Research Article

IDENTIFICATION, ISOLATION AND ANTICANCER ACTIVITY OF BIOACTIVE CONSTITUENTS FROM FRUIT EXTRACT OF *AVERRHOA CARAMBOLA* (LINN.)

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ABSTRACT

Research with plants and plant derivatives are increasing day by day for the discovery of therapeutic and nutraceutical agents from them and due to the versatile applications of those beneficial agents. The present study was thus designed to investigate the anticancer activity of biological constituents from hydro-alcoholic fruit extract of *Averrhoa carambola* (HEAC) and evaluated by *in-vitro* and *in-vivo* experimental models. Preliminary phytochemical screening of HEAC has confirmed the presence of alkaloids, glycosides, phenolics, flavanoids, carbohydrates etc. HEAC was subjected to column chromatography and yielded AC1. Mechanism of action of AC1 was determined through molecular docking study. The HEAC and AC1 were tested for oral toxicity study. The *in-vitro* study was carried out by MTT assay method using EAC cell lines. The *in-vivo* anticancer activity was evaluated against EAC tumour bearing mice by Liquid Tumour Model. AC1 possess better inhibitory action on CDK receptor than apigenin. The HEAC and AC1 don't show any sign of toxicity. AC1 showed good cytotoxic effect on EAC cell line than HEAC in MTT assay.

Keywords: *Averrhoa carambola* (L.), column chromatography, Molecular docking, MTT assay, Ehrlich Ascites Carcinoma, Liquid tumour model.

INTRODUCTION

In India, the people of different ethnic groups inhabiting various terrains possess their own distinct cultures, religious rites, food habit and a rich of knowledge about traditional medicines¹. Natural products especially plants have been used in the treatment of various diseases for thousands of years². In recent years, traditional medicinal plants are gaining popularity as alternative to the currently practiced medicines. This played a significant role in health care system through the discovery and synthesis of new drug products³⁻⁵. Cancer is a group of abnormal cells grow uncontrollably by disregarding the normal rules of cell division. In fact, almost 90% of cancer-related deaths are due to tumour spreading – a process called metastasis⁶. Natural products have long been a fertile source of cure for cancer, which is projected to become the major cause of death in this century. There are at least 2,50,000 species of plants out of which more than one thousand plants have been found to possess significant anticancer properties⁷. *Averrhoa carambola* generally called star fruit is an attractive, slow growing evergreen tree that belongs to Oxalidaceae family. It is commonly grown in Southern China, Taiwan and India^{8,9}. It is commonly used to treat headaches, vomiting, coughing and hangovers¹⁰. Furthermore, it is used as an appetite stimulant, a diuretic and as an anti diarrheal and febrifugal agent etc.¹¹. The objective of the present study was to investigate the anticancer activity of the bio-active constituents from the fruit extract of *Averrhoa carambola* (L.) by using *in-vitro* and *in-vivo* methods. A further aim of this study was to find out the mechanism of action of isolated compounds through molecular docking studies to confirm the activity.

MATERIALS AND METHODS

Chemicals and Reagents

Petroleum ether (HIMEDIA), Chloroform (HIMEDIA), Ethyl acetate (VETECK), Ethanol (CHEMIND), Trypan blue (LOBA CHEMIE), 5-Flourouracil (PDPI). All the chemicals and solvents used in this study were of analytical grade.

Software

Graph software (version 4.4.2), Graph Pad prism (version 4.03), Instat 3, Argus lab, Molegro Molecular viewer, Swiss PDB, and Chemscketch.

Instruments

Bruker Avance III 400 MHz spectrometer, LCMS-2010A SCHIMADZU, FTIR (Jasco), Vacuum Oven (Rotek), Hot air oven (LEICA), and UV Chamber (Rotek).

Plant material

The whole plant *Averrhoa carambola* was collected in the month of October and authenticated (Specimen No. 88452) by Dr. A. K. Pradeep, Herbarium Curator, Department of Botany, University of Calicut, Thenhipalam, Kerala, India. The specimen voucher was deposited in the Department of Botany, University of Calicut, itself.

