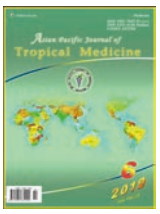

Original Article
Asian Pacific Journal of Tropical Medicine
journal homepage: www.apjtm.org

doi: 10.4103/1995-7645.261274 Impact factor:1.77

Annona muricata fruit extract protects against diethylnitrosamine-induced hepatocellular cancer in rats

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ARTICLE INFO

Article history:
Received 17 July 2018
Revised 1 May 2019
Accepted 13 May 2019
Available online 27 June 2019

Keywords:
HepG2-cells
Apoptosis
Liver cancer
Acetogenins
Rat liver cancer
Scratch wound assay

ABSTRACT

Objective: To evaluate the anticancer potentials of *Annona muricata* fruit by *in vitro* and *in vivo* methods.

Methods: The ethanolic extract of *Annona muricata* fruit was prepared by Soxhlet extraction method and further fractionated with petroleum ether, ethyl acetate and chloroform. The fractions were tested for cytotoxicity, apoptosis, scratch wound assay, and cell cycle analysis. IC_{50}, apoptotic index and percentage cell migration were determined using HepG2 cells. For the *in vivo* studies, hepatocellular carcinoma was induced by administering 0.01% diethylnitrosamine (DEN) in drinking water in Wistar rats. In pre-treatment, rats were co-administered 200 mg/kg of fruit extract with DEN for 14 weeks. In post-treatment, the extract was co-administered after 8-weeks of DEN-induction for 14 weeks. Liver function test, haematological test, oxidative stress markers, relative liver weight, number of cancer nodules and histopathological parameters were determined.

Results: *Annona muricata* fruit extract =significantly lowered cell proliferation counts. The chloroform-fraction possessed higher activity [IC_{50}=(53.7±4.3) μg/mL]. The chloroform fraction inhibited cell migration, which was significant compared to curcumin. Further investigations regarding the mode of anticancer activity revealed that the chloroform fraction induced apoptosis. The cell cycle analysis indicated that cells were being arrested at G_0/G_1. In the *in vivo* studies, the DEN-control group showed a significant decrease in body weights with increased mortality rate, hepatic nodules, and impairment of liver function compared to normal rats. The rats pre-treated and post-treated with the extract showed positive results with significant improvement in the parameters that were adversely affected by DEN. In addition, other adverse effects of DEN, such as blood dyscrasias and hepatic endogenous antioxidant, were significantly attenuated by *Annona muricata* fruit extract.

Conclusions: The *Annona muricata* fruit extract has anticancer activity when tested by *in vitro* and *in vivo* hepatocellular cancer models.

1. Introduction

Liver cancer is the 5th most common cause of cancer death with high fatality. Among liver cancers, 75% are primarily liver cell cancer[1]. Hepatocellular carcinoma (HCC) is highly malignant among the liver adenocarcinomas which are epithelial or glandular

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How to cite this article: Thomas SV, Paul J, Shrungeswara AH, Biswas S, Shah AA, Subraya CK, et al. *Annona muricata* fruit extract protects against diethylnitrosamine-induced hepatocellular cancer in rats. Asian Pac J Trop Med 2019; 12(6): 272-282.

cell origin. The aetiology of HCC includes food toxins such as fungal aflatoxins or chronic hepatitis B/C infection. HCC can result from the progression of cirrhosis due to chronic alcoholism, non-alcoholic steatohepatitis or due to alpha-1-antitrypsin deficiency[2]. Most importantly, HCC-patients have low survival rate after diagnosis and prognosis[3]. Thus the pathogenesis of HCC involves many causes and numerous risk factors. A generalized HCC-management plan involves chemotherapy, radiotherapy, radiofrequency ablation, hepatectomy and liver transplantation[4]. Present chemotherapy includes MAP-Kinase inhibitor sorafenib, which is generally well tolerated. Sorafenib frequently exhibits intolerable adverse drug reactions which include skin toxicities (hand-foot syndrome), GI-toxicities (diarrhoea), and hypertension, apart from the general toxicities such as anorexia, alopecia, weight loss, and voice changes[5,6]. Other chemotherapeutic agents used for palliative treatment are doxorubicin, 5-fluorouracil (5-FU) and cisplatin[7]. In comparison with all these modalities of treatments, the plant-based medicine has fewer side effects, cost-effective and many of them are efficacious[8].

Annona muricata (*A. muricata*; Family: Annonaceae) is a plant known to have different traditional uses and commonly known as 'Soursop', 'Graviola'', 'Guanabana', 'Paw-paw' and 'Sirsak'[9,10]. *A. muricata* is widely distributed in tropical and subtropical areas including India, Malaysia, and Nigeria. The bark, leaves, fruits, seeds, and roots of *A. muricata* were found to have different ethnomedical properties like antimalarial, smooth muscle relaxant, uterine stimulant, anti-crustacean, antiparasitic, cardiac depressant, antiamoebic, antibacterial, antifungal and insecticide[11]. *A. muricata* seeds are reported to be cytotoxic to 6 human cancer cell lines[12]. The leaves of this plant can lower the bilirubin level in jaundice and were reported for hepatoprotective activity[13,14]. Leaves were also reported for chemopreventive effects in chemically induced mice skin papilloma genesis[15]. Further, the antiulcerogenic property for leaves of *A. muricata* was also reported in animal models[16]. The edible fruits of this plant were used as a traditional medicine for arthritic pain, neuralgia, diarrhoea, dysentery, fever, malaria, parasites, rheumatism, skin rashes and worms[9]. Seeds of the fruits were used as an insecticide and larvicide for pest control[17]. Plant parts including fruits were reported for anticancer activity because of the presence of acetogenins, which is a new class of anticancer agents[18,19]. The recent report on this plant-leaves revealed the role of inhibiting the HepG2 cancer cell proliferation, but the results were not confirmed with animal models[20]. Though the fruits of *A. muricata* are mentioned in herbal medicine-books the anticancer activity has not been explored in HCC. Here we tried to explore its anticancer activity using HepG2-cell line and diethylnitrosamine (DEN) induced HCC in rats.

