Research Article

Evaluation of Cytotoxic Effect of *Limonia acidissima* on Ehrlich Ascites Carcinoma in Mice

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ABSTRACT

The ethanolic extract of *Limonia acidissima* (EELA) fruit pulp was evaluated for cytotoxic efficacy against Ehrlich ascites carcinoma (EAC)-inoculated Swiss albino mice. Tumor was induced in mice by intraperitoneal injection of EAC cells (1×10^6 cells/mouse). EELA 5-fluorouracil was administered to EAC-bearing mice at a dose of 400 mg/kg bw p.o. along with the standard 5-fluorouracil (20 mg/kg bw i.p.) after 24 h of tumor inoculation. Treatment schedule significantly incremented the survival of animals with ascites tumor, decreased the body weight induced by the tumor burden, and reduced the packed cell volume and viable cell count. The alterations in the hematological profile (RBC, Hb, PCV, TC, and DC), lysosome-specific cancer markers (cathepsin-D, β-d-glucuronidase, and acid phosphatase), liver-specific cancer markers (5′-nucleotidase and lactate dehydrogenase), and membrane-bound ATPases (Na^+/K^+-ATPases and Mg^{2+}-ATPases) were restored to a significant ($P < 0.05$) extent. The results of the study demonstrate the antitumor potential of EELA.

Keywords: Ehrlich ascites carcinoma, 5-fluorouracil, *Limonia acidissima*; packed cell volume, viable cell count.

INTRODUCTION

Plant-based medicine plays an important role in cancer treatment, and 60% of currently used anticancer agents are derived from plant resources. *Limonia acidissima* is a tropical fruit distributed in regions of Burma, India, Malaya, and Sri Lanka. The ripe fruit is popularly used as a dessert and a source of beverages, creams, and jellies. It is also used as a tonic to treat dysentery, stomatitis, asthma, leucorrhea, wounds, and ulcer. Ripe fruit of this plant contains tyramine derivatives, acidissimol, acidissiminin, epxide N-benzoyl tyramine, and stigmasterol.

The plant possesses a wide range of biological activities such as adaptogenic activity, removal of blood impurities, and treatment of dyspepsia, jaundice, and liver disorders. The fruit pulp is also applied externally as a remedy for certain insect bites.[1] Fruits, leaves, and stem bark of *L. acidissima* possess larvicidal[2] and antimicrobial activities.[3]

Preliminary phytochemical investigation of fruit pulp of this plant revealed the occurrence of alkaloids, flavonoids, tannins, and terpenoids.[4] Several reports have proved the antimutagenic and antimalignant effects of flavonoids. Moreover, flavonoids have chemopreventive role through their effects on signal transduction in cell proliferation.[5] No reports are available in the literature for the *in vivo* antitumor potential of this plant. The present investigation was undertaken to evaluate the antitumor effect of ethanolic extract of *Limonia acidissima* (EELA) against Ehrlich ascites carcinoma (EAC) in Swiss albino mice.

MATERIALS AND METHODS

Chemicals

P-nitrophenyl-β-glycerophosphate,adenosine-5'monophosphate, nicotinamide adenine dinucleotide, adenosine triphosphate, deoxyribonucleic acid, and ribonucleic acid were purchased from Himedia Laboratories Ltd . All the other chemicals used were of analytical grade.

Preparation of plant extract

The fruit of *L. acidissima* was procured from a local market in Coimbatore, Tamilnadu, India. The plant was authenticated by a botanist in Botanical Survey of India, Coimbatore. Freshly collected material was chopped, shade dried, and coarsely powdered in a mechanical grinder; 100 g of dried powder was extracted with 150 ml of ethanol–water (1:1) several times at room temperature. The total extract was recovered by distillation under reduced pressure in a rotary evaporator. The yield of the plant extract was noted to be 6%.

Tumor cells

EAC cells were obtained through the courtesy of Amala Cancer Research Center, Kerala (Thrissur), India. The EAC cells were maintained *in vivo* in Swiss albino mice by intraperitoneal (i.p.) transplantation of 1×10^6 cells/mouse after every 10 days.

Animals and treatment schedule

Male Swiss albino mice weighing 20 ± 2 g were procured from the animal house of PSG Institute of Medical Sciences and Research, Coimbatore. The procured mice were categorized into five groups with nine animals each and maintained under standard laboratory conditions.