Extraction and Isolation

The collected fruits of *Averrhoa carambola* was cleaned and dried under shade at room temperature and powdered to get a coarse powder using a mixer grinder. The air dried powdered material (1 Kg) was subjected to continuous hot extraction for 72 hrs by using 70% ethanol. The extract was concentrated under reduced pressure and percentage yield was calculated¹². The hydro-alcoholic extract of *Averrhoa carambola* (HEAC) was subjected to phytochemical screening, adapted as per the standard procedure¹³. The HEAC was subjected to column chromatography and eluted with solvents in the increasing polarity (Petroleum ether, Chloroform, Ethyl acetate and Ethanol). Fractions with similar R_f value and identification test, were pooled together and solvents evaporated to get residues. The isolated compound was characterised using FT-IR Spectra, ¹H NMR, ¹³C NMR and Mass spectra.

Molecular Docking

The molecular docking approach can be used to model the interaction between a small molecule and a protein at the atomic level, which allow us to characterize the behaviour of small molecules in the binding site of target proteins as well as to elucidate fundamental biochemical processes¹⁴. In order to fulfil the aim of the present study, molecular docking analysis was used for the determination of mechanism of action of isolated compound in cancer treatment.

Methodology of docking studies include,

Preparation of protein molecule

- Crystallographic structures of the targets of interest were obtained from PDB.
- The protein molecule was prepared by using software Swiss PDB viewer.

Preparation of ligand

- The ligand compounds were drawn using ACD/Chemskech.
- 3D structure of ligand downloaded from CORINA software (PDB format).

Molecular docking

- The docking of ligand and target were done in Argus Lab Software.
- The energy score, hydrogen bond interaction and hydrophobicity were noted and tabulated.

Pharmacological studies

Animals

Albino mice of Swiss strains were used for the pharmacological and toxicological studies. These animals were purchased and stock maintained in the animal house of Devaki Amma Memorial College of Pharmacy, Malappuram District, Kerala, India. Female mice selected were nulliparus and non-pregnant. Female mice weighing 25-30 g were used for the study. All the animals were kept under standard laboratory conditions with a 12 hr light-dark cycle. These animals were fed with pellet diet (Amrut Laboratory, Maharashtra) and drinking water *ad libitum*. The animal experimental protocol was approved by our IAE (Reg. No. 1527/PO/Re/S/11/CPCSEA), with Proposal Number: DAMCOP/IAEC/013.

Acute oral toxicity study

The acute oral toxicity study was carried out on Swiss Albino mice as per the guidelines No. 423 given by the Organisation for Economic Co-operations and Development (OECD 423, 1988). A Limit test at one dose level of 2000 mg/Kg. b. wt. was carried out with six animals (Three animals per step) were fasted over night. Animals were observed individually after dosing at least once during the first 30 min periodically during the first 24 h, and daily thereafter for 14 days¹⁵.

In-vitro anticancer study

MTT assay was employed for the *in-vitro* anticancer study of the HEAC and isolated compound. MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] is a pale yellow substrate that is cleaved by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even freshly dead cells do not cleave significant amount of MTT. Thus the amount of MTT cleaved is directly proportional to the number of viable cells present, which is quantified by colorimetric methods. Briefly, the compounds were dissolved in dimethyl sulphoxide (DMSO) and serially diluted with complete medium to get the concentrations a range of test concentration. DMSO concentration was kept <0.1% in all the samples. Cell lines maintained in appropriate conditions were seeded in 96 well plates and treated with different concentrations of the test samples, and incubated at 37°C, 5% CO₂ for 96 h. MTT reagent was added to the wells and incubated for 4 h; the dark blue formazan product formed by the cells was dissolved in DMSO under a safety cabinet and read at 550 nm¹⁶.

In-vivo anticancer study

Thirty six mice were divided into six groups, containing six animals in each group and treated as given below:

- Group I: Normal control (oral dose of 10 ml/Kg.b.wt. Sodium CMC suspension 0.5%)
- Group II: EAC control (oral dose of 10 ml/Kg.b.wt. Sodium CMC suspension 0.5%)
- Group III: EAC induced + Reference drug (oral dose of 20 mg/Kg.b.wt. 5-Fluorouracil)
- Group IV: EAC induced + hydro-alcoholic extract (200 mg/Kg.b.wt) treated mice
- Group V: EAC induced + hydro-alcoholic extract (400 mg/Kg.b.wt) treated mice
- Group VI: EAC induced + Isolated compound (oral dose of 20mg/Kg.b.wt.)