2. Materials and methods

2.1. Materials

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) and curcumin were purchased from Sigma-Aldrich, USA. DEN was purchased from TCI, India. Dulbecco's Modified Eagle's Medium (DMEM) and other chemicals required for the cell culture were obtained from HiMedia, Mumbai. Solvents for the extractions and phytochemical processing were purchased from Merck, India. The 5-FU was the gift from Cipla Pharmaceuticals, India. All the solvents, reagents and chemicals used were of laboratory grade.

2.2. Collection of fruits, extraction, and phytochemical standardization

The fruits of *A. muricata* were collected from Anchal and Channapetta near Kollam district, Kerala, India. The plant fruits were taxonomically identified by Dr. Alexander T, Research Associate (Environmental science), Environmental Resource Research Center, Trivandrum, India. A sample herbarium was prepared under the guidance of Dr. Richard Lobo, Professor, Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences and preserved with voucher number PP621. Fresh ripened fruits pulp was separated from the seeds, peels and dried in a hot-air oven at a temperature of 50 ℃. The dried material was then subjected to coarse powdering. The powdered material was subjected to Soxhlet extraction using ethanol. The concentrated and dried *A. muricata* fruit (AMF)-extract was used for the *in vitro* and *in vivo* assays. The *A. muricata* fruit extract was further subjected to fractionation with petroleum ether, chloroform, and ethyl acetate. Each fraction was dried in a desiccator. The petroleum ether fraction, chloroform fraction (CHL-AMF) and ethyl acetate fraction were further investigated for anticancer activity in HepG2 cells. The fraction of the *A. muricata* fruit extract was analyzed to determine the presence of active constituents such as phenolics, flavonoids, tannins, saponins, terpenoids, steroids, glycosides, anthraquinone, lipids, reducing sugar, amino acids, and peptides using appropriate standard methods for each of the constituents determined[21]. Further, the extract was standardized for quantitative consistency prior to use in the study by its ash value and total phenolic contents[22].

2.3. Assessment of in vitro anticancer activity

HepG2 cells were procured from National Center for Cell Sciences, Pune, India. The cells were sub-cultured in a tissue culture flask (T25 or T75) containing DMEM media supplemented with 10% FBS and 4.0 mg/L gentamycin (complete DMEM). The cells were maintained at 37 ℃ in a CO_2 incubator in an atmosphere of 95% air and 5% CO_2. The anticancer activity of the extract was determined by the MTT assay on HepG2 cells as per the standard protocol and 50% inhibitory concentration (IC_{50}) was determined[23]. The

induction of apoptosis was assayed by acridine orange/ethidium bromide staining (AO/EB staining). The changes such as chromatin condensation, membrane blebbing and nuclear fragmentation were observed under Nikon eclipse TS100 inverted-microscope with excitation filter 480/530 nm and emission filter 535/540 nm. The apoptotic index was determined for crude extract, its fractions and they were compared with the standard curcumin at their IC_{50} concentrations. The anti-metastatic activity was assessed by the scratch-wound assay[23]. The cell cycle analysis was determined as per the standard protocol using flow cytometer (Accuri C 6- Flow cytometer, BD Biosciences, USA)[24]. The ability of CHL-AMF to inhibit HepG2 cell proliferation *in vitro* was determined and during different cell cycle phases and the resultant inhibition was considered as representative for the anticancer potential of CHL-AMF extract. The results were compared to that of the standard inhibition by curcumin at dose ranges (1-100) μg/mL.

2.4. Animals

All the animal studies were conducted after obtaining the ethical clearance from the institutional animal ethics committee (No. IAEC/KMC/68/2015), Manipal Academy of Higher Education, Manipal India. Experiments were carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals. The inbred Wistar rats of both gender from Central Animal Research Facilities, Manipal Academy of Higher Education, were acclimatized to the room condition for a period of 7 d prior to the experiments. The rats were maintained in a room with controlled humidity and temperature at (23±3) ℃. The 12 h/12 h dark and light cycles were maintained. They were fed with standard rat-chow food and water ad libitum.

2.5. Acute oral toxicity in rats

An acute oral toxicity study was conducted as per OECD guidelines 425. Female Wistar rats were used for this study. Considering *A. muricata* extract is from the edible fruit, the limiting test was performed as per guideline 425 with 2 000 mg/kg dose. Animals fasted for a period of 4 h with water ad libitum and *A. muricata* fruit extract (2 000 mg/kg) was administered orally with a volume >2.0 mL. The animals were observed intensively for the first 30 min and then for 2 h each for a period of 2 d. Each rat was dosed with the extract by an interval of 72 h observed[25].

2.6. Induction of HCC in rats by DEN and assessment of anticancer activity

Male Wistar rats of 12-week old and (90-100) g weight were used for the DEN-induced HCC model. Rats were randomized as per their body weight into 4 groups with 6 rats each except in the Group 2 where 10 rats were kept. The purpose of keeping 10 rats was to see the induction of HCC after sacrificing one rat each on 4th, 8th, 12th and 16th week. The procedure for the induction of HCC in the rat was modified from the reported literature[26]. Briefly, Group 1: Normal control (NC), rats were fed with standard food and water. Group 2: was disease control (DC), rats were fed with 0.01% of DEN in drinking water for 14-weeks. Group 3: Pre-treatment group (AMF-Pre), rats were co-administered AMF-extract (200 mg/kg, *p.o.*) from the first day (0th week) with DEN in drinking water for 14 weeks. Group 4: Post-treatment group (AMF-Post), rats were induced HCC by administering DEN in drinking water for 8-weeks and then AMF-extract (200 mg/kg, *p.o.*) was co-administered with DEN in drinking water till the 14th week.

2.7. Parameters assessed for anticancer activity in HCC induced rats

2.7.1. Body weight and mortality rate

The body weights of rats in all the groups were monitored and the percentage increase in body weight was compared between the groups. The mortality in the rats was noted on each day. The survival rate in each group was noted and compared between the groups.

