration globally. Many hope that the traditional herbal medicine research will play a critical role in global health. Industry has also invested millions of US dollars looking for promising medicinal herbs and novel chemical compounds.[6,7]

The high demand for drugs from plant sources therefore requires systematic evaluation of plants used in traditional medicine for various ailments. Hence, it is necessary to evaluate medicinal plants for promising biological activity.[8,9] With this view, the plant *Kedrostis rostrata* was selected for our research. As a part of the investigation, in our previous effort, the aerial parts of the plant was extracted by soxhlation with the solvents of petroleum ether, chloroform, ethyl acetate and methanol. The extracts obtained were subjected to preliminary phytochemical evaluation and screened for antimicrobial activity.[5] Now the present study was designed to evaluate the anticancer activity of the extracts by *in vitro* and *in vivo* methods an attempt to provide a platform for further research.

MATERIALS AND METHODS

Preparation of plant extracts

The whole plant of *Kedrostis rostrata* was collected, identified and authenticated properly, the aerial parts of the plant was separated, dried, powdered and extracted with the solvents of increasing polarity viz., petroleum ether, chloroform, ethyl acetate and methanol in the soxhlet apparatus assembly. Each extract was filtered, distilled and the dried extract obtained was preserved for experiments.

***In vitro* anticancer activity**

Sub culturing and maintenance of cell line

In vitro anticancer activity was evaluated by MTT assay in accordance with the standard procedure [10-12] against MCF7 and SKMEL cell lines procured from NCCS, Pune, India. The procured cell lines were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat inactivated foetal bovine serum (FBS) and incubated at 37°C in a humidified atmosphere of 5% CO_2.

MTT assay

Cells were transferred in to 96-well flat bottom plates at the concentration of 1×10^4 cells/ml and incubated at 37°C in a humidified incubator (5% CO_2) for 24h followed by exposure to the extracts of *K. rostrata* (500µg/ml) for 48h. Then 20µl of MTT (3-[4,5-dimethyl thiazol-2yl]-2,5- diphenyl tetrazolium bromide) reagent dissolved in PBS (phosphate buffered saline, pH 7.4) was added to each well and mixed and incubated for an additional 4h. Subsequently, the supernatant was removed, 150µl DMSO (dimethyl sulphoxide) was added to each well for dissolving the MTT- formazan crystals. Finally absorbance was recorded at 540nm using a micro plate reader with DMSO as a blank and the percentage viability was calculated by

$$\text{Cell viability (\%)} = \frac{\text{Mean OD}}{\text{Control OD}} \text{ x } 100$$

***In vivo* anticancer activity**

Plant extract, animal and approval of the study

Based on the results obtained from the *in vitro* anticancer evaluation, the selected extracts were subjected to *in vivo* evaluation in reference with standard procedure [13] with slight modification. Healthy male Swiss albino mice having 6-8weeks age weighing 20-25gm obtained from the Central animal house, Cape Bio Lab & Research Centre, Marthandam, Kanyakumari, Tamil Nadu, India, was used for the experiments.The study was conducted as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The institutional animal ethical committee approved the study (CBLRC/IAEC/04/01-2020).

Acute toxicity study

The LD_{50} of the selected extracts was determined as per the guidelines of OECD 423 and in reference with the standard procedure.[14, 15] Required animals were randomly selected, marked facilitating identification, and kept in the standard environmental conditions like ambient temperature (25±1°C), relative humidity (55±5%) and 12h light/dark cycle for five days prior to dosing for adaptation with laboratory conditions. The animals were fed with standard pellet diet and water *ad libitum*. Following the overnight fasting with free access to water, the test extracts were administered in a single dose by gavage using stomach tube. 50mg.kg^{-1} was selected as initial dose and administered to three animals and after 24h, the animals were observed for the number of death. The same procedure was repeated with next doses 300mg.kg^{-1} and 2000mg.kg^{-1}.

Tumour cell line and cancer induction

Ehrlich ascites carcinoma cells obtained from the Amala cancer research centre, Thrissur, Kerala, India was used. The cell line is maintained by weekly intraperitoneal injection of tumour cell suspension of 106 cells/mouse. The carcinoma cells were aspirated from the peritoneal cavity of mice using saline. After the cell counting further dilutions were made to adjust the total cells to 1 x 106 cells/ml. Selected animals were randomly divided in to five groups of six each. Carcinoma cells (1x106 cells/ml) were administered intraperitoneally to all the animals in four groups and the remaining one group is normal control group. The tumour was allowed to grow in the mice for minimum seven days before starting treatments.

Treatment protocol

Group 1 served as normal control. Group 2 served as tumour control. Group1 and Group 2 received normal diet and water. Group 3 served as positive control was treated with injection 5-fluorouracil (20 mg.kg^{-1}, i.p.). Group 4 served as treatment control received 200mg.kg^{-1} of selected extract administrated orally. Group 5 served as treatment control received 400mg.kg^{-1}of selected extract administered orally.

