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Research Article

Anti-Tumour Activity of *Limonia acidissima* L. Methanolic Extract in Mice Model of Dalton's Ascitic Lymphoma

Jagadeesh Reddy Eluru[1*], Dr. Taranalli A D[2], Simmy Kawatra[3]

[1]*Department of Clinical Research and Development, Shantha Biotechnics Private Limited, Hyderabad – 500004, Telangana, India.*
[2]*Department of Pharmacology, KLE University's College of Pharmacy, KLE University, Belgaum – 590010, Karnataka, India.*
[3]*Department of Pharmacology, Central Council for Research in Ayurvedic Sciences, New Delhi - 110058, New Delhi, India.*

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ABSTRACT
Ripe fruits of *Limonia acidissima* L. (Rutacae) were being used by traditional practitioners to treat diarrhoea, dysentery, tumours, asthma, vomiting, ulcers, and cardiac debility without scientific rationale. In order to provide the scientific basis for the antitumor activity, methanolic extract of *Limonia acidissima* L. (LAME) fruits was administered orally at a dose of 570mg/kg in the present study against mice model of Dalton's Ascitic Lymphoma (DAL). *In vitro* cell cytotoxicity, *Solid tumour* and *Liquid tumour* models of DAL were used in this study to assess the anti-tumour activity. Depending on the parameters to be assessed *Liquid tumour* model was sub divided into *group A* and *group B*. Depending on the treatments to be received, animals in the *Solid tumour*, the *group A* and *group B* of *Liquid tumour model*s were categorized into 4 groups consisting of six animals each i.e. *Normal, Tumour Bearing, Tumour Bearing + 5-FU, Tumour Bearing + LAME*. *Solid tumour* model was observed for solid tumour mass and Percentage increase in solid tumour (%IST). Post treatment changes in body weight, percentage increase in body weight, mean survival time (MST) and percentage increase in life span (%ILS) in group A; whereas tumour volume, tumour cell growth, haematological parameters, serological parameters and liver antioxidants in group B were observed. *Tumour Bearing + LAME* groups showed significant ($P<0.05$) positive changes with respect to all parameters among all the groups in comparison to *Tumour Bearing* group.

Key Words: Tumour, *Limonia acidissima*, Solid tumour, Liquid tumour, Dalton's Ascitic Lymphoma.

INTRODUCTION

A tumour or tumor is the name for a neoplasm or a solid lesion formed by an abnormal growth of cells (termed neoplastic) which looks like a swelling. Cancer (medical term: malignant neoplasm) is a class of diseases in which a group of cells display uncontrolled growth, invasion that intrudes upon destroys adjacent tissues, sometimes metastasise to other locations in the body via lymph or blood. Lymphoma is a cancer in the lymphatic cells. Typically, lymphomas present as a solid tumours[1].

The prevalence of lymphoma is on the rise and posing a serious health concern. In 2010 an estimated malignant Hodgkin's, Non-Hodgkin's lymphoma (NHL) cases were 62,000 and 2, 90,000 respectively[2]. Approximately 60,000 and 7,000 subjects are diagnosed with NHLs and Hodgkin's disease in the United States annually. Hodgkin's disease accounts for only one percent whereas, NHL represent four percent of all cancers in the United States[3]. The Indian scenario is not clearly known.

Lymphoma treatment involves chemotherapy, radiotherapy and bone marrow transplantation. It can be curable depending on the histology type and stage of the disease. The treatment programmes are costly, intense, technically demanding and associated with considerable secondary complications. Long-term toxicities with standarrd chemotherapy regimens have been well documented[4].