2.7.2. The relative liver weights and cancerous nodules

At the end of 14th week, rats were sacrificed, the liver was perfused with saline. The perfused rat livers were dried and weighed. The number of nodules present on the isolated liver was counted manually. The total cancer nodules were calculated based on the relative liver weight of rats. The results of each group animals were then compared.

2.7.3. Haematological parameters

Blood was withdrawn from the tail vein before sacrificing the rats and collected in anticoagulated vacutainers. The haematological parameters such as blood haemoglobin, total WBC, total RBC, the percentage of lymphocytes and monocytes were determined using veterinary cell counter (ERMA Inc, Tokyo). The results were compared between the groups.

2.7.4. Liver function test and oxidative stress

The liver function test was assessed by determination of serum aspartate aminotransferase (AST), alanine transaminase (ALT) and total bilirubin using commercially available assay kits (Aspen Laboratory, New Delhi, India) and semi-autoanalyzer (Star 21). The endogenous antioxidants (total proteins, superoxide dismutase (SOD) catalase, glutathione (GSH) and oxidants (lipid peroxidation end-product, thiobarbituric acid reactive substances were determined by the standard protocol in our laboratory[27]. The results were compared between the treatment groups.

2.7.5. Liver histopathology

The isolated livers were fixed in 10% buffered formalin and 3 liver lobes of one animal from each group were further processed for histopathological investigations. The liver histology from different treatment group rats were compared.

2.8. Statistical analysis

All the data in the treatment groups were presented as mean±SEM and statistical analysis was carried out by one-way ANOVA followed by *post hoc* Tukey's multiple comparison tests using Graph pad prism 6.0. (Trial version). The $P<0.05$ was considered statistically significant.

3. Results

3.1. Phytochemical standardization of AMF-extract

The yield of the AMF-extract was 55.3% of the dry fruits. The extract exhibited positive test for phenolics, flavonoids, tannins, terpenoids, and lipids. The extract did not show any reactions for other phytochemicals tested. The CHL-AMF exhibited lipid content, phenolic, and flavonoids as active constituents. The AMF-extract had ash-value of 7.15% W/W. In this, 1.25% W/W was water soluble and 0.25% W/W was acid soluble ash values. The total phenolic content in AMF-extract was 113.5 mg Gallic Acid Equivalent per 100 g of dry fruits extract.

3.2. In vitro anticancer activity of AMF-extract and its fractions

The anticancer activity was assessed by *in vitro* assay in HepG2 cells after 48 h of incubation of extract. The IC_{50} was calculated for *A. muricata* fruit extract, its fractions, standard 5-FU, and curcumin. The results are shown in Figure 1. The IC_{50} was found to be (274.9±8.3), (53.7±4.3), (341.4±6.7), (928.8±10.5), (292.0±7.4), (0.26±0.20) μg/mL, for AMF, CHL-AMF, EA-AMF, PE-AMF, 5-FU and Curcumin respectively. *A. muricata* fruit extract had dose-dependent and graded inhibition of HepG2-cell proliferation. The CHL-AMF was found to be most potent compared to other fractions, petroleum ether fraction and ethyl acetate fraction.

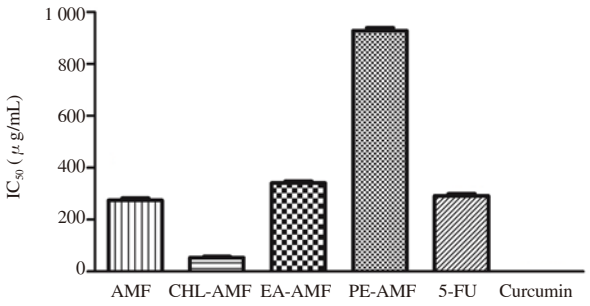


Figure 1. Actions of *Annona muricata* fruit extract and fractions compared to standards on HepG2 cells. Data represents 50% inhibitory concentration IC_{50} (μg/mL). Values are expressed as mean±SEM (n=3); AMF: *Annona muricata* fruits; CHL-AMF: Chloroform fraction of AMF; EA-AMF: Ethyl acetate fraction of AMF; PE-AMF: Petroleum ether fraction of AMF; 5-FU: 5-fluorouracil.

3.3. Anti-metastatic potency of CHL-AMF in the scratch-wound assay

The photo-microgram of the scratch wound and cell migration after 24 h and 48 h are represented in Figure 2. The percentage of cell migration and its inhibition by AMF-CHL are represented in graph inserted in Figure 2. The fraction CHL-AMF at 55 μg/mL dose had significant inhibition of cancer cell migration compared to curcumin at a dose of 0.81 μM (0.3 μg/mL).

3.4. Induction of apoptosis by CHL-AMF: AOEB staining of HepG2 cells

The photo-microgram of the apoptotic cells are shown in Figure 3 (A, B and C). The apoptotic index was represented by graph inserted in Figure 3D. The fraction CHL-AMF at a dose of 55 μg/mL exhibited significant apoptotic index which can be comparable to that of standard curcumin at dose 0.3 μg/mL on HepG2 cells.

