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Research Article

Evaluation of Cell Viability Effects and Antioxidant Activity of *Caryota urens Linn.* and *Couroupita guianensis aublet* Leaves in a Model of Ehrlich Ascites Carcinoma as a Xenograft model

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ABSTRACT

Plants used in traditional medicine have been identified as the primary source of anticancer agents. One of the cancer-related parameters is the cell viability parameter. Current study aimed to evaluate cell viability effects and antioxidant activity of *Caryota urens Linn.* and *Couroupita guianensis aublet leaves* using xenograft model by transplantation of ehrlich ascites carcinoma (EAC) cells into mice. Animals were randomly divided into seven groups of six animals each. For 10 days, EAC cells (2×10^6 cells/mouse) were injected i.p. into each mouse in each group except the normal control and vehicle control groups. The treatment drugs were compared with standard 5-fluorouracil. Group I received water as a control, group II received 0.9% normal saline, group III received 0.5% CMC, group IV received EAC cells as a model control group, group V received EAC cells with 5-flourouracil treatment, group VI received EAC cells with *C. urens linn* extract, and group VII received EAC cells with *C. guianensis aublet.* After 10 days of treatment, animals were sacrificed, ascitic fluid was collected for evaluation of cell viability effects, blood collected for hematological parameter estimation, and liver tissue was collected for histopathological study and evaluation of antioxidant activity. Study results of *C. urens linn* and *C. guianensis aublet* both shows significant positive effects on cell viability and antioxidant activity. In the study visible differences were observed in liver tissue of different groups. Disease control groups showed damaged liver cells, while the treatment group showed less damage than disease control. Both plants produced positive effects on cell viability and antioxidant activity, resulting in a decrease in viable cell count.

Introduction

According to WHO cancer is the leading cause of morbidity and mortality worldwide.[1] In the last decades, there is rise in the number of cancer patients globally. Neoplasia means an abnormal proliferation of a group of cells which shows invasion as well as sometimes metastasis.[2] 5-FU is an aromatic heterocyclic organic compound having a structure similarity with the pyrimidine molecules of DNA and RNA. It is an analog of uracil through a fluorine atom at the C-5 position instead of hydrogen atom.[3] It quickly enters the cell and shows a facilitated transport mechanism as uracil. 5-FU inhibiting essential biosynthetic processes, or by being incorporated into macromolecules like fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP), such as DNA and RNA, and inhibiting their normal function.[4] Due to its structure, 5-FU hamper nucleoside metabolism and can be included into RNA and DNA, leading to cytotoxicity and apoptosis of cell.[5-6] Despite of potent antitumor potential, in normal cells, it is a harmful cancerous agent for various tissues and organs and causes different adverse effects such as headache, weakness, muscle aches, coordination, irritated eyes, increased tears, watering eyes, blurred

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vision, heart problems, confusion, tingling, numbness, or swelling in the hands and feet, severe allergic reaction.[7] It also showed toxic effects such as apoptosis induced by p53,[8] metabolic encephalopathy,[9] cardiotoxicity, and pulmonary hypertension.[10]

Reactive oxygen species (ROS) generation is an outcome of oxidative stress and/or inhibition of the antioxidant defense system.[11] The stimulation of ROS after the harm has been reported in numerous studies connected to FU toxicity.[8-10] To recover the toxic effects of chemotherapy such as 5-FU, numerous investigators have explored safe medicinal plants for their antioxidant as well as anticancer potential.[12]

In current scenario, more attention has to be given to use of herbal medicinal plants for therapeutic effects or to reduce adverse drug impacts.[13-15] *Caryota urens* is a palm tree, which belongs to Palmae family. This plant is native to Sri Lanka, India and Nepal.[16] *C. urens* is a plant very much useful in traditional medicine as an antiiflamamtory, antimicrobial, antiparasitic, antidiabetic, anticancer, neuroprotective, antioxidant and analgesic.[17] *Couroupita guianensis aubl* belong to family Lecythidaceae and commonly known as cannon ball tree, locally known as Kailashpati. It has been useful in traditional medicine for antiulcer, antihypertensive, anti-inflammatory, antimicrobial, antioxidant, antiasthmatic, and healing properties.[18]