The secondary complications and high cost involved in the treatment of lymphoma fostered our attempts to evaluate the effect of plant products against it, as they are less likely to cause serious side effects. India is a rich source of medicinal plants and a number of plant extracts have been used in various systems of medicines such as Ayurveda, Sidda, Unani, etc. to cure multiple ailments. Many Indian plants like black pepper, asafoetida, pippali and garlic are quoted to be useful in different types of cancer[5,6]. One such plant is "*Limonia acidissima* L." belongs to family Rutacae is well known for its astringent, appetizing and stimulant properties[7-9]. Traditionally it was used for treating tumours without scientific rationale[7]. The main constituents reported are benzamide derivatives, coumarins and flavonoids[10-12]. Plants containing flavonoids and antioxidant principles are constantly being screened for antitumor activity. It

**Author for Correspondence*

Table 1: Experimental Design

Group	S.No	Category	Treatment (Daily)	Description	Parameters measured
Solid tumour	I	Normal	0.9% Saline, p.o.[#]	Treatments were given for 10 days and sacrificed on day 11 using ether anaesthesia	Solid Tumour
	II	Tumour Bearing	0.9% Saline, p.o.[#]		
	III	Tumour Bearing + 5-FU*	5-FU 20mg/Kg I.P.[$]		
	IV	Tumour Bearing + LAME[$$]	LAME, p.o.[#] 570 mg/kg		
Liquid tumour, Group A	I	Normal	0.9% Saline, p.o.[#]	Treatment were given for 10 days and observed for 40 days.	Body Weight Analysis % increase in body weight Mean survival time (MST) % increase in lifespan (% ILS)
	II	Tumour Bearing	0.9% Saline, p.o.[#]		
	III	Tumour Bearing + 5-FU*	5-FU 20mg/Kg I.P.[$]		
	IV	Tumour Bearing + LAME[$$]	LAME, p.o.[#] 570 mg/kg		
Liquid tumour, Group B	I	Normal	0.9% Saline, p.o.[#]	Treatments were given for 10 days; on the day 14 all the animals were sacrificed.	Tumour volume Tumour cell growth Haematological parameters
	II	Tumour Bearing	0.9% Saline, p.o.[#]		
	III	Tumour Bearing + 5-FU*	5-FU 20mg/Kg I.P.[$]		
	IV	Tumour Bearing + LAME[$$]	LAME, p.o.[#] 570 mg/kg		

#: p.o. – per oral; $: I.P. – intraperitoneal; *: 5-FU- 5-Fluoro Uracil; $$: LAME – *Limonia acidissima* L. methanolic extract

Table 2: Preliminary Phytochemical analysis of Methanolic extract of *Limonia acidissima* L. ripe fruits

Phytochemical constituents	Methanolic extract of *Limonia acidissima*
Carbohydrates	+
Proteins	-
Steroids	+
Flavonoids	+
Alkaloids	-
Tannins	+
Glycosides	+
Saponins	+
Amino acids	-
Acidic compounds	+

Table 3: Effect of fruits of LAME on *In vitro* cell cytotoxicity

Concentration (µg/ml)	Percentage Dead Cells	
	5-FU	LAME
10	35±2.21	17±1.32[ns]
100	64±1.56	32±4.25[ns]
1000	80±4.35	47±3.96[ns]

possesses diuretic, antifungal, wound healing, antioxidant, hepatoprotective, and antidiabetic properties[13-17]. In previous studies methanolic extract of fruits of *Limonia acidissima* L., was used to prove most of the reported activities. Based on that, the present study was planned to evaluate the anti-tumour activity of methanolic extract of *Limonia acidissima* L. fruits against mice model of Dalton's Ascitic Lymphoma.

MATERIALS AND METHODS

Plant material and sample extracts and Preliminary phytochemical analysis

The fruits of *Limonia acidissima* L. were collected from vidavalur village, nellore district, andhra pradesh, India. They were identified and authenticated by Dr. Harsha hegde, scientist B, regional medical research centre (RMRC), Indian council of medical research (ICMR), belgaum (Voucher specimen no. RMRC-552). The voucher specimens were preserved in the Department of Pharmacology, K.L.E university's College of Pharmacy, Belgaum for future reference. The ripe fruits were shade dried; pulp was taken out and coarsely powdered. The coarsely powdered fruit pulp was defatted with hexane using soxhlet apparatus (approx. 8 hrs). The defatted marc was pressed, dried and further subjected to methanolic extraction (approx. 18 hrs). The obtained extract was concentrated in a rotary flash evaporator and subjected to qualitative phytochemical analysis.