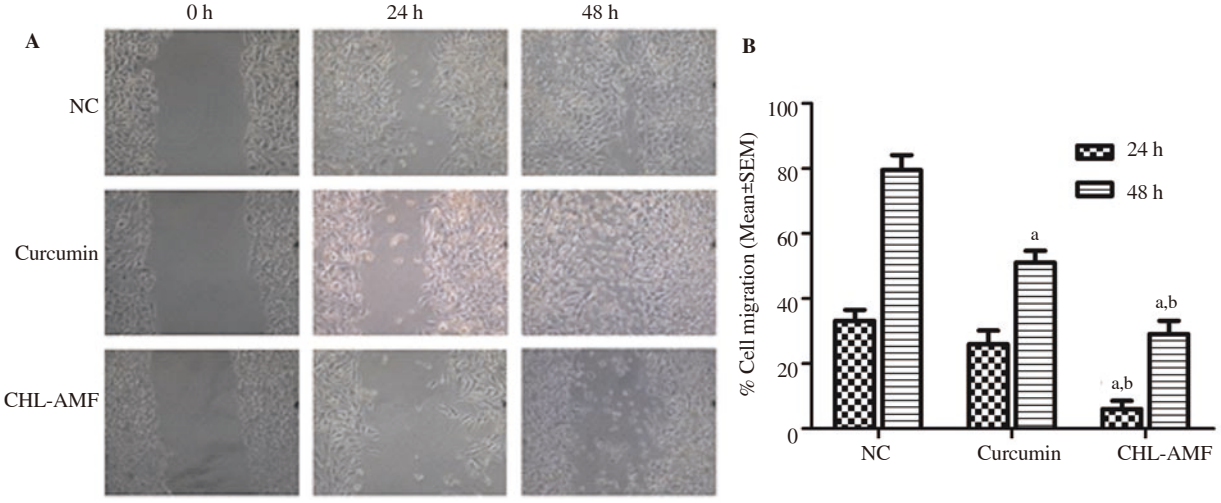


Figure 2. Effects of CHL-AMF on scratch wound antimetastatic activity. A: Photographs of the scratch wound and cell migration after 0, 24 and 48 h of exposure to CHL-AMF (55 μg/mL). B: Representation of % cell migration after 24 and 48 h of exposure to curcumin and CHL-AMF. Data were analysed by one-way ANOVA followed by *post hoc* Tukey's multiple comparison tests; [a]$P<0.05$ compared to normal control and b: compared to curcumin at dose of 0.3 μg/mL (NC: Normal control; CHL-AMF: Chloroform fraction of *Annona muricata* fruits).

3.5. Actions of CHL-AMF on of HepG2 cell cycle

Cell cycle analysis for fraction CHL-AMF, 5-FU and curcumin-treated HepG2 cell lines are shown in Figure 4. The table inserted in Figure 4, represented the percentage of cells in a different phase of the cell cycle. The fraction CHL-AMF arrested cells maximally in G_0/G_1 phases compare to S and G_2/M phase indicated that the extract acts at the preparatory phase of DNA synthesis.

3.6. Acute oral toxicities of AMF-extract

Upon oral acute toxicity studies, *A. muricata* fruit extract at a dose of 2.0 g/kg did not produce any mortality and visible toxicity such as signs of lacrimation, urination, tremors, oedema and change in skin colour or locomotion in female rats. Also, there was no mortality when these rats were observed for 14 d. Based on these findings and the available literature for the activity we have fixed the dose of 200 mg/kg, *p.o.* daily for all further studies in rats[28].

3.7. Effect of AMF-extract on DEN-induced HCC rats model

3.7.1 A. muricata fruit extract protected the body-weight loss and survival rate due to DEN-induced HCC in rats

The percentage of body weight gain of rats upon treatment are shown in Figure 5. There was a significant reduction in body weight in DC-group compared to the NC-group. The body weight loss was significantly seen after 8 weeks of induction with 0.01% DEN in drinking water. The reduction in body weight was seen in all the groups. The *A. muricata* fruit extract pre-treatment and post-treatment significantly prevented the loss of body weight.

The HCC-induction by DEN (0.01% in drinking water) produced fatality in rats and the deaths were observed after 8 weeks. In DC-group, one rat each was sacrificed in 4th, 8th and 12th weeks respectively to see the liver nodules and to analyse by histopathological observation. After 4-weeks of DEN administration, we could not find any nodules in the liver, confirmed by histopathological observations and there was no significant change in serum AST-ALT compared to normal animals. After 8-weeks, the plasma AST, ALT, and direct bilirubin were significantly increased in DC-group inferring the hepatic damage and carcinogenesis. Further, the post-mortem observation of the liver confirmed the presence of nodules and the death was due to cancer morbidity in DC-group.

After 8-weeks, there was 30.0% mortality in the DC-group, by 14-weeks the survival rate was 40.0%. In AMF pre-treatment group, the survival rate was 100% till 14th week, indicats that the *A. muricata* fruit extract pre-treated group protected death due to DEN-induced HCC. Whereas, post-treatment produced 71.5% survival after 14-weeks.

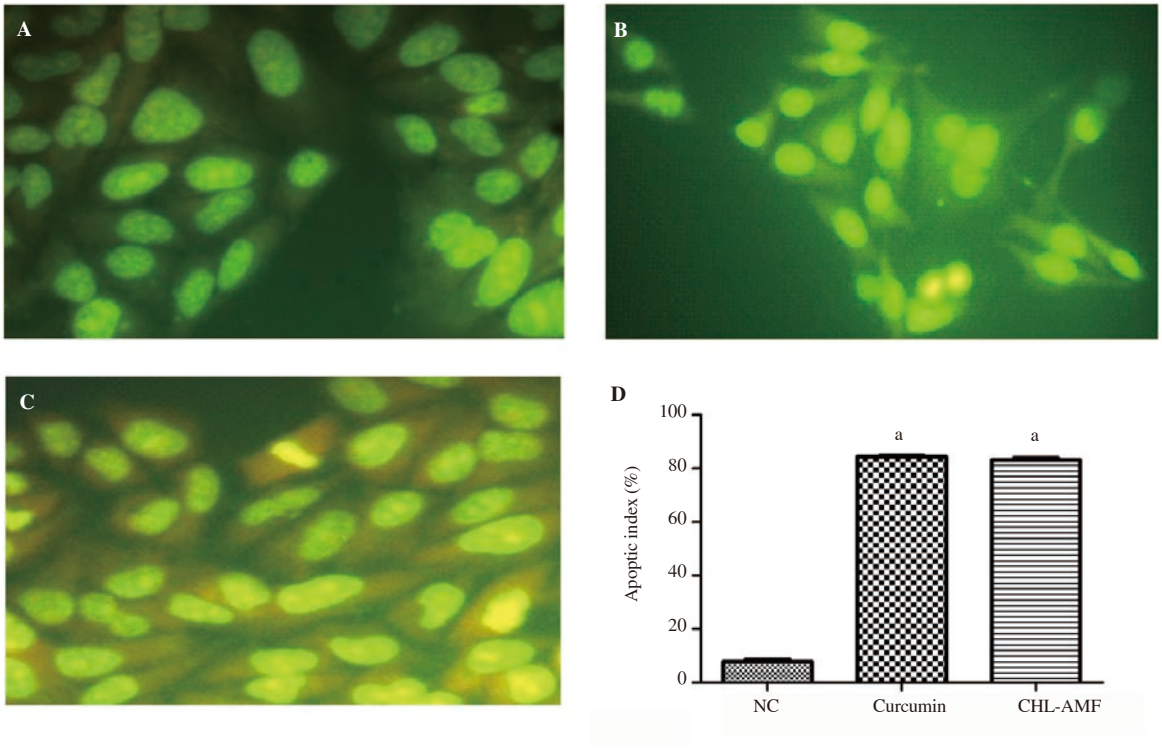
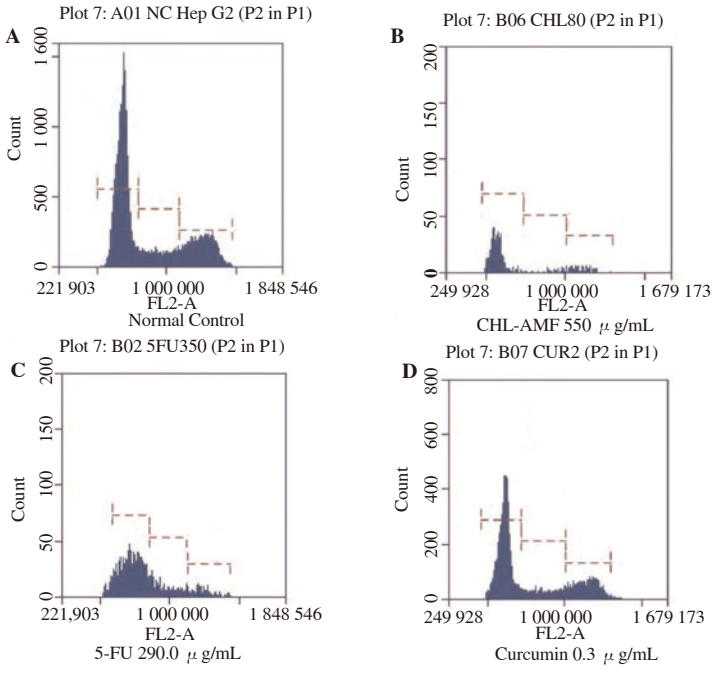