All the treatments were given orally at 24 h after tumour inoculation and continued once daily for 14 d. On the 15th day, half of the animals from each group are anesthetized with Ketamine xylazine and blood was collected by retro-orbital puncture for the evaluation of haematological parameters, which include Hb, RBC and WBC. The remaining animals in each of the groups were kept to check the MST and percent increase in life span of the tumour bearing hosts¹⁷.

Anti tumour effect of the extract was assessed by observation of change with respect to body weight, ascitic tumour volume and viable tumour cell count, Mean Survival Time and percentage increase in Life Span (% ILS). The mice were dissected for collecting ascitic fluid from peritoneal cavity. The transplantable murine tumour was carefully collected and measured the fluid volume. Viable and non viable cell count of ascitic cells were performed by dye exclusion test using Trypan blue stain (0.4% in normal saline). The cell counts were

determined in a Neubauer counting chamber. The effect of extract on tumour growth was observed by MST and % ILS. MST of each group containing 6 mice was monitored by recording the mortality daily for 6 weeks and % ILS was calculated by using following equation¹⁸⁻²¹.

$$\text{Death MST} = \text{Day of first death} + \text{Day of last} / 2$$

Statistical analysis

The experimental results will be expressed as mean $\pm$ SEM. Data is assessed by ANOVA followed by the Dunnett's multiple comparison post hoc tests. Value of $p < 0.05$ was considered as statistically significant. The IC_{50} concentrations were calculated using Graph software (4.4.2). The statistical analysis will be performed using Graph Prism software (version 4.03).

In-vitro antioxidant study

Nitric Oxide Free Radical Scavenging Activity

Two ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (p^H 7.4) was mixed with 0.5 ml of extract at various concentrations and the mixture was incubated at 25°C for 150 min. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1 ml of naphthyl ethylene diamine dihydrochloride, 0.1% w/v) was mixed and incubated at room temperature for 30 min and the absorbance was measured at 540 nm with a spectrophotometer²². The nitric oxide radical scavenging activity was calculated as:

$$S\% = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Where A_{control} = absorbance of the blank control (containing all reagents except the extract solution) and
 A_{sample} = absorbance of test sample.

Linear regression analysis was used to calculate IC_{50} values.

Hydrogen Peroxide Free Radical Scavenging Activity

Scavenging activity of Hydrogen Peroxide (H_2O_2) by the plant extract was determined by the method of Ruch. Plant extract (4 ml) prepared in distilled water at various concentrations was mixed with 0.6 ml of 40 mM H_2O_2 solution prepared in phosphate buffer (0.1 M p^H 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm. Ascorbic acid was used as a positive control compound. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples using following equation²³.

$$S\% = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Where A_{control} = absorbance of the blank control (containing all reagents except the extract solution) and
 A_{sample} = absorbance of test sample.

Linear regression analysis was used to calculate IC_{50} values.

Reducing Power Assay

The Fe^{3+} reducing power of the extract was determined by the method of Oyaizu. The extract (0.75 ml) at various concentrations was mixed with 0.75 ml of phosphate buffer (0.2 M, p^H 6.6) and 0.75 ml of potassium hexacyanoferrate [$K_3Fe(CN)_6$] (1% w/v), followed by incubating at 50°C in a water bath for 20 min. The reaction was stopped by adding 0.75

ml of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 3000 r/min for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride ($FeCl_3$) solution (0.1%) for 10 min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power²⁴.

RESULTS

Phytochemical screening

The percentage yield of the hydro-alcoholic extract of the fruits of *Averrhoa carambola* (Linn.) was found to be 29.4% w/w. The phytochemical identification was carried out as per the standard procedure and the HEAC showed the presence of Alkaloids, Glycosides, Flavanoids, Carbohydrates, Tannins and Phenolics, Proteins and Amino acids, Steroids and Terpenoids. Two major fractions (Fraction I and Fraction II) were obtained from the analytical column [Pet.ether: Chloroform (80: 20) and Ethyl acetate: Ethanol (60:40)]. Compound (AC1) was isolated from Fraction II by preparative column method by using ethyl acetate: ethanol (60: 40) as the solvent system. Out of the various trials, the suitable mobile phase for the AC1 was found to be ethyl acetate: methanol (7: 3). The isolated compound (AC1) is pale yellow in colour, soluble in DMSO, methanol, ethanol and reported to be a flavanoid and possessing the melting point of 140°C. The R_f value was found to be 0.66.