Recent scientific research on plants used in ethinomedicine has led to the discovery of many valuable drugs such as vincristine, camptothecin, podphylotoxin, taxol, combretastatin.[19]

Preliminary phytochemical test of methanolic extract for fruits of *C. urens Linn.* (CUME) indicated presence of carbohydrates, steroids, glycosides, saponins, alkaloids, flavonoids, tannins and phenolic compounds. Ethanolic extract of *C. guianensis aublet* leaves (CGEE) indicated presence of carbohydrates, steroids, flavonoids, tannins and phenolic compounds. It was found from a literature survey that flavonoids and phenolic compounds helps to inhibit inflammation by simulating the production of immunogenic cells like interleukins that helps to the destruction of endogenous molecules like tumor cells. There are no particular for curative treatments available for cancer nowadays. Herbal is the best source of phytoconstituents. Plants used in traditional medicines have been accepted as main source of anticancer agents. Fruits of *C. urens Linn.* and Leaves of *C. guianensis aublet* yet not evaluated for anticancer activity. Present study involves the evaluation of the anticancer activity of *C. urens Linn.* fruits and *C. guianensis aublet* leaves.

Materials and Methods

Plant Material and Preparation of Plant Extract

The fruits of *C. urens Linn.* were collected from the botanical garden of A. R. College of Pharmacy, Vallabh Vidyanagar, Gujarat, and the leaves of *C. guianensis aublet* were collected from Navsari, Gujarat. Dr. Sasidharan N., Professor & Head of Department of Seed Science and Technology, B.A. College of Agriculture, Anand Agriculture University, Anand-388110, performed taxonomic identification and authentication. A voucher specimen (AAU/BACA/SST/SN/216/16) of the plant was deposited in our laboratory, the Department of Pharmacology of A. R. College of Pharmacy, Vallabh Vidyanagar.

The plant material, *C. urens linn* fruits, was dried under normal conditions to maintain its active principles and reduced to a coarse powder using a mixer grinder. These powdered fruits were stored in a tight container. *C. urens Linn.* fruit's dried powder was extracted with methanol using the soxhlet apparatus. The liquid was then filtered and kept in a hot air oven at 65°C for 8 hours to get a more solid extract. Then the methanol extract was weighed. The dry extract was stored at 4°C until used.[20] Leaves of *C. guianensis aublet* were collected from Navsari, Gujarat, and washed with water and allowed to shade-dry. The dry leaves were powdered and extracted by static macerations with ethanol at room temperature. The liquid was then filtered, and the ethanol extract of *C. guianensis aublet* leaves was evaporated to determine the yield.[21]

Experimental Animals

Female Swiss albino mice weighing about 20 to 25 g were obtained from the Zydus Research Centre, Ahmedabad, Gujarat, India. Before the commencement of the study, all animals were acclimatized for 1-week. Standard commercial normal pellet diet (NPD) and water were provided *ad libitum* for the animals during the course of an experiment. The animals were maintained at a controlled temperature (22 ± 2°C) and relative humidity (55 ± 5%) with a 12:12 hours light and dark cycle. The study was approved by the A. R. College of Pharmacy's Institutional Animal Ethics Committee (Protocol No: CPCSEA/IAEC/ARCP/2015-16/01), which was formed under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India. The established public guidelines in the Guide for the Care and Use of Laboratory Animals were strictly followed.[22]

Preliminary Phytochemical Tests

A preliminary phytochemical test was used to perform quantitative analysis on the plant extract.[23] Alkaloids, phenols, flavonoids, terpenoids, proteins, tannins, and saponins were all tested for.