Selection of the dose

The LD_{50} value of the methanolic extract has been reported 2000 mg/kg and 400 mg/kg dose was found to be effective in hepatoprotective, antioxidant and wound healing activities in rats[15,16]. Hence 570 mg/kg was selected for the assessment of antitumor activity against Dalton's ascitic lymphoma in mice (according to dose conversion from rat to mice)[18]. *Chemicals and Materials*

Tryphan Blue was obtained from Crest Biosystems, Goa, India. Adrucil inj. was used as a source for 5-FU. All other chemicals used in the experiment were of analytical grade. Female Swiss albino mice weighing 20-25 g were procured form sri venkateshwara enterprises, bangalore. DAL (Dalton's Ascitic Lymphoma) cells were procured from Amala Cancer Research Institute, Thrissur, Kerala.

Dalton's ascitic lymphoma (DAL) cells

Table 4: *Solid tumor model* - Effect of Fruits of LAME on Solid Tumour & Percentage Increase in Solid Tumor (% IST)

Group	Solid Tumour (mm^3)				Percentage increase in solid tumour (%IST)		
	0 day	15th day	20th day	25th day	15th day	20th day	25th day
Normal	97.08±5.34	122.8±8.27	139.8±7.69	169.2±11.16	26.49± 8.22 a*	13.80± 6.27 a*	21.03± 8.37 a*
Tumour Bearing	100.1±5.04	150.2±7.56	255.3±12.8	382.9±19.29	62.50± 10.54	83.04± 8.732	60.69± 11.45
Tumour Bearing + 5-FU$$	101.5±4.94	131.9±6.42	171.5±8.35	222.9±10.86	35.30± 18.32 a*	35.22± 12.36 a*	34.98± 16.34 a*
Tumour Bearing + LAME$	95.27±5.56	133.4±7.79	186.7±10.91	284.7±17.94	51.69± 16.47 a*b*	45.33± 8.65a*b*	47.32± 15.35 a*b*

All the values were expressed as Mean ± SEM using one way ANOVA followed by Newman-Keuls Multiple range test, where n=6

a-when compared with Tumour Bearing; b- when compared with Tumour Bearing + 5-FU

*-P<0.05; $: 5-FU- 5-Fluoro Uracil; $$: LAME – *Limonia acidissima* L. methanolic extract

Dalton's Ascitic Lymphoma (DAL) originally derived from DBA/2 mice by serial transplantation[19]. DAL is reported to be a transplantable T-cell lymphoma that can be induced through ascitic fluid[20]. Following transplantation of DL ascites cells (AC) into the abdominal cavity of healthy mice, tumour genesis will start immediately. The recipient or transformed mice usually survive up to 2–3 weeks. Dalton's lymphoma (DL) ascites tumour genesis is a convenient model to study antitumor effects within a short time.

Experimental animals

The female Swiss albino mice weighing 20-25 g were housed in sterile polypropylene cages with a stainless steel grill on top with bedding of clean paddy husk, at ambient temperature, humidity with 12 hour light dark cycle. The mice were kept on *ad libitum* feed and water. The experimental protocol was approved by the institutional animal ethics committee (IAEC Reg No. 221/CPCSEA) Belgaum. All the experiments were conducted in strict adherence to the protocol and are compliant with the ethical principles and guidelines provided by committee for the purpose of control supervision of experiments on animals (CPCSEA).

LAME test extracts

Test extract was prepared in a concentration of 57 mg/ml using distilled water.

Dose administration

LAME and saline were administered orally to the animals through 22 G, 1/2" curved, ball ended stainless steel needle. 5-FU was administered through intraperitoneal route using 26 G, 1/2" stainless steel needle.

Blood withdrawal

Blood was collected through Retro-orbital puncture in heparinized tubes.