Spectral characterization

IR: OH stretch at 3377.71 cm^{-1} , Ar CH stretch at 3017.09 cm^{-1} , sp^3 CH stretch at 2916.81 cm^{-1} , 2851.24 cm^{-1} , Ar C=O stretch at 1728.87 cm^{-1} , C-O stretch at 1070.3 cm^{-1} , 1258.32 cm^{-1} , Ar C=C stretch at 1400.07 cm^{-1} , 1458.89 cm^{-1} , Ar sp^2 CH bend at 713.533 cm^{-1} and 773.315 cm^{-1} . **¹H NMR:** Peak at δ 7.01, δ 6.68 (s) indicates H of Aromatic ring. Peak at δ 4.68, δ 4.65 (d) indicates H of Aromatic OH. Peak at δ 1.23 (s) indicates the H of methyl group. Peak at δ 1.99 (s) indicates H of glycone OH group. Peak at δ 4.21 (s), δ 3.44, δ 3.50 (d) indicates H of glycone moiety. **¹³C NMR:** Peak at δ 181.92 indicates C of Aromatic ketone. Peak at δ 161.09, δ 158.43 C attached to OH group of Aromatic ring. Peak at δ 14.09 indicates C of methyl group. Peak at δ 118.61, δ 125.09 indicates C of Aromatic ring. Peak at δ 63.12, δ 71.92, and δ 81.92 indicates C attached to OH group of glycone moiety. **Mass:** The spectrum shows base peak at 269.00 and molecular ion peak [M^+] at 416. Molecular mass of the AC1 was found to be 416. Molecular formula of AC1 was found to be $C_{21}H_{20}O_9$. From the IR, NMR, Mass spectral characterizations, the structure of isolated compound AC1 is shown in Figure 1.

Molecular docking

Since the isolated compound AC1 possess a flavone nucleus (Apigenin), it showed an inhibitory effect on the cyclin dependent kinase (CDK) receptor²⁵. So Apigenin was used as standard for the docking studies. The target used for the docking studies of AC1 was found to be CDK. The crystallographic structure of CDK was obtained from protein data bank (PDB ID- 2XNB). Docking studies of ligand and protein molecule were carried out by using Argus lab software. The docking scores, hydrogen bond and hydrophobic interactions were studied. Least docking energy of AC1 (-9.039) indicates its better stability with the receptor than the Apigenin (-7.411). Four strong hydrogen bond interaction of AC1 with the Phenyl alanine and Alanine residues of the target protein indicate very effective drug receptor interaction than Apigenin (weaker hydrogen bond interactions). Stronger hydrogen bonding causes

better hydrophobic interaction in the AC1 confirms the effective drug receptor interaction with target protein than in Apigenin (Fig. 2 to Fig. 5).

Pharmacological studies

Acute oral toxicity study

The HEAC and AC1 were showed no mortality even at 2000 mg/Kg. All the animals were found to be normal and there were no gross behavioural and weight changes till the end of the observation period of 14 days.

In-vitro anticancer study

The HEAC and AC1 were subjected to MTT assay against the EAC cell lines. HEAC showed an IC_{50} concentration at 186.4 μ g/ml whereas AC1 having an IC_{50} concentration at 134.8 μ g/ml (Table 1).

In-vivo anticancer study (Ehrlich Ascites Carcinoma (EAC) tumour cells induced anti-tumour study)

Effect on tumour growth

The average life span of EAC tumour control animals was found to be 22 days. When EAC control group compared to other treated groups, AC1 at 20 mg/Kg.b.wt showed a significant change in the average life span of animals than the HEAC treated groups. The average life span of 5-FU treated animals was found to be 28 days, which indicates its potential anticancer nature (Table 2). The anti-tumour nature of AC1 at 20 mg/Kg.b.wt. was evidenced by the significant reduction in the

body weight of animals than HEAC treated groups when compared to the EAC control mice. The treatment with AC1 also showed a significant decrease in the tumour volume, tumour weight, viable cell count and a significant increase in the non-viable cell count as compared to the EAC bearing mice (Table 3).

