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IN VITRO AND IN VIVO ANTICANCER EVALUATION OF THE EXTRACTS OF CNIDOSCOLUS ACONITIFOLIUS

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ABSTRACT

Several studies were reported the therapeutic potential of the plants of genus *Cnidoscolus.* In our previous effort, a preliminary phytochemical evaluation and *in vitro* evaluation of antimicrobial activity of the different extracts of *C. aconitifolius* was done successfully. As continuation, in the present study, the *in vitro* anticancer evaluation of the petroleum ether, chloroform, ethyl acetate and methanol extracts of *C. aconitifolius* was done by MTT assay against MCF7 and HCT116 cell lines. The methanol extract revealed a significant activity in the *in vitro* evaluation which was selected for the *in vivo* anticancer evaluation on Swiss albino mice induced with Ehrlich ascites carcinoma cells. Two doses of methanol extract, 200 and 400 $mg.kg^{-1}$ fixed based on the acute toxicity study was employed for the *in vivo* evaluation. Standard drug, 5-fluorouracil (20 $mg.kg^{-1}$) was used for the comparative evaluation. Several parameters such as changes in the body weight of the tested animals, their average life span, cancer cell count, various hematological parameters such as WBC, RBC, hemoglobin, platelet count and packed cell volume and biochemical parameters such as aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), total cholesterol (TC) and triglyceride (TG) levels were analyzed in the *in vivo* evaluation. The methanol extract (400 $mg.kg^{-1}$) showed significant activity in all these tested parameters comparing the standard drug, 5-fluorouracil. Results of the present study supported the anticancer property of the methanol extract of *C. aconitifolius.* In our previous study, the preliminary level phytochemical analysis done on these extracts revealed the presence of variety of phytochemicals including alkaloids that maybe responsible for the activity currently found. Further studies with focus on the compound level studies and toxicity analysis in the future may give more significant results which are useful for the development of novel chemotherapeutic agents.

KEYWORDS: *Cnidoscolus aconitifolius* extracts, MTT assay, *In vivo* anticancer activity, Swiss albino mice.

INTRODUCTION

Globally, plants have been widely used as the source of medicines for the treatment of diverse ailments and disorders since prehistoric period.[1-3] The herbal oriented treatment strategies of different traditional healing practices plays an important role in the search for novel therapeutics and it inspire and guided the researchers.[2,4] Many hope that the research on traditional herbal medicines will play a critical role in global healthcare. Industry has also invested millions of US dollars looking for promising novel chemical compounds from medicinal herbs.[5] Certain recent reports stated that approximately two-thirds of the medicines approved globally are plant originated.[6] The active chemical constituents otherwise known as secondary metabolites are termed as phytochemicals are responsible for their definite physiological activity which include alkaloids, glycosides, flavonoids, essential oils, saponins, resins, phenols etc. They are chemically distinct compounds present in the different parts of the plants viz., root, stem, flower, fruit, seed and exudates. They can be used to treat different types of chronic as well as infectious diseases.[7-10]

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] With this view the plant *Cnidoscolus aconitifolius* was selected for our research. As a part of the investigation, in our previous effort, the aerial parts of the plant was extracted. The extracts thus obtained were subjected to preliminary phytochemical evaluation and screened for antimicrobial activity.[9] Now the present study was aimed 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

Plant extract

In our previous effort,[9] the aerial parts of the plant, *C. aconitifolius* was collected, authenticated, dried in shade and made into a coarse powder by using mechanical grinder. Extraction of powdered material was done by soxhlation using the different solvents of increasing polarity viz., petroleum ether, chloroform, ethyl acetate and methanol. The extracts thus obtained were preserved for further studies.

In vitro anticancer evaluation – MTT assay

Sub culturing and maintenance of cell line

In vitro anticancer activity was evaluated by MTT assay against MCF7 and HCT116 cell line in reference with the standard procedure.[11-13] The cell lines were procured from NCCS, Pune, India. They were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with heat inactivated foetal bovine serum (10%) and incubated (37°C; 5% CO_2).

MTT assay

Cells (1×10^4 cells/ml) were transferred to 96-well flat bottom plates and incubated in humidified incubator (5% CO_2) at 37°C for 24h, then exposed to the extracts of *Cnidoscolus aconitifolius* (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 evaluation

Plant extract, Animal and Approval of the study

Based on the results obtained from the *in vitro* anticancer evaluation, the selected extract were subjected to *in vivo* evaluation in reference with standard procedure.[14] 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/10/01-2020).

Acute toxicity study

The LD_{50} of the selected extract was determined as per the guidelines of OECD 423 and in reference with the standard procedure.[15,16] 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 extract was 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, Switzerland and MAX MAT.

RESULTS AND DISCUSSION

***In vitro* anticancer evaluation**

In vitro anticancer evaluation of the *C. aconitifolius* extracts by MTT assay was done on MCF7 and HCT116 cell lines. Effect of *C. aconitifolius* extracts on MCF7 cell line is presented in Table 1 and Figure 1. In this evaluation, the petroleum ether extract showed 62.2% cell viability, while the chloroform extract showed 50.9% cell viability. In case of ethyl acetate extract, it showed 39.3% and the methanol extract showed 28.8%, a significant score comparing with other tested extracts.