Experimental Design

Method for Induction of EAC Cells

- *Tumor Cells*

Ehrlich ascites carcinoma cells (EAC) were obtained from the National Cancer Institute, Pune, India. The EAC cells

were maintained in Swiss albino mice by intraperitoneal (i.p.) transplantation of 2×10^6 cells per mouse; each animal received approximately 0.1 mL of cell suspension intraperitoneally. After 9 days, ascitic fluid was removed from the peritoneal cavity to assess cell viability.[24-25]

- *Treatment Groups*

Eighty four adult female swiss albino mice were distributed into 7 groups (6 animals /group) as follows: Group I received water as a normal control, while Group II received 0.9% normal saline as a vehicle control-1, and group III was given 0.5% carboxymethyl cellulose as vehicle control 2, group IV was given an EAC cell line on the first day as model control group, and group V was given 5-FU treated as a standard. Standard received 1st day (2×10^6 cells/mice) EAC cell line and 2nd–9th days of 5 fluorouracil (20 mg/Kg). group VI is CUME treated and received 1st days EAC (2×10^6 cells/mice) and 2nd–9th days CUME (2 mg/Kg) while group VII is CGEE treated and received 1st days EAC (2×10^6 cells/mice) and 2nd–9th days CGEE (100 mg/Kg). EAC cells (2×10^6 cells/mice) were injected i.p. into each mouse in each group except the normal saline group. That was taken as day 0. The extract and reference drug treatment was extended for nine days beginning on day 1. On the 10th day, 24 hours after the last dose, mice were sacrificed from each group. After sacrificing the animals, blood was collected to evaluate the haematological and biochemical parameters. Liver tissue was collected for histopathology evaluation and the evaluation of antioxidant activity.

Evaluation of EAC Volume, Cytology and Viability

The ascetic fluid was aspirated from mice, for EAC volume determination, viability test and cytological examination. The volume of ascetic fluid was collected from the peritoneal cavity. The volume was measured by using a graduated centrifuge tube. The counting of total live and dead EAC cells was done by diluting the collected fluid (9 vol) with tryptan blue 1%. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber, and the numbers of cells in the 64 small squares were counted. Within 5 minutes, the total number of cells, dead (stained), and alive (unstained) cells were observed and counted.

Hematological Parameters

An automated haematology analyzer was used to perform a complete blood analysis (RBCs, WBCs, and Hb).

Biochemical Aparameters

Serum biochemical enzymes such as serum glutamic oxaloacetic (SGOT) and glutamic pyruvic transaminase (SGPT) and alkaline phosphatase (ALP) activities were estimated.

Antioxidant Parameters

The antioxidant parameters MPO, MDA, SOD, and NO levels were evaluated.

Histopathological Evaluation

The mice were sacrificed, and liver tissue was rapidly excised, followed by fixing it for 48 hours in 10% formalin, and was dehydrated by passing successively in different mixtures of ethyl alcohol and water (50, 80, and 95%) and finally in absolute alcohol, cleared in xylene, and embedded in paraffin. Thick sections (4–5 Mm) were cut and stained with haematoxylin and eosin dye for microscopic examination of cell necrosis and fatty change.

Results

Phytochemical Analysis

The methanolic extract of *C. urens Linn. fruits* shows the presence of carbohydrates, steroids, glycosides, saponins, flavonoids, alkaloids, tannins, and phenolic compounds. The ethanolic extract of *C. guianensis aublet* leaves shows the presence of carbohydrates, steroids, glycosides, saponins, flavonoids, tannins, and phenolic compounds.

For Cell Viability Effect Parameters

Body Weight

The effects of the fruits of *C. ursina linn.* and leaves of *C. guianensis aublet were* evaluated during the study. For ten days, the body weight of all animals was recorded, and the change in body weight of animals from different groups was shown. As shown in Fig. 1, the change in body weight of animals in the EAC control group is increased. After induction of EAC cells, in the EAC control group, body weight increased by only 1.61 ± 0.83 compared with the normal group. The difference was found to be statistically significant ($p<0.001$; $p<0.05$). 5-FU fluorouracil (20 mg/Kg) pre-treatment results in a significant increase in body weight 28.49 ± 0.77. The changes in body weight observed in CUME treated mice were 29.99 ± 1.63 and 29.33 ± 0.88 in CGEE treated mice.