Effect on haematological parameters

EAC tumour bearing mice showed a significant decrease in the Hb, RBC count and a significant increase in the WBC count as compared to the normal control. Treatment with AC1 at the dose of 20 mg/Kg.b.wt. significantly reversed EAC tumour-induced changes in the haematological profiles (Table 4). The standard (5-FU) also showed the similar observations.

In-vitro antioxidant study

Nitric Oxide Free Radical Scavenging Activity: The IC_{50} values for HEAC and Ascorbic acid were found to be 71.44 μ g/ml and 48.60 μ g/ml respectively. HEAC significantly inhibited nitric oxide in a dose dependent manner (Table 5).

Hydrogen Peroxide Free Radical Scavenging Activity: The IC_{50} values for HEAC and Ascorbic acid were found to be 77.20 μ g/ml and 42.21 μ g/ml respectively. The HEAC significantly inhibited the H_2O_2 in a dose dependent manner (Table 5).

Reducing Power Assay: The result showed that the HEAC possess greater reducing power (Table 6).

Table 1: Percentage inhibition and IC_{50} of HEAC and AC1 in *in-vitro* anticancer study

Samples	% inhibition (mean±SEM)					IC ₅₀ (µg/ml)
	Concentration (µg/ml)					
	12.5	25	50	100	200	
5-FU	23.73± 1.57	34.46± 0.75	56.1± 0.87	81.83± 1.18	87.86± 1.13	42.96
HEAC	4.08± 0.08	15.13± 0.12	23.58± 0.73	38.12± 0.12	53.45± 0.57	186.4
AC1	7.70± 0.57	19.19± 0.57	30.51± 0.46	43.54± 0.16	62.11± 0.85	134.8

Table 2: Effect of survival time of tumour bearing mice in *in-vivo* anticancer study

Parameter	EAC control	5- FU 20mg/kg	HEAC 200mg/kg	HEAC 400mg/kg	AC1 20mg/kg
Mean Survival Time	22 days	28 days	24 days	25 days	26 days
Percentage Life Span	-	27.27%	9.09%	13.63%	18.18%

Table 3: Effect of body weight and other parameters in *in-vivo* anticancer study

Parameter	EAC control	5-FU 20mg/kg	HEAC 200mg/kg	HEAC 400mg/kg	AC1 20mg/kg
Body weight (g)	42.0 $\pm$ 0.8944****a	32 $\pm$ 0.5774***b	39.00 $\pm$ 0.7303 ns c	37.83 $\pm$ 1.493**d	34.17 $\pm$ 0.6831***e
Tumour weight (g)	23 $\pm$ 0.5774	9 $\pm$ 0.5774***b	18 $\pm$ 0.5774 ns c	15.33 $\pm$ 2.906*f	12.33 $\pm$ 1.202**g
Tumour volume (ml)	23.5 $\pm$ 0.7638	8.567 $\pm$ 0.348***b	20.5 $\pm$ 0.866 ns c	16.33 $\pm$ 1.922**d	11.93 $\pm$ 1.027***e
Viable cell count ($\times 10^7$ cells/ml)	5.093 $\pm$ 0.0648	2 $\pm$ 0.0264***b	4.33 $\pm$ 0.1069*h	3.427 $\pm$ 0.0176**d	2.327 $\pm$ 0.1302***e
Non-viable cell count ($\times 10^7$ cells/ml)	0.1433 $\pm$ 0.0290	1.54 $\pm$ 0.0231***b	0.53 $\pm$ 0.0493 *h	0.74 $\pm$ 0.0378*f	1.233 $\pm$ 0.0352**g

(Values are mean $\pm$ S.E.M; (n=6), a- ****p<0.0001 - Normal compared with EAC control; b- ***p<0.001- EAC with 5-FU; c- ns - EAC with HEAC 200mg/kg; d- **p<0.01 - EAC with HEAC 400mg/kg; e- ***p<0.001 - EAC with AC1 20mg/kg; f- *p<0.05- EAC with HEAC 400mg/kg; g- **p<0.01- EAC with AC1 20mg/kg; h- *p<0.05- EAC with HEAC 200mg/kg).