Group I served as the normal mice and received normal saline. Group II served as the EAC-bearing mice (1×10^6 cells/mouse i.p.). Group III was treated with EELA (400 mg/kg bw p.o.) after a day of inoculation. Group IV consisted of EAC-inoculated mice treated with the standard drug 5-flurouracil (20 mg/kg bw i.p.) after 24 h of inoculation. Group V served as the plant control and received a dose of EELA (400 mg/kg bw p.o.). The treatment schedule was followed for a period of 14 days. After the last dose and 18 h of fasting, six mice from each group were killed for various hematological and liver biochemical parameters. The rest of the animals in groups II, III, and IV were kept to check the survival time of EAC-tumor bearing hosts. The clearance of the ethical committee for experimentation on animals was obtained before the start of the experiment (No: 158/1999/CPCSEA).

Measurement of biochemical parameters

The ascitic fluid was collected from the peritoneal cavity and used for counting the number of viable cells using tryphan blue exclusion assay.[6] The blood collected after killing was anticoagulated by adding EDTA and used for determining various hematological parameters such as RBC, Hb, PCV, total WBC count, and differential count (neutrophils, lymphocytes, and monocytes).[7]

One gram of liver tissue was taken and homogenized with 10 ml of 0.1 M cold Tris-buffer, pH 7.4. Lysosome-specific cancer marker enzymes, namely cathepsin-D,[8] β-d-glucuronidase,[9] and acid phosphatase (ACP),[10] were determined. Liver-specific cancer marker enzymes were assessed by measuring 5′-nucleotidase[11] and lactate dehydrogenase (LDH).[12] Membrane-bound ATPases, Na^+/K^+-ATPase and Mg^{2+}-ATPase,[13] were also estimated.

Statistical analysis

All results were expressed as mean ± SD. For analyzing the variations in the observation of tumor growth between plant-treated EAC mice and EAC control, student's 't' test was used. The significance of the *in vivo* data was analyzed by the one-way analysis of variance (ANOVA), followed by the *post hoc* LSD comparison test using SPSS version 10.0. $P < 0.05$ was considered as statistically significant.

RESULTS

Monitoring of tumor growth

Administration of EELA and 5-fluorouracil incremented the life span of tumor-bearing mice by 56.12% and 89.13%, respectively, whereas EAC-bearing mice died after 18 days of tumor inoculation. The ascitic fluid volume, packed cell volume, and viable cell count were found to be remarkably ($p<0.05$) increased, and nonviable cell count was significantly decremented in EAC control when compared with group III. Post treatment of mice with EELA brought back the levels to near normal (table 1).

Hematological parameters

In EAC-bearing mice, RBC count, packed cell volume, and Hb content were markedly ($P < 0.05$) declined, whereas total WBC count was enhanced as compared to the normal mice. Among the various white blood cells analyzed, neutrophils were found to be elevated, while the lymphocytes and monocytes were decremented in EAC-bearing mice when compared to normal mice. Supplementation of EELA and 5-fluorouracil to diseased animal has restored the above alterations to a significant extent (Table 2 and 3).

Lysosomal marker enzymes

Table 4 demonstrates the effect of EELA on the activities of lysosomal marker enzymes in liver of normal and experimental group of mice. In EAC-bearing animals, the activity of cathepsin-D was elevated twice (41.08 ± 1.92) than the normal mice (21.55 ± 1.19). β-D-glucuronidase activity was incremented by 57.80% in EAC control when compared to normal mice. The increase in the activity of ACP was threefold when compared to the normal mice. Oral feeding of EELA extract to EAC control was found to stabilize the lysosomal integrity and retrieve the normal functioning of lysosomes (Table 4).

Liver-specific cancer markers

The activity of 5′-nucleotidase in EAC-transplanted mice was elevated thrice (6.34 ± 0.28) when compared to normal mice (2.31 ± 0.11). LDH activity in the liver of EAC-inoculated mice was markedly declined (0.57 ± 0.12) in comparison to normal mice (1.28 ± 0.10). Upon treatment with EELA and 5-fluorouracil, the activity was restored toward normal (Table 5).

Membrane-bound ATPases

Na^+/K^+-ATPase activity was decreased by twofold, and the decrement in Mg^{2+}-ATPase was found to be 56.22% in comparison to the normal mice. By treating with plant extract and 5-fluorouracil, the membrane-bound ATPase activities were regained to a significant extent (Table 6).

Table 1: Effect of EELA on monitoring the tumor growth in normal and experimental group of mice

Groups	Mean survival time (days)	% Increase in lifespan (%ILS)	Ascitic fluid volume (ml)	Packed cell volume (ml)	Tumor cell count (1×10^7 cells/ml)	
					Viable	Nonviable
EAC control (1×10^6 cells/mouse)	18.05 ± 0.78	—	2.7 ± 0.16	1.2 ± 0.09	10.32 ± 0.87	0.34 ± 0.03
EAC control + EELA (400 mg/kg bw p.o.)	28.18 ± 0.91	56.12	1.8 ± 0.09*	0.6 ± 0.02*	7.62 ± 0.25*	0.68 ± 0.038*
EAC control + 5-fluorouracil (20 mg/kg bw i.p.)	34.17 ± 2.18	89.30	—	—	—	—

Values are mean ± SD (n = 6).