Table 1: Evaluation of *C. aconitifolius* extracts by MTT assay in MCF7 cell line

Extracts	Optical density	% Viability
Control	0.683	100
Petroleum ether	0.425	62.2
Chloroform	0.348	50.9
Ethyl acetate	0.269	39.3
Methanol	0.197	28.8

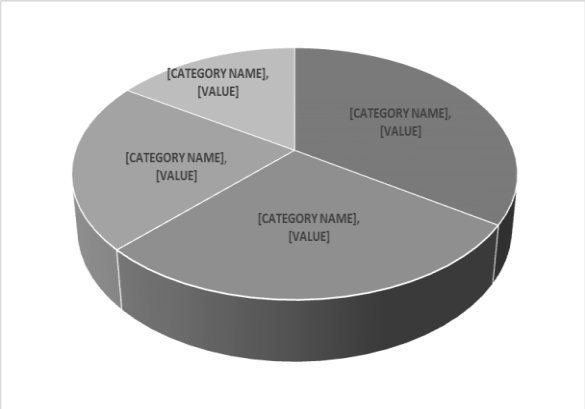

Figure 1: Effect of *C. aconitifolius* extracts on the cell viability in MCF7 cell line.

The results obtained in the analysis of effect of *C. aconitifolius* extracts on HCT116 cell line is presented in Table 2 and Figure 2. In this analysis, the petroleum ether extract showed 60.1% cell viability, while the chloroform extract showed 49.9% cell viability. In case of ethyl acetate extract, it showed 39.3% and the methanol extract showed 28.7%, a significant score comparing with other tested extracts.

Table 2: Evaluation of *C. aconitifolius* extracts by MTT assay in HCT116 cell line.

Extracts	Optical density	% Viability
Control	0.547	100
Petroleum ether	0.329	60.1
Chloroform	0.273	49.9
Ethyl acetate	0.215	39.3
Methanol	0.157	28.7

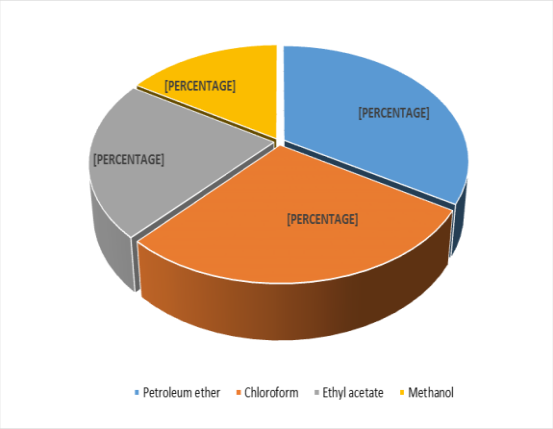

Figure 2: Effect of *C. aconitifolius* extracts on the cell viability in HCT116 cell line.

***In vivo* anticancer evaluation**

Collectively, from the results it was found that the methanol extract of *C. aconitifolius* showed a significant activity in both MCF7 and HCT116 cell lines comparing with other tested extracts followed by the ethyl acetate extract showed a significant activity. 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 *C. aconitifolius*.

In the body weight analysis, the animals treated with the methanol extract showed a significant gain in body weight comparing with tumour control animals. In case of average life span, the tumour control animals showed the life span of 45% whereas in group 4 and 5, animals treated with the methanol extract of *C. aconitifolius* at the dose of 200 and 400 mg.kg^{-1} body weight showed the life span of 67% and 83% respectively. The animal group treated with fluorouracil showed the life span of 92%. Regarding with cell count, the animals treated with methanol extract of *C. aconitifolius* 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 *C. aconitifolius* 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	2.25±0.30	-
G 2	6	45	8.12±0.58^{a**}	2.38±0.15 ^{b**}
G 3	6	92	4.20±0.62 ^{b**}	1.20±0.60 ^{b**}
G 4	6	67	5.95±0.16 ^{b**}	1.63±1.42 ^{b**}
G 5	6	83	5.13±0.11 ^{b**}	1.55±0.10 ^{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 *C. aconitifolius* 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 & Figure 3).

Table 4: Effect of methanol extract of *C. aconitifolius* on hematological parameters.

Animal group	WBC (cells/ml×10^3)	RBC (Mill/cumm)	Haemoglobin (G/dl)	Packed cell volume (%)	Platelets (Lakhs/cumm)
G 1	12.15±1.02	4.15±0.10	11.50±1.08	29.11±3.40	4.20±0.55
G 2	16.32±2.55 ^{a**}	2.20±0.35 ^{a**}	6.14±0.55 ^{a**}	15.26±2.30 ^{a**}	1.30±0.60 ^{a**}
G 3	13.10±1.40 ^{b**}	4.02±0.25 ^{b**}	11.04±1.72 ^{b**}	25.43±2.55 ^{b**}	2.90±0.75 ^{b**}
G 4	14.23±1.25 ^{b**}	3.16±0.45 ^{b**}	10.13±1.20 ^{b**}	18.10±1.35 ^{b**}	2.10±0.12 ^{b**}
G 5	14.02±1.30 ^{b**}	3.55±0.50 ^{b**}	10.40±0.56 ^{b**}	22.36±1.60 ^{b**}	2.50±0.60 ^{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 $P < 0.01$