Table 4: Effect of haematological parameters in *in-vivo* anticancer study

Parameter	Normal control	EAC control	5-FU 20mg/kg	HEAC 200mg/kg	HEAC 400mg/kg	AC1 20mg/kg
WBC (10 ³ /cu.mm)	8.800± 0.650	26.400± 1.098***a	11.40± 0.5508***b	21.183± 0.7132*c	19.30± 0.4619**d	13.317± 2.132***e
RBC (million/cu.mm)	7.283± 0.127	3.127± 0.285 ***a	6.723± 0.049***b	4.093± 0.089*c	4.17± 0.295**d	6.33± 0.145***e
Hb (g %)	14.33± 0.218	9.367± 0.120****f	13.8± 0.416***b	10.9± 0.057**g	11.03± 0.088**d	13.67± 0.290***e

(Values are mean±S.E.M; (n=6); a- *** p<0.001 - normal compared with EAC control; b- *** p<0.001 - EAC with 5-FU; c- *p<0.05 - EAC with HEAC 200mg/kg; d- **p<0.01 - EAC with HEAC 400mg/kg; e- *** p<0.001 - EAC with AC1 20mg/kg; f- **** p<0.0001-Normal compared with EAC control; g- **p<0.01 - EAC with HEAC 200mg/kg).

Table 5: IC₅₀ values of samples in Nitric oxide and Hydrogen peroxide free radical scavenging assay

Samples	IC ₅₀ (µg/ml)	
	Nitric oxide free radical scavenging assay	Hydrogen peroxide free radical scavenging assay
Ascorbic acid	48.60	42.21
HEAC	71.44	77.20

Table 6: Percentage inhibition of samples in reducing power assay

Samples	% inhibition (mean±SD)					
	Concentration (µg/ml)					
	12.5	25	50	100	150	200
Ascorbic acid	0.112±0.01	0.336±0.014	0.518±0.005	0.774±0.00	-	-
HEAC	-	-	0.301±0.019	0.511±0.01	0.693±0.02	0.806±0.008

Figure 1: Structure of AC1

Figure 2: Hydrogen bond interaction of AC1 with CDK 9 receptor

Figure 3: Hydrophobic interaction of AC1 with CDK 9 receptor

Figure 4: Hydrogen bond interaction of Apigenin CDK 9 receptor

Figure 5: Hydrophobic interaction of Apigenin with CDK 9 receptor

DISCUSSION

The present investigation was carried out to evaluate the anticancer activity of isolated compound (AC1) isolated from fruit extract of *Averrhoa carambola* (L.). The mechanism of action of AC1 was determined through the molecular docking studies. The AC1 showed a better inhibitory effect on the CDK receptor than apigenin and which thereby prevents the over proliferation of cells in cancer treatment as a good anticancer agent.

In-vitro anticancer study by using EAC cell lines, showed better activity with AC1 than HEAC. In order to compare and confirm the activity of AC1 with HEAC, both samples were used for the *in-vivo* study using EAC tumour in mice. The prolongation of life span of tumour bearing animals indicates the potent nature of anti-cancer drugs. There is also a significant increase in the body weight of EAC tumour bearing mice due to the regular and rapid increase in the ascitic tumour fluid²⁶. The EAC tumour mice when orally treated with AC1 showed a significant increase in the life span of animals and also significantly prevented the increase in body weight that was observed in the EAC control mice. The AC1 treatment showed significant changes in the tumour parameters which indicate its anticancer effect on cells. In cancer chemotherapy the major problems are myelosuppression and anaemia²⁷⁻²⁸. The causes of anaemia in the tumour bearing mice is due to the reduction in the RBC or Hb level and which may be due to the iron deficiency or due to the haemolytic or myelopathic conditions. AC1 treatment restored the haematological profiles in animals when compared to the EAC mice. This indicates AC1 possess protective action on the hemopoietic system.

Excessive production of free radical causes oxidative stress, that leads to the damage of macromolecules such as lipids and which induce lipid peroxidation *in-vivo*. Lipid peroxide formed in the primary site would be transferred through the circulation and propagating the process of lipid peroxidation. Thus the increased peroxidation causes the degeneration of tissues. Malondialdehyde (MDA) formed as the end product of lipid peroxidation and it was reported to be higher in cancer tissues than in non diseased organs²⁹. The HEAC possess dose dependent free radical scavenging activity, could be responsible for the anticancer activity.

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