Figure 3. Effect of CHL-AMF on apoptosis: AO/EB staining of HepG2 cells and comparison with the effects of curcumin for apoptotic index. A: Normal HepG2 cell lines (40×); B: HepG2 cells treated with 55 μg/mL of CHL-AMF (10×); C: HepG2 cells treated with 0.3 μg/mL of curcumin (10×); D. Comparison of apoptotic index with that of *Curcumin* (graph insert). Data was analysed by one-way ANOVA followed by *post hoc* Tukey's multiple comparison tests; $^aP<0.05$ compared with NC.



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Group	G_0/G_1 phase(%)	S phase(%)	G_2/M phase (%)
NC	59.9±2.3	14.9±1.3	25.7±2.0
CHL-AMF	71.9±3.1	10.7±2.0	17.8±1.6
5-FU	63.4±2.1	20.9±1.9	11.2±1.1
Curcumin	59.3±1.8	15.7±1.2	24.8±2.4

Figure 4. Effect of CHL-AMF fraction on cell cycle of HepG2 cell proliferation by flow cytometry. A: Normal control without any treatment; B: Treated with CHL-AMF (55.0 μg/mL); C: Treated with 5-FU (290.0 μg/mL); D: Treated with curcumin (0.3 μg/mL); E: Table shows percentage cells in different phases of cell cycle (n=3; Values are expressed as mean±SD); NC: Normal control; CHL-AMF: Chloroform fraction of *Annona muricata* fruit; 5-FU: 5-fluorouracil.

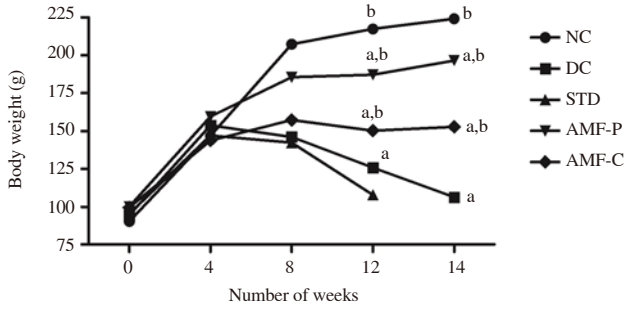

Figure 5. Effect of *Annona muricata* fruit extract on body weight of rats with HCC induced by DEN; NC: Normal control; DC: Disease control (DEN 0.01% in drinking water); AMF-Pre: AMF-extract pre-treatment (200 mg/kg, *p.o*); AMF-Post: AMF-extract pot-treatment (200 mg/kg, *p.o*); AMF: *Annona muricata* fruit extract; DEN: Diethylnitrosamine; Data was analysed by one-way ANOVA followed by *post hoc* Tukey's multiple comparison tests; [a]P<0.05 compared with NC; [b]P<0.05 compared with DC.

3.7.2. A. muricata fruit extract protects from HCC assessed by relative liver weight and number of liver nodules

The relative liver weight of DC-group and AMF post-treatment group were significant higher than that of the control group (Figure 6A). AMF pre-treatment significantly prevented the increasing liver weights nearly to normal as there was no significant difference compared to NC-group rats. The number of nodules were found to be significantly increased in rats of all groups compared to NC-group which did not show any liver nodules (Figure 6B). Both pre- and post-treatment by AMF-extract prevented the increase in number of liver nodules. But, compared to the NC-control rats the difference in the nodule was significant hence the extract could not completely protect the liver nodule formation and normalise the rats.

3.7.3. A. muricata fruit extract modulates haematological parameters and liver function test

The DEN-administration for 14-weeks in DC-group elevated the total-WBCs by 2.2-fold or induced leukocytosis (total WBC in Table 1). The pre-treatment with *A. muricata* fruit extract significantly protected the leukocytosis whereas, post-treatment could not significantly protect the leukocytosis induced by DEN. The DEN for 14-weeks reduced the RBCs and Hb in rats of group DC, AMF-pre and AMF-post compare to the NC-group. The AMF pre- and post-treatment prevented the decreased in RBC and Hb but there was significant difference compared to NC-group indicats that the treatment is not

Table 1. Effect of *Annona muricata* fruit extract on haematological parameters in DEN-induced HCC in rats.