Table 2: Antitumor activity of EELA on hematological parameters in normal and experimental group of mice

Groups	RBC (millions/cu.mm)	Hb (g/dl)	PCV (mm)
Normal	4.57 ± 0.13	11.90 ± 0.11	35.47 ± 1.29
EAC control (1 × 10^6 cells/mouse)	2.06 ± 0.08[a]	6.84 ± 0.28[a]	21.32 ± 0.88[a]
EAC control + EELA (400 mg/kg bw p.o.)	4.10 ± 0.16[b]	10.34 ± 0.54[b]	31.35 ± 1.14[b]
EAC control + 5-fluorouracil (20 mg/kg bw i.p.)	4.17 ± 0.16[b]	11.11 ± 0.51[b]	33.47 ± 1.08[b]
Plant control (400 mg/kg bw p.o.)	4.59 ± 0.17	11.42 ± 0.63	35.35 ± 1.16

Values are mean ± SD (n = 6).

Table 3: Antitumor activity of EELA on hematological parameters in normal and experimental group of mice

Groups	WBC (10^3/cu.mm)	Differential count (%)		
		Neutrophils	Lymphocytes	Monocytes
Normal	9.27 ± 0.35	16.40 ± 0.63	81.48 ± 3.12	1.49 ± 0.11
EAC control (1 × 10^6 cells/mouse)	15.37 ± 0.63[a]	61.17 ± 2.71[a]	35.24 ± 1.24[a]	0.84 ± 0.03[a]
EAC control + EELA (400 mg/kg bw p.o.)	11.44 ± 0.55[b]	51.27 ± 2.31[b]	72.42 ± 2.79[b]	1.08 ± 0.06[b]
EAC control + 5-fluorouracil (20 mg/kg bw i.p.)	10.39 ± 0.59[b]	55.62 ± 1.97[b]	77.11 ± 1.98[b]	1.30 ± 0.04[b]
Plant control (400 mg/kg bw p.o.)	9.45 ± 0.25	17.04 ± 0.62	80.92 ± 2.44	1.47 ± 0.06

Values are mean ± SD (n = 6).

Table 4: Effect of EELA on the activities of lysosomal marker enzymes in liver of normal and experimental group of animals

Groups	Cathepsin-D (µmoles of tyrosine liberated/hr/mg protein)	β-D-glucuronidase (µmoles of *p*-nitrophenol formed /min/mg protein)	Acid phosphatase (µmoles of *Pi* liberated/min/mg protein)
Normal	21.55 ± 1.09	24.46 ± 0.79	3.13 ± 0.11
EAC control (1 × 10^6 cells/mouse)	41.08 ± 1.75[a]	38.56 ± 1.25[a]	9.92 ± 0.24[a]
EAC control + EELA (400 mg/kg bw p.o.)	27.30 ± 1.10[b]	26.30 ± 0.70[b]	5.85 ± 0.20[b]
EAC control + 5-fluorouracil (20 mg/kg bw i.p.)	25.12 ± 1.27[b]	24.90 ± 0.59[b]	4.92 ± 0.14[b]
Plant control (400 mg/kg bw p.o.)	22.52 ± 0.77	24.32 ± 0.82	3.38 ± 0.09

Values are mean ± SD (n = 6)

Table 5: Effect of EELA on the activities of liver marker enzymes in normal and experimental group of animals

Groups	5′-Nucleotidase (µmoles of *Pi* liberated/min/mg protein)	Lactate dehydrogenase (µmoles of pyruvate liberated/min/mg protein)
Normal	2.31 ± 0.10	1.28 ± 0.10
EAC control (1 × 10^6 cells/mouse)	6.34 ± 0.25[a]	0.57 ± 0.12[a]
EAC control + EELA (400 mg/kg bw p.o.)	3.38 ± 0.16[b]	1.08 ± 0.08[b]
EAC control + 5-fluorouracil (20 mg/kg bw i.p.)	3.04 ± 0.17[b]	1.18 ± 0.08[b]
Plant control (400 mg/kg bw p.o.)	2.36 ± 0.16	1.22 ± 0.09

Values are mean ± SD (n = 6)

Table 6: Effect of EELA on the activities of Na^+/K^+ ATPase and Mg^{2+} ATPase in liver of normal and experimental group of animals