Withdrawal of DAL cells from peritoneal cavity

0.3 ml of PBS was taken in 1 ml syringe which is attached with 24 G needle and gently inserted into the peritoneal cavity of DAL mice. More than 0.2ml (< 0.3 ml) of the PBS was injected into the peritoneal cavity and peritoneal fluid was collected by withdrawing the syringe plunger towards back. These cells were subjected to washing, where 0.2 ml of peritoneal fluid and 0.5 ml of ice cold PBS were taken in a 2 ml eppendorf tube, centrifuged for 10 min. at 2000xg. The resultant supernatant liquid was removed and the procedure was repeated for 3 times.

Counting of DAL cells

At the end of third centrifugation, supernatant liquid was removed and the sediment was subjected to cell count using WBC counting method. The sediment was adjusted to the concentration of 10^6 cells/0.2 ml using ice cold PBS depending on the DAL cell count.

Tumour Induction

0.2 ml of (10^6 DAL cells/0.2 ml) peritoneal fluid was injected intraperitoneally or into the thigh muscle to induce liquid and solid tumours respectively and that day was considered as day 0. Tumour was induced to the animals belongs to *Tumour Bearing, Tumour Bearing + 5-FU, Tumour Bearing + LAME* category.

Experimental design

We used 2 major models namely *Solid tumour* model (n=24) and *Liquid tumour* model. As the parameters measured in liquid tumour necessitates the need of 2 sub groups, we kept two sub groups named *group A* and *group B.* Animals in the *Solid tumour,* the *group A* and *group B* of *Liquid tumour model*s were categorized into 4 groups containing six animals each, depending on the treatments to be received i.e. *Normal, Tumour Bearing, Tumour Bearing + 5-FU, Tumour Bearing + LAME*. Experimental design of each group was described in Table 1.

Parameters measured

In vitro cell cytotoxicity[21]

We performed *in vitro* cell cytotoxicity using trypan blue dye exclusion test, where only dead cells will take up the dye due to lack of intact membranes. In this procedure 0.2 ml of DAL cells (10^6 cells/ 0.2 ml), 1 ml of ice cold PBS (phosphate buffer saline pH 7.4) and 0.2 ml of extracts ranging from 10 to 1000 µg/ml (10, 100 & 1000 µg/ml) were taken in a eppendorf tube. The contents were

Table 5: *Liquid tumor, Group A* - Effect of Fruits of LAME on Body Weight, Percentage Increase in Body Weight (% IBW), mean survival time (MST) and percentage increase in life span (% ILS)

Group	Body Weight (g)				Percentage Increase In Body Weight (%)			Mean Survival Time (MST) (Days)	Percentage increase in life span (% ILS)
	0 day	5th day	10th day	15th day	By 5th day	By 10th day	By 15th day		
Normal	20.75± 0.31	22.88 ±0.44	25.13± 0.29	27.00 ± 0.37	10.23± 1.4 a*	9.93± 1.21 a*	7.11± 0.78 a*	39.0± 1.0 a*	>>40 days
Tumour Bearing	21.25± 0.36	24.38 ±0.46	28.13± 0.44	35.13 ± 0.44	14.75± 1.74	14.96± 0.78	23.42± 3.40	18.75± 0.49	-
Tumour Bearing + 5-FU$$	22.38± 0.59	26.13 ±0.58	28.00± 0.46	31.75 ± 0.36	16.87± 1.13 ans	7.26± 1.22 a*	14.74± 2.31 a*	28.88± 0.66 a*	54.0± 1.01 a*
Tumour Bearing + LAME$	21.75± 0.31	25.38 ±0.41	28.38± 0.41	33.63 ± 0.49	16.63± 1.25 ans bns	17.85± 0.83 a* b*	18.5± 1.17 ans bns	25.0± 0.53 a*b*	33.33± 1.05 a* b*

All the values were expressed as Mean ± SEM using one way ANOVA followed by Newman-Keuls Multiple range test, where n=6

a-when compared with Tumour Bearing; b- when compared with Tumour Bearing + 5-FU

*-P<0.05 ; ns-no significance; $: 5-FU- 5-Fluoro Uracil; $$: LAME – *Limonia acidissima* L. methanolic extract

subjected to incubation in CO_2 incubator for 3 hours at 37^0C with continuous flow of 5% CO_2. Then 0.2 ml of previous mixture, 0.3 ml of ice cold PBS and 0.5 ml of trypan blue solution (0.4% in normal saline) were taken in a eppendorf tube and kept aside for 5 to 15 min. at room temperature. Then % dead cells were calculated with the using neubauer chamber (Same as WBC counting method). 5-FU was taken as a standard drug in the concentration range of 10 to 1000 µg/ml (10, 100 & 1000 µg/ml). All the procedures were done in triplicate.