Treatment groups	WBC ($\times 10^3$ μL)	RBC ($\times 10^6$ μL)	Hb (g/dL)	% Lymphocyte	% Monocyte
NC	7.57±1.06	9.59±0.18	14.13±0.20	79.30±2.49	9.80±1.27
DC	17.03±2.10[a]	6.29±0.23[a]	8.6±0.12[a]	46.80±8.65[a]	20.05±1.67[a]
AMF-Pre	10.33±0.50[b]	7.88±0.28[ab]	12.10±0.39[ab]	70.88±1.51[b]	11.53±0.64[b]
AMF-Post	14.28±0.11[a]	7.21±0.33[a]	10.75±0.49[ab]	71.03±0.91[b]	12.85±0.2[b]

Values expressed as mean±SEM; NC: Normal control; DC: Disease control (DEN 0.01% in drinking water); AMF: *Annona muricata* fruit extract; AMF-Pre: AMF-extract pre-treatment (200 mg/kg, *p.o*); AMF-Post: AMF-extract post-treatment (200 mg/kg, *p.o*); Data was analysed by one-way ANOVA followed by *post hoc* Tukey's multiple comparison tests; [a]$P<0.05$, When compared with NC; [b]$P<0.05$, When compared with DC.

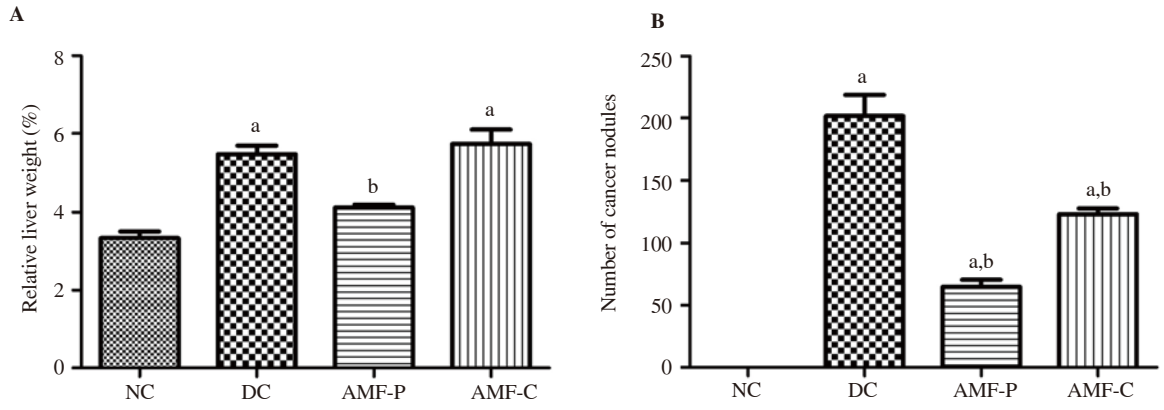

Figure 6. Effect of *Annona muricata* fruit extract on relative liver weight in DEN-induced HCC model. A: Relative liver weight; B: Number of cancer nodules; NC: Normal control; DC: Disease control (DEN 0.01% in drinking water); AMF: *Annona muricata* fruit extract; AMF-Pre: AMF-extract pre-treatment (200 mg/kg, *p.o*); AMF-Post: AMF-extract post-treatment (200 mg/kg, *p.o*); Data was analysed by one-way ANOVA followed by *post hoc* Tukey's multiple comparison tests; [a]$P<0.05$ compared with NC; [b]$P<0.05$ compared with DC.

effective in boosting the RBC synthesis (total RBC in Table 1). DEN significantly lowered the % lymphocytes in DC-control group compared to rats in NC-group. The pre and post-treatment by *A. muricata* fruit extract significantly normalised the % lymphocytes in blood. Similar results are obtained for % monocyte (Table 1).

The liver function test is a direct marker of liver damage and the induction of hepatocellular carcinoma by DEN. The liver enzyme such as AST ALT and direct bilirubin are listed in Table 2. There was a significant increase in plasma AST and ALT in rats of DC-group compared to NC-group. In pre-treatment group, there was a significant difference in AST compared to both NC and DC. This indicates that the pre-treatment protected the AST but could not normalise. In the post-treatment group, there was a significant difference compared to NC-group but there was no significant difference between DC-group rats. This indicates that the AST was not protected by post-treatment with *A. muricata*. The ALT levels were lowered upon *A. muricata* treatment in both pre-treatment and post-treatment group rats comparing with the DC group but ALT levels were not significantly normalised compared to the NC-control group rats (Table 2). The plasma bilirubin elevated significantly by the administration of DEN in DC-group compared to NC-group rats. The pre and post-treatment with *A. muricata* fruit extract could not significantly protect the elevation in bilirubin (Table 2).

Table 2. Effect of *Annona muricata* fruit extract on liver function in DEN-induced HCC in rats.

Treatment groups	AST (U/L)	ALT (U/L)	Direct bilirubin (mg/dL)
NC	124.9±1.90	95.24±1.99	0.368±0.04
DC	262.8±2.49[a]	181.6±6.45[a]	2.12±0.015[a]
AMF-Pre	217.3±7.86[ab]	120.1±2.69[ab]	1.78±0.10[ab]
AMF-Post	258.0±9.40[a]	129.6±5.60[ab]	1.88±0.09[a]

Values expressed as mean±SEM; Data was analysed by one-way ANOVA followed by *post hoc* Tukey's multiple comparison tests; [a]$P<0.05$, When compared with NC; [b]$P<0.05$, When compared with DC.