Treatment

In this study drug treatment was given, once daily for 14 days. After the last dose all mice from each group were sacrificed and the blood was withdrawn from each mouse by retro orbital puncture bleeding and utilized for the analysis of clinical parameters such as cancer cell count, hematological parameters and serum enzyme and lipid profile and derived parameters.

DERIVED PARAMETERS

Body weight

All the mice were weighed, from the beginning to 15th day of the study. Average increase in body weight on the 15th day was determined.

Percentage increase in life span (ILS)

Survival time of treated groups were compared with those of control using the formula

$$\%\text{ILS} = \frac{\text{Life span of treated group}}{\text{Life span of control group}} - 1 \text{ x } 100$$

CLINICAL PARAMETERS

Cancer cell count

The ascetic fluid (0.1ml) from the peritoneal cavity of each mouse was withdrawn by sterile syringe and diluted with 0.8ml of ice cold normal saline and 0.1ml of tryphan blue (0.1mg /ml) and total number of the living cells were counted using hemocytometer.

Cell count = No. of cells x dilution / Area x thickness of liquid film

Hematological parameters

The collected blood was analyzed for WBC, RBC, hemoglobin, platelets count and packed cell volume. These investigations were carried out in COBAS MICROS OT 18 Roche, Switzerland.

Biochemical parameters

The serum was analyzed for aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), total cholesterol (TC) and triglyceride (TG) levels. All biochemical investigations were done by using COBAS MIRA PLUS – S auto analyzer Roche, Switzer land and MAX MAT.

RESULTS AND DISCUSSION

In the present study, the whole plant *Kedrostis rostrata* was collected and the aerials parts of the collected material was made into coarse powder after proper drying and extracted with solvents of increasing polarity viz., Petroleum ether, Chloroform, Ethyl acetate and Methanol. The results of the *in vitro* anticancer evaluation of the *K. rostrata* extracts by MTT assay is shown in Table 1 & 2. From the results it was found that the methanol extract of aerial parts of *K. rostrata* showed a significant activity in both MCF7 and SKMEL cell lines comparing with other tested extracts followed by the ethyl acetate extract showed a significant activity.

Table 1: *In vitro* anticancer evaluation of *K. rostrata* extracts by MTT assay in MCF7 cell line.

Extracts	Optical density	% Viability
Control	0.771	100
Petroleum ether	0.406	52.6
Chloroform	0.350	49.9
Ethyl acetate	0.285	36.9
Methanol	0.205	26.5

Table 2: *In vitro* anticancer evaluation of *K. rostrata* extracts by MTT assay in SKMEL cell line.

Extracts	Optical density	% Viability
Control	0.509	100
Petroleum ether	0.357	70.1
Chloroform	0.319	62.6
Ethyl acetate	0.283	55.5
Methanol	0.226	44.4

Based on the results obtained from the *in vitro* evaluation, the methanol extract was selected for the *in vivo* anticancer evaluation. In the acute toxicity evaluation, initially animal death was not observed after the administration of first two doses of the extract. But, two out of three animals died within the 24h of administration of third dose 2000mg.kg^{-1}. So, the third dose was concluded as LD_{50} and from this the ED_{50} was fixed as 200mg.kg^{-1} for the selected methanol extract of *K. rostrata*.

In the body weight analysis, the animals treated with the methanol extract showed a significant reduction in body weight comparing with tumour control animals. In case of average life span, the tumour control animals showed the life span of 47% whereas in group 4 and 5, animals treated with the methanol extract of *K. rostrata* at the dose of 200 and 400 mg.kg^{-1} body weight showed the life span of 73% and 78% respectively. The animal group treated with fluorouracil showed the life span of 94%. Regarding with cell count, the animals treated with methanol extract of *K. rostrata* showed a significant reduction in the viable tumor cell count comparing with tumour control group animals (Table 3).

Table 3: Effect of methanol extract of *K. rostrata* on the life span, body weight and cancer cell count.

Animal group	No. of animals	% ILS life span	Body weight (g)	Cell count (ml ×10^6)
G 1	6	>>30 Days	2.23±0.50	-
G 2	6	47	7.52±0.18^{a**}	2.48±0.65 ^{b**}
G 3	6	94	3.70±0.62 ^{b**}	1.30±0.10 ^{b**}
G 4	6	73	4.85±0.36 ^{b**}	1.75±0.46 ^{b**}
G 5	6	78	4.10±0.12 ^{b**}	1.68±2.11 ^{b**}

G_1 – Normal control; G_2 – Tumour control; G_3 – Positive control; G_4 – Treatment control (low dose); G5 – Treatment control (High dose). All values are expressed as mean ± SEM for 6 animals in each group.

^{a}** – Values are significantly different from control (G_1) at $P < 0.01$

^{b}** – Values are significantly different from cancer control (G_2) at $P < 0.01$

In the analysis of hematological parameters such as WBC, RBC and platelet count, hemoglobin level and packed cell volume, the results showed that the level of RBC, hemoglobin and platelets were decreased and WBC count was significantly increased in the tumour control group comparing with normal control group. The treatment control group received the methanol extract of *K. rostrata* at 200 and 400mg.kg^{-1} dose showed a significant increase in RBC, hemoglobin, packed cell volume and platelets and significant reduction in WBC count comparing with positive control group received fluorouracil, which showed a better result in all these parameters (Table 4).