Solid tumour[22]

Solid tumour was measured on every 5th day for a period of 25 days starting from the 15th day of tumour induction.

Percentage increase in solid tumour (%IST)

The %IST was calculated using following formula.

$$\%IST = \frac{\text{present solid tumour – previous solid tumour x 100}}{\text{previous solid tumour}}$$

Body Weight

Body weights of all the animals were recorded from day 0 to day 15 for 3 times with 5 day intervals.

Percentage increase in body weight

From the recorded body weights, % increase in body weight for every 5days was calculated using the following formula.

$$\text{Percentage increase in body weight} = \frac{\text{present body weight – previous body weight x 100}}{\text{previous body weight}}$$

Mean survival time (MST)[23]

All the animals were observed for their survival time from day 0 to day 40. MST was calculated using the following formula.

$$MST = \frac{1^{st} \text{ death} + \text{last death}}{2}$$

Percentage increase in lifespan (% ILS)

From the recorded MST, % ILS was calculated using the following formula.

$$\%\ ILS = \frac{\text{MST of respective group - MST of tumour bearing group x 100}}{\text{MST of tumour bearing group}}$$

Haematological parameters

RBC, WBC, Haemoglobin, and Leucocyte counts were estimated using standard methods.

Tumour volume[24]

Peritoneal fluid was withdrawn from the tumour induced animals using 5 ml syringe which was attached with 24 G needle. The withdrawn peritoneal fluid was measured and represented as tumour volume.

Tumour cell growth[25]

0.2 ml of peritoneal fluid was taken from the pool of peritoneal fluid, which was used for tumour volume measurement and subjected to washing followed by trypan blue dye exclusion test as described previously. The live cell counts were recorded and represented as Tumour cell growth.

Statistical Analysis

Results were expressed as mean ± SEM. Differences among data were determined using one way ANOVA followed by Newman-keuls Multiple Comparison Test (Graph Pad Prism software, version V) except for *in vitro* cell cytotoxicity, where independent "t" test has been used[26]. $P \leq 0.05$ was considered significant.

RESULTS AND DISCUSSION

Plant material and extraction

The yield of the Methanolic extracts of *Limonia acidissima* ripe fruits was found to be 23 % w/w.

Preliminary phytochemical studies

Preliminary Phytochemical studies of Methanolic extracts of *Limonia acidissima* ripe fruits reveal the presence of steroids, flavonoids, tannins, glycosides, saponins and

Table 6: *Liquid tumor, Group B* - Effect of Fruits of LAME on Tumour volume, Tumour cell growth and Haematological parameters

Group	Tumour Volume (ml)	Tumour Cell Growth (cells/ml x10^6)	Haematological parameters RBC (cells/cmmx10^6)	WBC (cells/cmmx10^3)	Haemoglobin (g%)
Normal	-	-	9.909±0.054 a*	7.875±0.1161 a*	14.26±0.092 a*
Tumour Bearing	17.53±0.21	37.40±0.82	6.863±0.070	29.88±0.4135	10.26±0.088
Tumour Bearing + 5-FU$^{\$\$}$	7.4±0.24 a*	16.17±0.66 a*	8.649±0.076 a*	10.60±0.4247 a*	12.14±0.059 a*
Tumour Bearing + LAME$^{\$}$	15.06±0.11 a*b*	18.21±0.22 a*b*	7.968±0.22 a*b*	19.43±0.6050 a*b*	11.29±0.148 a*b*

All the values were expressed as Mean ± SEM using one way ANOVA followed by Newman-Keuls Multiple range test, where n=6

a-when compared with Tumour Bearing; b- when compared with Tumour Bearing + 5-FU

*-P<0.05; $: 5-FU- 5-Fluoro Uracil; $$: LAME – *Limonia acidissima* L. methanolic extract

acidic compounds. The results obtained are shown in Table 2.