3.7.4. A. muricata fruits extract on the enhancement of liver proteins and endogenous antioxidant deference

The results of protein estimation and endogenous antioxidant enzymes are shown in Table 3. The proteins are decreased significantly in all the groups compared to NC-group rats. The enzyme catalase, SOD, and GSH were significantly reduced and TBARS significantly increased in the liver upon administration of DEN for 14-weeks in DC-group compared to NC-group rats. The *A. muricata* fruit extract could significantly protect catalase, SOD, and GSH in the liver both in pre-treatment and post-treatment group. Whereas, the oxidative stress marker TBARS was significantly lowered in pre and post-treatment groups compared to DC-group but it was not normalised when compared to NC-group rats.

3.7.5. Protection from HCC by A. muricata fruits extract in rat liver supported by histopathology

The photograph of histopathology is presented in Figure 7. The histopathology of NC-group rat liver had normal hepatic architecture such as hepatocytes with granular cytoplasm taken up the acidophilic stain and centrally located nuclei. The central vein, bile ducts are seen clearly with H&E staining. The DC-group histology looks distorted architecture, cellular swelling or blabbing, hyperplasic and dysplasia in hepatocytes with inflammatory cells infiltration. The arrow marks in Figure 7 represents the distorted features in histology indicats carcinogenesis. Whereas, in the histology of AMF pre-treatment and post-treatment groups, the dotted arrow indicates the protection from distorted architecture and carcinogenesis.

4. Discussion

The use of *A. muricata* fruits in protecting liver carcinogenicity by DEN was tested and reported in this manuscript. In the *in vitro* study, the chloroform fraction of the extract showed a better

Table 3. Effect of *Annona muricata* fruits extract on liver antioxidants in DEN-induced HCC model.

Treatment groups	Total protein (mg/dL)	Catalase (U/mg protein)	SOD (μmol/mg protein)	TBARS (nmol/mg)	GSH (μmol/mg)
NC	710.4±70.78	3.63±0.294	194.6±4.84	35.60±2.92	1.11±0.12
DC	601.0±36.86[a]	1.12±0.19[a]	110.2±8.43[a]	81.63±3.93[a]	0.69±0.09[a]
AMF-Pre	619.8±33.37[ab]	3.63±0.55[b]	192.1±17.2[b]	61.02±2.82[ab]	1.19±0.05[b]
AMF-Post	633.8±26.93[ab]	3.19±0.53[b]	190.5±8.72[b]	66.48 ±1.45[ab]	1.14±0.07[b]

Values were expressed as mean±SEM; Data was analysed by one-way ANOVA followed by *post hoc* Tukey's multiple comparison tests; [a]$P<0.05$ compared with NC; [b]$P<0.05$ compared with DC.

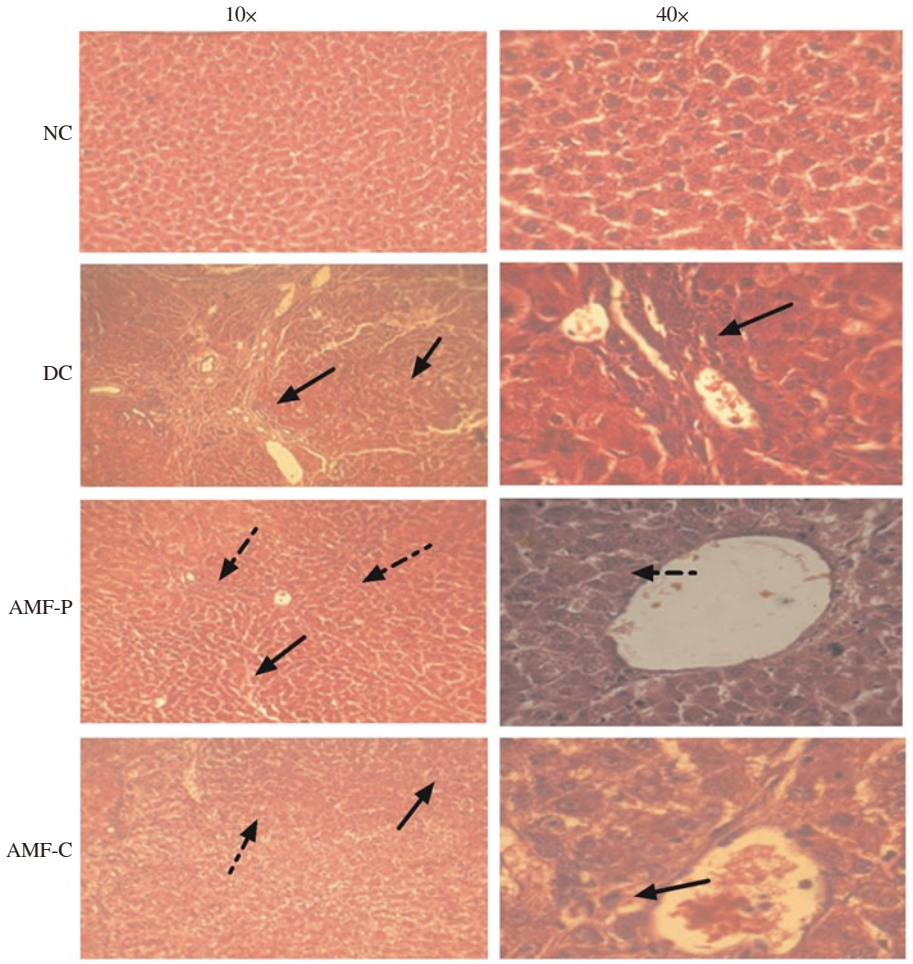

Figure 7. Histopathology of liver isolated from Wistar rats with DEN-induced HCC. Black arrow indicates the damage to the tissue and dotted arrow indicate the protection of tissue; NC: Normal control; DC: Disease control (DEN 0.01% in drinking water); AMF: *Annona muricata* fruit extract; AMF-P: AMF-extract prophylactic treatment (200 mg/kg, *p.o*); AMF-C: AMF-extract curative treatment (200 mg/kg, *p.o*).