Data are expressed as a Mean ± SEM, (n=6), *p<0.05, **p< 0.001, One way ANOVA repeated measurement followed by Dunnet's test., $p^{\#}$=EAC control group (model

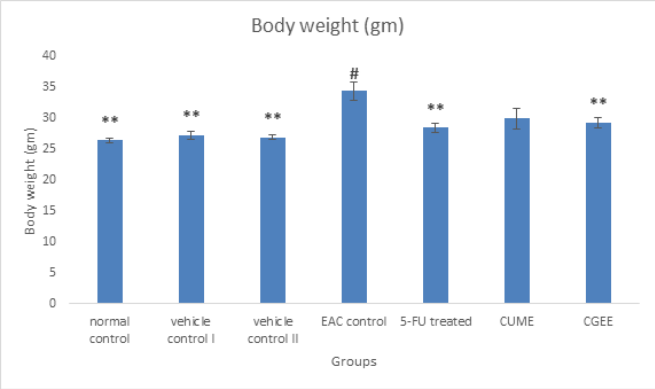

Fig. 1: Effect of *C. urens Linn.* and *C. guianensis aublet* leaves on Body weight

control), *p<0.05 = Significant as compared to EAC control group, **p<0.01 = Highly significant as compared to EAC control group.

Cell Viability Parameters

After sacrificing mice from all groups, tumors parameters were evaluated. Ascitic fluid volumes in the EAC control group, STD group (5-FU treated) group, CUME treated group, and CGEE treated group were evaluated. Figs. 2-5 depict the evaluation of ascitic fluid volume, total cell count, viable cell count, and non-viable cells. Ascitic fluid volume, total cell count, and viable cell count were all found to be significantly lower (p<0.05). The non-viable cell count was found to be statistically significant (p<0.05) higher in 5-FU treated mice compared to the control group.

Data are expressed as a Mean ± SEM, (n=6), *p<0.05, **p<0.001, One way ANOVA repeated measurement followed by Dunnet's test., $p^{\#}$ = EAC control group (model control), *p<0.05 = Significant as compared to EAC control group, **p<0.001 =Highly significant as compared to EAC control group.

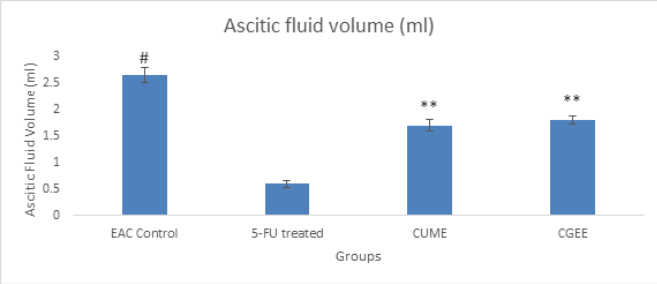

Fig. 2: Effect of *C. urens linn* and *C. guianensis aublet* leaves on ascitic fluid volume

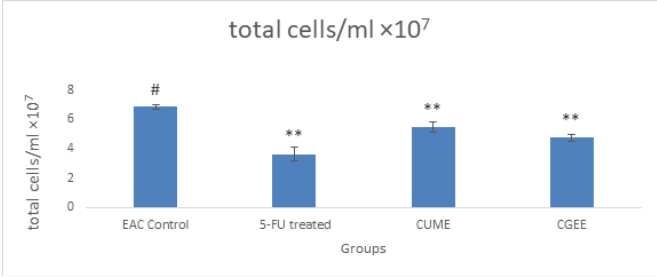

Fig. 3: Effect of *C. urens Linn.* and *C. guinanensis aublet* on total cell count

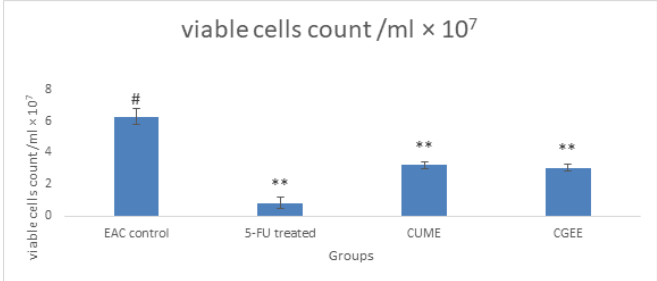

Fig. 4: *C. urens linn* and *C. guianensis aublet* leaves' effect on viable cell count