Pharmacological Investigations

The major criteria to be taken into consideration for any potential anticancer drug are its efficacy in prolongation of lifespan, decrease of tumour volume and viable tumour cell count, and disappearance of leukemic cells from blood. LAME showed significant ($P \leq 0.05$) positive changes in all the parameters when compared with *Tumour Bearing* group whereas 5-FU showed significant antitumor activity than LAME fruits.

In vitro cell cytotoxicity

In the present study, fruits of LAME showed dose dependent increase in the amounts of % dead cells in *in vitro* cell cytotoxicity; although it was significantly less when compared with 5-FU. Treatment with fruits of LAME was found to enhance nonviable cell counts in peritoneal exudates and decrease the viable cell count. It might be due to the absorption of extract by viable cells which leads to lysis of cell through the activation of macrophages or some cytokine production n peritoneal cavity. The results were shown in Table 3.

All the values were expressed as Mean ± SEM using "t" test. ns-non significant when compared with same concentration of 5-FU.

Solid tumour & Percentage increase in solid tumour (%IST)

In the present study, intramuscular (to thigh muscle) injection of DAL cells to the mice produced an increase in cancer cell count via proliferation, which is an indicative of cancer progression and is represented by solid tumour volume (size). The decrease in the solid tumour volume & %IST observed after treatment with LAME indicates that it has significant inhibitory effect on the tumour cell proliferation. The results were shown in Table 4.

Body Weight Analysis & Percentage increase in body weight

Intraperitoneal inoculation of DAL cells produced a marked increase in the cancer cell count and is represented by gain in body weight. The increase in body weight was may be due to accumulation of peritoneal fluid as suggested by an abnormal enlargement of peritoneal cavity in tumour- induced mice. The decrease in the body weight & %IBW observed in the LAME treated group indicates that it possess significant inhibitory effect on the tumour cell proliferation. The results were shown in Table 5.

Mean survival time (MST) and Percentage increase in lifespan (% ILS)

Post treatment with LAME significantly increased MST & %ILS which may be due to flavonoid content or antioxidant properties of the plant which were earlier reported[12,15]. The results were shown in Table 5.

Tumour volume & Tumour cell growth

A reduction in the number of ascitic cells (tumour volume) noticed in LAME treated group is because of its' direct cytotoxic effect or due to its 'effect on peritoneal macrophages (activation) or other components of the immune system further enhancing their tumour cells engulfing capacity. The results were shown in Table 5.

Haematological parameters

An increase in RBC count and a decrease in elevated WBC especially lymphocyte count were reported as markers for the protection against DAL[27]. Haematological investigations were carried out and the results confirmed the rise in WBC in DAL induced animals, might be a defensive mechanism against cancer cells. Significant reduction in the raised WBC count was noticed after treatment with LAME. Myelosuppression is one of the major problems that are encountered in cancer chemotherapy. The results of the present study have clearly shown that fruits of LAME have restored both the RBC and the haemoglobin content towards normal without any myelosuppression. This suggests that the extract does not affect the normal function of bone marrow. The results were shown in Table 6.

CONCLUSION

The results of the present study clearly indicate that, methanolic extract of *Limonia acidissima* L. fruits when administered at a dose of 570mg/kg shows significant antitumor activity against DAL induced tumour genesis in mice. The antitumor effect can be attributed to its' cytotoxic and antioxidant properties. The active

ingredients responsible for the activity were not isolated. The fruits are known to contain flavonoids and coumarins. The antitumor potential might be due to them. It needs to be explored further. As expected 5-FU showed significant antitumor activity than LAME fruits. It should be noted that 5-FU is a pure compound whereas the extract is a crude one containing numerous compounds.

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DECLARATION OF INTEREST

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