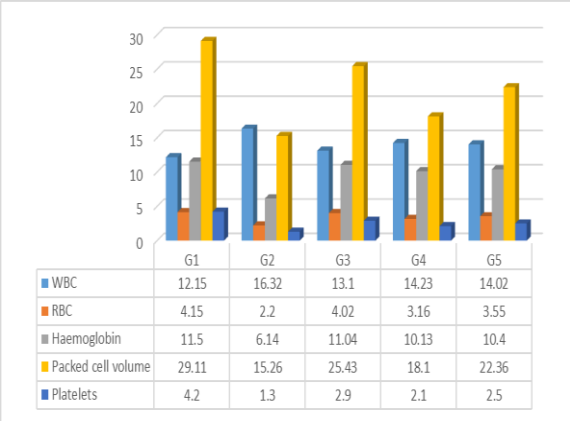

Figure 3: Effect of test Extract and Standard on the hematological profile of experimental animals.

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 *C. aconitifolius* 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 & Figure 4).

Table 5: Effect of methanol extract of *C. aconitifolius* on biochemical parameters.

Animal group	Cholesterol (mg/dl)	TGL (mg/dl)	AST (U/L)	ALT (U/L)	ALP (U/L)
G 1	110.14±2.20	136.25±5.10	43.10±2.09	35.55±1.20	125.05±2.10
G 2	145.32±5.10 [a**]	226.65±3.20 [a**]	85.40±2.57 [a**]	65.10±2.55 [a**]	240.55±3.20 [a**]
G 3	117.45±1.32 [b**]	160.10±1.50 [b**]	57.20±1.21 [b**]	42.12±1.60 [b**]	163.02±2.50 [b**]
G 4	124.05±1.25 [b**]	175.32±2.10 [b**]	65.30±1.70 [b**]	51.30±1.05 [b**]	190.10±2.42 [b**]
G 5	120.40±2.15 [b**]	167.10±2.23 [b**]	62.25±2.10 [b**]	49.25±1.31 [b**]	185.20±1.30 [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 $P < 0.01$

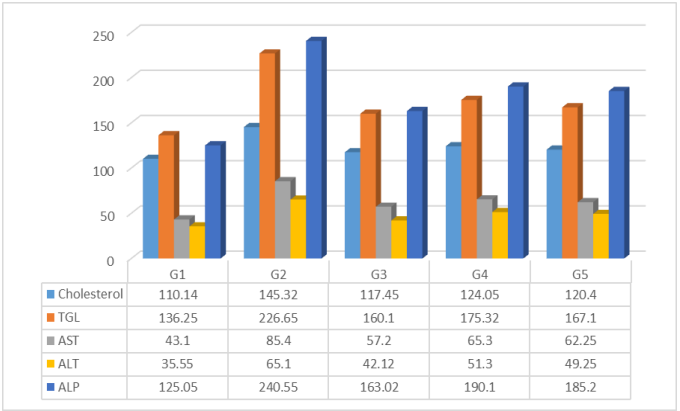

Figure 4: Effect of test Extract and Standard on the biochemical profile of experimental animals.

Results of the present study supported the anticancer property of the methanol extract of *C. aconitifolius*. In the preliminary level phytochemical analysis on these extracts was done in our previous study[9] 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.[17]

CONCLUSION

In the present study, the *in vitro* anticancer evaluation of the petroleum ether, chloroform, ethyl acetate and methanol extracts of *C. aconitifolius* was done by MTT assay against MCF7 and HCT116 cell lines. Comparing with other tested extracts, the methanol extract revealed a significant activity in the *in vitro* evaluation which was selected for the *in vivo* anticancer evaluation on Swiss albino mice induced with Ehrlich ascites carcinoma cells. In the acute toxicity study, 200mg.kg^{-1} of the methanol extract was fixed as ED_{50}. Two doses 200 and 400 mg.kg^{-1} of methanol extract was employed for the evaluation of anticancer activity. Standard drug, 5-fluorouracil (20 mg.kg^{-1}) was used for the comparative evaluation. Several parameters such as changes in the body weight of the tested animals, their average life span, cancer cell count, various hematological parameters such as WBC, RBC, hemoglobin, platelet count and packed cell volume and biochemical parameters such as aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), total cholesterol (TC) and triglyceride (TG) levels were analyzed in the *in vivo* evaluation. The methanol extract (400 mg.kg^{-1}) showed significant activity in all these tested parameters comparing the standard drug, 5-fluorouracil. Results of the present study supported the anticancer property of the methanol extract of *C. aconitifolius.* In our previous study, the preliminary level phytochemical analysis done on these extracts revealed the presence of variety of phytochemicals including alkaloids that maybe responsible for the activity currently found. Further studies with focus on the compound level studies and toxicity analysis in the future may give more significant results which are useful for the development of novel chemotherapeutic agents.

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