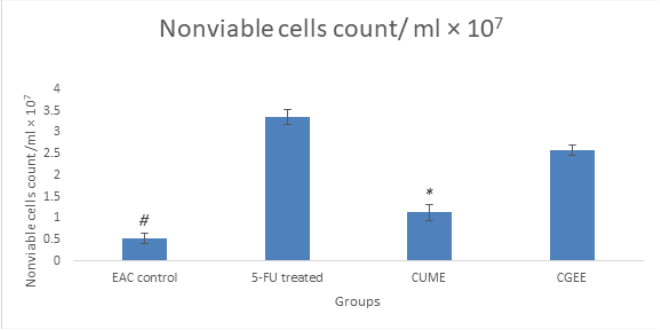

Fig. 5: Effect of *Caryota urens linn* and *C. guianensis aublet* leaves on non-viable cell count

Haematological Parameters

Cell viability studies examined haematological parameters such as RBC, WBC, and haemoglobin. The haemoglobin levels of all groups were evaluated, and their significance was checked. After evaluation of haematological parameters, it was found that there was a reduction in haemoglobin (6.10 ± 0.75), and RBC count (1.40 ±0.12) in EAC control animals, but an increase in WBC count (13.38 ± 0.17) when compared with normal). Where p<0.001. The WBC count was found to be significant when compared to the EAC control group (13.38 ± 0.17). The WBC count was found to be increased when compared to the normal group (5.80 ±0.14), as shown in Table 1.

Data are expressed as a Mean ± SEM, (n=6), *p<0.05, **p<0.001, One way ANOVA repeated measurement followed by Dunnet's test, $p^{\#}$=EAC control group (model control), *p<0.05 = Significant as compared to EAC control group, **p<0.001 = Highly significant as compared to EAC control group.

Biochemical Parameters

Liver enzymes are most important in diagnosing liver damage. Liver enzymes were found to have increased. SGPT, SGOT, and ALP levels were increased as compared to the EAC control group, as shown in Table 2.

Data are expressed as a Mean ± SEM, (n=6), *p<0.05, **p<0.001, One way ANOVA repeated measured followed by Dunnet's test., $p^{\#}$ =EAC control group (model control), *p<0.05 = Significant as compared to EAC control group, **p<0.001 = Highly significant as compared to EAC control group.

Antioxidant Parameters

Antioxidant parameters are evaluated MPO, MDA, SOD and NO levels were measured. When compared with EAC control group (3.06±0.10) NO levels were found to be highly significant. CUME treated mice shows 2.01 ± 0.305 and CGEE treated mice shows 2.33 ± 0.269 U/mg. MDA levels were increased as compared to CGEE treated group 19.71±0.28 when compared to EAC control group 23.25±0.40, the values were found to be significant where *p<0.05 is significant. Table 3 shows that **p<0.001 is highly significant.

Table 1: Effect of CUME and CGEE on haematological parameters

Groups	*Haemoglobin g/dl*	*RBC count 10^6cells/mm^3*	*WBC count 10^3cells/mm^3*
Normal control	9.09 ± 0.17 **	3.74 ± 0.33**	5.80 ± 0.14
Vehicle control I	9.05 ± 0.10 **	3.46 ± 0.31**	6.6 ± 0.13
Vehicle control II	7.53 ± 0.59**	3.39 ± 0.27**	6.36 ± 0.12
EAC control	6.10 ± 0.75$^{\#}$	1.40 ± 0.12$^{\#}$	13.38 ± 0.17$^{\#}$
5-FU treated	8.88 ± 0.24**	3.09 ± 0.06	7.22 ± 0.35
CUME	7.60 ± 0.48	2.19 ± 0.25**	8.13 ± 0.23
CGEE	6.44 ± 0.34	2.25 ± 0.31*	9.33 ± 0.36

Table 2: Effect of CUME and CGEE on Liver enzymes

Groups	*SGPT (IU/ L)*	*SGOT (IU/L)*	*ALP (uniits/dl)*
Normal	27.60 ± 2.57	29.07 ± 2.17	7.03 ± 0.16
Vehicle i	23.68 ± 1.24	27.29 ± 2.14	6.89 ± 0.32
Vehicle ii	22.08 ± 0.91	28.20 ± 2.32	6.89 ± 0.43
Eac	56.03 ± 0.92$^{\#}$	68.11 ± 2.56$^{\#}$	19.92 ± 0.45$^{\#}$
5-Fu	48.88 ± 1.92	50.54 ± 1.94**	10.73 ± 0.32
Cume	40.72 ± 1.44**	41.50 ± 2.61**	13.30 ± 0.23
Cgee	38.17 ± 2.09**	44.17 ± 0.99**	12.13 ± 0.28