Table 4: Effect of methanol extract of *K. rostrata* on hematological parameters.

Animal group	WBC (cells/ml×10^3)	RBC (Mill/cumm)	Haemoglobin (G/dl)	Packed cell volume (%)	Platelets (Lakhs/cumm)
G 1	10.25±1.32	4.55±0.20	12.60±1.18	30.31±3.50	3.20±0.65
G 2	13.22±2.15 ^{a**}	2.60±0.15 ^{a**}	7.24±0.65 ^{a**}	14.46±2.10 ^{a**}	1.60±0.70 ^{a**}
G 3	11.20±1.60 ^{b**}	4.05±0.85 ^{b**}	11.43±1.82 ^{b**}	24.33±2.51 ^{b**}	2.80±0.85 ^{b**}
G 4	12.10±1.45 ^{b**}	3.25±0.15 ^{b**}	10.45±1.30 ^{b**}	19.20±1.65 ^{b**}	2.15±0.22 ^{b**}
G 5	12.02±1.50 ^{b**}	3.10±0.20 ^{b**}	10.12±0.76 ^{b**}	21.76±1.10 ^{b**}	2.45±0.70 ^{b**}

G_1 – Normal control; G_2 – Tumor control; G_3 – Positive control; G_4 – Treatment control (low dose); G5 – Treatment control (High dose); All values are expressed as mean ± SEM for 6 animals in each group.
^{a}** – Values are significantly different from control (G_1) at $P < 0.01$
^{b}** – Values are significantly different from cancer control (G_2) at $P < 0.01$

The biochemical parameters such as total cholesterol, triglycerides, aspartate amino transferase, alanine transaminase and alkaline phosphatase are increased in tumor control group animals comparing with normal control group animals. The treatment with methanol extract of *K. rostrata* at a dose of 200 and 400mg.kg^{-1} body weight reversed these changes towards the normal level. The treatment with standard 5- FU also gave similar results (Table 5).

Table 5: Effect of methanol extract of *K. rostrata* on biochemical parameters.

Animal group	Cholesterol (mg/dl)	TGL (mg/dl)	AST (U/L)	ALT (U/L)	ALP (U/L)
G 1	112.15±3.55	134.55±2.60	41.40±1.10	34.35±1.60	127.15±2.30
G 2	144.82±4.50 ^{a**}	221.75±4.40 ^{a**}	87.30±2.67 ^{a**}	63.50±2.75 ^{a**}	242.30±4.20 ^{a**}
G 3	120.35±3.62 ^{b**}	162.40±2.30 ^{b**}	55.10±1.41 ^{b**}	42.40±1.60 ^{b**}	161.22±2.30 ^{b**}
G 4	125.15±3.55 ^{b**}	177.12±2.60 ^{b**}	67.50±1.80 ^{b**}	48.10±1.55 ^{b**}	192.50±2.12 ^{b**}
G 5	121.10±3.25 ^{b**}	169.70±2.13 ^{b**}	61.20±2.50 ^{b**}	46.35±1.81 ^{b**}	184.10±1.20 ^{b**}

G_1 – Normal control; G_2 – Tumor control; G_3 – Positive control; G_4 – Treatment control (low dose); G5 – Treatment control (High dose); All values are expressed as mean ± SEM for 6 animals in each group.
^{a}** – Values are significantly different from control (G_1) at $P < 0.01$
^{b}** – Values are significantly different from cancer control (G_2) at $P < 0.01$

Results of the present study supported the anticancer property of the methanol extract of *K. rostrata*. In our previous study[5], a preliminary level phytochemical analysis on these extracts was done which revealed the presence of variety of phytochemicals including alkaloids that maybe responsible for the activity currently found. It is worth to note here that the anticancer property of alkaloids was documented in previous literatures.[16]

CONCLUSION

In summary, the systematic evaluation of plants used in the traditional medicinal system is playa an important role in the novel drug development. With this view, the plant *Kedrostis rostrata* was selected for our research. As a part of the investigation, in our previous study, the aerial parts of the *Kedrostis rostrata* was extracted by solvents viz., petroleum ether, chloroform, ethyl acetate and methanol by soxhlation and the extracts obtained were subjected to preliminary phytochemical evaluation and screened for antimicrobial activity successfully. In the present study, the anticancer activity of these extracts were screened by MTT assay using MCF7 and SKMEL cell lines. From the results, it was found that the methanol extract of *K. rostrata* possessed a significant activity comparing with other tested extracts. Based on this result, the methanol extract was selected for *in vivo* anticancer evaluation in Swiss albino mice with tumour induced by Ehrlich ascites carcinoma cells. Standard drug 5-fluorouracil was used for the comparative evaluation. The results of *in vivo* evaluation also strongly supported the anticancer activity of the methanol extract of *K. rostrata*. From these studies, it is clear that further works needed to be done in the future for the development of clinically useful chemotherapeutic agents.

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