Groups	Na^+/K^+ ATPase (µmoles of *Pi* liberated/min/mg protein)	Mg^{2+} ATPase (µmoles of *Pi* liberated/min/mg protein)
Normal	1.88 ± 0.11	2.71 ± 0.12
EAC control (1 × 10^6 cells/mouse)	0.93 ± 0.15[a]	1.22 ± 0.07[a]
EAC control + EELA (400 mg/kg bw p.o.)	1.57 ± 0.08[b]	2.46 ± 0.08[b]
EAC control + 5-fluorouracil (20 mg/kg bw i.p.)	1.61 ± 0.08[b]	2.58 ± 0.08[b]
Plant control (400 mg/kg bw p.o.)	1.83 ± 0.08	2.67 ± 0.09

Values are mean ± SD (n = 6).

DISCUSSION

EAC is one of the experimental breast tumor derived from spontaneous mouse adenocarcinoma. Intraperitoneal injection of the tumor emulsion produces ascites.[14] The body weight of mice inoculated with EAC cells incremented due to the enhanced ascites volume by actively proliferating peritoneal cells.[15] Decreased lifespan is due to the low Hb levels observed in cancerous condition.[16] The change in the body weight and increased lifespan of animals in the treated group suggest the tumor growth inhibitory property of the plant extract.

Tumor growth is generally associated with marked changes in hematopoiesis, immuneresponse, myelosuppression, and anemia. The reduction in RBC and Hb percentage may be due to the deficiency of iron or due to the hemolytic or myelopathic conditions in EAC mice.[17] The significant increase in total WBC count and neutrophils in tumor-bearing mice is due to its primary defense mechanism.[18] The perturbation in the hematological profile was restored, which evidenced the protective action of EELA on the hematopoietic system.

Lysosomes are a group of cytoplasmic organelles which are characterized by their content of acid hydrolases that are capable of digesting the macromolecules such as polysaccharides, nucleic acids, and lipids.[19] The enormous production of free radicals in the cancerous condition leads to the abnormal fragility of the lysosomes and in turn results in the elevated levels of lysosomal enzymes.[20] Oral feeding of *L. acidissima* lowered the leakage of lysosomal marker enzymes, most likely via stabilizing the membrane architecture. This could be attributed to the presence of flavonoids in the extract that have an inhibitory property on lysosomal membranes.[21]

Cathepsin-D plays a proteolytic role in the digestion of extracellular matrix (ECM) components and is implicated in tumor invasion and metastasis. Numerous studies have reported the elevated activities of cathepsin-D in various types of cancers.[22]

β-D-glucuronidase is considered both a microsomal and a lysosomal enzyme. They are glycosidase family of enzymes that catalyze the breakdown of complex carbohydrates. It is shown to be a sensitive marker of lysosomal integrity.[23] Karunairatnam *et al.*[24] and Cohen and Bittner[25] have reported the enhanced activity of β-D-glucuronidase enzyme. ACP is also a cytoplasmic enzyme that has been considered to be associated with the lysosomes, which catalyze the hydrolysis of organic phosphate.

5'-Nucleotidase is a glycoprotein having phosphatase activity. It is widely distributed throughout the tissues of the body and is principally localized in the cytoplasmic membrane of cells. Nucleotidase activity is increased when tumor occludes the bile ducts. LDH is a tetrameric enzyme recognized as a potential tumor marker in assessing the progression of the proliferating malignant cells. Liver marker enzymes were brought back to normal by the presence of flavonoids in the plant extract, exerting antiproliferative action on cancer cells.[26]

Biological membranes encompass a group of ATPases, which maintain ionic gradients between aqueous intracellular and extracellular phases. They are lipid-dependant membrane-bound enzymes, and any alterations in the lipid bilayer may affect the activities of ATPases and in turn the normal cellular functions get affected.[27] Membrane-bound enzymes such as $Na^{+/}K^{+}$-ATPase and Mg^{2+}-ATPase are responsible for the transport of sodium/potassium and magnesium across the cell membranes at the expense of ATP by hydrolysis.[28]

The significant decrease in the activities of $Na^{+/}K^{+}$-ATPase and Mg^{2+}-ATPase in liver of cancer-bearing mice may be due to the increased production of free radicals, which exerts their cytotoxic effects by causing peroxidation of membrane phospholipids.[29] Flavonoids in EELA influence the permeability of biomembranes by interacting with ATPase pumps in the animal cell, thereby regain their normal efficiency and assume normal properties.

CONCLUSION

The present study concludes that the phytochemicals in ethanolic extract of *L. acidissima* inhibited the tumor induced by EAC.

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