Data are expressed as a Mean ± SEM, (n=6),), *p<0.05, **p<0.001, One way ANOVA repeated measured followed by Dunnet's test, p$^{\#}$ =EAC control group (model control), *p<0.05 Significant as compared to EAC control group, **p<0.001 = Highly significant as compared to EAC control group

Histopatholgical Parameters

Fig. 6 shows, the normal group has normal lobular structure and normal central veins, the vehicle group I has similar structure to the normal group, the vehicle group II has clear central veins, the EAC control group has destructed lobular structure and central veins, the 5-FU treated group has less damage than the EAC control group, the CUME treated group has less damage than the 5-FU treated group but more than the 5-FU treated In all groups, histopathological analysis of liver sections revealed changes. The normal control group exhibited lobular structure with a normal central vein and no inflammation. The EAC control section showed inflammatory regions and destructed lobular structure. The standard 5-FU-treated group achieves comparable results to the control group. When compared to the CGEE group, the CUME group has less vein damage and an inflamed area.

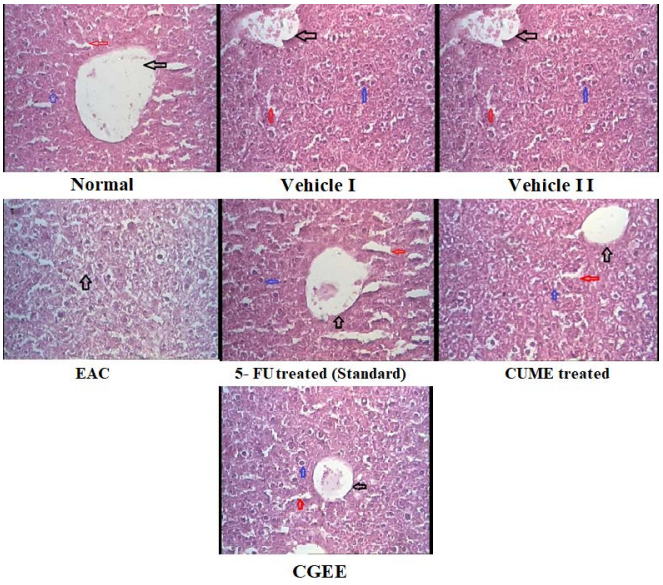

Fig. 6: Photomicrographs of sections of liver tissues from mice of various groups.

Discussion

Cancer is the abnormal growth of cells. Up until 2016, 1.22 million new cases were estimated in India. Cell viability effects can be evaluated by body weight, RBC count, WBC count, etc. Ehrlich ascites carcinoma (EAC) cells are used for evaluating the cell viability effects of various plants. In the EAC model for evaluating cell viability effects intraperitoneal (I.P.) EAC cell transplantation causes local inflammation caused by changes in vascular permeability, resulting in ascitic fluid formation and accumulation in the peritoneal cavity. Ascitic fluid is the primary source of nutrition for EAC cell development and growth. In the EAC-induced model for assessing cell viability, radicals were produced that bound to membrane proteins, resulting in the destruction of red blood cells and a decrease in haemoglobin levels. EAC cells: erythroquin as a novel inhibitor of LDH in cancer cells and as a combinatory drug to increase the efficacy of cisplatin[25] Diallyl disulfide may have anticancer effects mediated via modulation

Table 3: Effect of CUME and CGEE on antioxidant parameters

Groups	*MPO (U/mg)*	*MDA (nM/mg)*	*SOD (U/mg)*	*NO (nM/mg)*
Normal control	12.05 ± 0.38	5.17 ± 0.39	62.31 ± 0.80	1.41 ± 0.02
Vehicle control I	11.17 ± 0.38	5.09 ± 0.19	65.39 ± 0.70	1.45 ± 0.01
Vehicle control II	9.27 ± 0.19	5.75 ± 0.15	71.95 ± 0.61	1.58 ± 0.12
EAC control	21.05 ± 0.38$^{\#}$	23.25 ± 0.40$^{\#}$	30.85 ± 0.71#	3.06 ± 0.10#
5-FU treated	12.76 ± 0.23	7.26 ± 0.31	88.25 ± 0.67	1.84 ± 0.20*
CUME	10.47 ± 0.59	18.15 ± 0.37	51.56 ± 0.99	2.01 ± 0.30**
CGEE	9.02 ± 0.28	19.71 ± 0.28*	83.82 ± 0.70	2.50 ± 0.26**

of apoptosis and cell cycle arrest.[26] Subcutaneous EAC mouse model used for studying cancer-induced cardiomyopathy[27] Tetrodotoxin is part of a successful therapeutic regimen against cancer.[28] Encapsulating sorafenib in a nanoemulsion has decreased its toxicity to the heart and blood.[29]

Ethnomedicine has played an increasingly important role in the evaluation of drug effects. Herbal plants having different chemical constituents have effects on different activities. Methanolic extract of its rhizome (MEZZR) possesses promising antiproliferative efficacy against EAC cells.[30] Grape seed extract ameliorated Ehrlich solid tumor-induced hepatic tissue and DNA damage with reduction of PCNA and P53 protein expression in mice. [31] *Androctonus amoreuxi* has cytotoxic potential effects on tumor cells via anti-proliferative, apoptotic, and anti-angiogenic activities.[32] For these reasons, the plants are used in research.

A preliminary phytochemical test indicates methanolic extract of fruits of *C. ursina Linn.* (CUME) indicate the presence of carbohydrates, steroids, glycosides, saponins, alkaloids, flavonoids, tannins, and phenolic compounds. An ethanolic extract of *C. guianensis aublet* leaves (CGEE) indicates the presence of carbohydrates, steroids, flavonoids, tannins, and phenolic compounds. According to a literature review, flavonoids and phenolic compounds help to inhibit inflammation by simulating the production of immunogenic cells such as interleukins, which aid in the destruction of endogenous molecules such as tumor cells.

In present study, the effect of cell viability and antioxidant activity was found. The body weight of EAC control mice increased due to an increase in ascitic fluid volume. It can be related to each other that EAC cell-induced mice have increased body weight due to an increase in ascitic fluid volume. It is also found in the present study that an increase in the total cell count of ascitic fluid was observed in the EAC control group when compared to the normal group. There was a reduction in viable cell count and an increase in non-viable cell count, showing that cells were viable more in the EAC control group; this suggests that treatment groups CUME and CGEE have also had an effect on cell viability when compared to the control group.

As side effects of cancer treatment, myelosupression and anaemia are major ones, which are mainly because of an iron deficiency or other conditions that may lead to a reduction in RBC and haemoglobin levels. Related to this present experiment and evaluation, there was a reduction in RBC count and haemoglobin levels. WBC levels were found to be elevated in the study.

Serum enzymes are the most important parameter to evaluate disease conditions like inflammation or any other condition. A number of studies have found that tumour cells or EAC cells cause liver damage and disrupt hepatic cell metabolism, resulting in changes in liver enzyme activity. In the present work, elevated levels of SGPT, SGOT, and ALP were found. The increase levels of biochemical estimation may be expressed as a result of hepatocellular damage by EAC cells. Both drug treatment and biochemical estimation levels indicate protection against EAC cells

Several studies have found that when EAC cells are injected into mice, MDA levels rise. Similar to that, there was a change in MDA levels in the EAC control group that was found to be higher than any other group. Levels of superoxide dismutase (SOD) and myeloperoxidase (MPO) were also measured and found to be relevant to the current study.

EAC cells injected into mice cause inflammatory and degenerative changes in the liver that were observed in a histopathological study. Scavenging the toxic free radical causes damage to hepatic cells, central veins, and other tissues. Treatment groups showed less damage to liver tissue, which indicates the effects of both plant extracts on mice.

Conclusion

It can be concluded from present study that fruits of *C. urens Linn.* and leaves of *C. guianensis aublet* shows progressive effect on cell viability and antioxidant activity against EAC cells induced xenograft model due to chemical constitutes like flavonoids and phenolic compounds.

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