cytotoxic activity compared to petroleum ether and ethyl acetate fractions. This activity was comparatively less than Curcumin. The active phytochemicals in *A. muricata* fruits such as acetogenins are more soluble in chloroform fraction, hence the chloroform fraction exhibited better activity among the fractions. Acetogenins are the polyketide compounds having anticancer activity and they are reported in *A. muricata* fruits[29]. Scratch wound healing assay is generally used to determine the antimetastatic potential of extract or synthetic compounds[23]. It explains that the *A. muricata* fruit extract can prevent spreading or migration of cells from the confluent area to the wound area. Further, the extract fraction could inhibit the apoptosis studied by AO/EB staining. Thus, it can be confirmed that the cancer cells are undergoing apoptosis when they are exposed to chloroform fraction of the extract. Further studies warrant to investigate the pathways for the induction of apoptosis. In cell cycle analysis, G_0/G_1 phases of the cell cycle are said to be the quiescent phase and preparatory phase for DNA synthesis respectively. G_1 phase is the preparatory phase for the DNA synthesis and it was checked by the G_1 checkpoint. Cell size, proteins needed for DNA synthesis and any mutations in the proteins are checked by the G_1 checkpoint, in case of any kind of dysfunctions or errors the cell growth-process will get arrested in the G_1 check-point through inhibiting cyclins to bind with a cyclin-dependent kinase[30]. Further studies required to establish the actions of *A. muricata* fruit extract to inhibit cyclin-dependent kinase or the mutation of proteins required for DNA synthesis.

Initially, when we tested the fractions for cytotoxicity by MTT assay, both 5-FU and curcumin were used as the standard for comparison. Further, both standards were used for cell cycle analysis. We found the curcumin was superior to 5-FU may because of its action at multiple targets[31]. The CHL-fraction fo *Annona muricata* fruits have multiple chemical components which are similarly inducing apoptosis when tested by AO/EB staining and inhibiting the cell migration in scratch wound healing assay.

Weight loss is a symptom of cancer induction by DEN[32]. We found, the pre- and post-treatment with *A. muricata* extract could significantly prevent body weight loss. The prevention of body weight loss could also because of increases in the appetite or through preventing the action of DEN-induced liver damage and inflammation, require further investigation. DEN is a liver toxicant that induces cancer of the liver, which disturbs the normal functioning of the liver. The continuous contact of the toxicant to the liver causes inflammation, cirrhosis scarring of the liver and finally cancer. The end stage of liver cancer the liver function gets disturbed, liver enzymes parameters elevated and death at the end. As per the literature, 4-weeks of induction the animals started showing the symptoms of liver cancer. Further, the literature has a varied opinion on the induction period by DEN in animals[33]. In the present study, the survival rate of rats was in accordance with the protection from a decrease in body weight during the treatment period. The mortality rate could be correlated to the decrease in body weight gain. The crude *A. muricata* fruit extract can be correlated with improving the quality of life and survival rate in a human being if it is investigated in clinical trials.

The *A. muricata* fruit extract will be beneficial in preventing relative liver weight gain which happens in hepatocellular cancer. In this study, the relative liver weight was significantly high in DEN-induced HCC in rats. This finding was also reported in the earlier literature[34]. The cytochrome-P450 and NADPH-P450 reductase will convert DEN into α-hydroxyl nitrosamine and then dissociate into acetaldehyde and electrophilic ethyldiazonium ion[35]. This product undergoes alkylation reaction and forms DNA-adduct with nucleophilic bases of DNA leads to mutation and carcinogenicity. In the process, superoxide and hydroxyl radical generated cause membrane lipid peroxidation which triggers arachidonic acid cascade thereby activating epoxyeicosatrienoic acids pathway in the presence of cytochrome P450-epoxygenase. This pathway triggers cell proliferation, which aids increase in liver weight and nodules[36]. The AMF-extract has the ability to protecting the induction of cancer can also be contributed to scavenging the free radicals and by preventing the mutation of DNA. In this direction, the *A. muricata* fruit extract can be further studied for the NRF2 mediated oxidative stress modulation and immune modulation to prevent cancer[37]. Most of the literature reports leucocytosis are due to chronic inflammation in the liver induced by DEN. This remarkable increase could also be due to the initiation of leukaemia by DEN. There are no reports on the induction of leukaemia in Wistar rats by DEN. This discussion requires further studies to have conclusive evidence. The prophylactic *A. muricata* fruit extract significantly protected the leukocytosis whereas, curative treatment could not protect the leukocytosis induced by DEN compared to DC. Hence, *A. muricata* fruit extract is a good immunomodulator along with the anticancer activity.

DEN for 14-weeks reduce the RBCs and Hb levels in rats. The prophylactic treatment *A. muricata* fruit extract could protect the erythropenia induced by DEN whereby, the RBCs and Hb were in normal range. Whereas, the post-treatment was not found to be effective in maintaining RBC count. DEN significantly lowered the % lymphocytes in rats compared to untreated rats. The pre-treatment by *A. muricata* fruit extract significantly normalised the % lymphocyte and % monocyte.

The liver function test is a direct marker of liver damage and the induction of hepatocellular carcinoma by DEN. There was a significant increase in plasma AST and ALT in HCC rats. Pre-treatment or post-treatment by *A. muricata* fruit extract did not had conclusive results if the plasm AST, ALT and bilirubin are considered. Increased bilirubin in the blood is one of the markers for liver damage. In the present study, plasma bilirubin elevated

significantly by administration of DEN. But the pre or post-treatment with *A. muricata* fruit extract did not had any conclusive results in current study. From our experiment, we found the *A. muricata* fruit extract modulates SOD, catalase and GSH activity in liver. These actions require further studies to establish the nuclear mechanism of action such as NRF2-signaling[37]. The TBARS is an indicator for lipid peroxidation. The elevated MDA can induce DNA adduct formation, which results in a mutation that ultimately leads to cancer. For this reason, *A. muricata* fruit extract can be the best alternative antioxidant supplement for cancer treatment[38].

The current pharmacotherapy of hepatocellular cancer has limited options. The *A. muricata* fruit extract could be an alternative which requires further exploration. The fruit's pulp of *A. muricata* is edible[9]. Hence, it can also be developed into a dietary supplement to the liver cancer patient.

We have concluded that the *A. muricata* fruits are a good source of anticancer agent. We found the fruit pulp extract and its chloroform-fraction possessing significant anticancer activity in HepG2 cells and in DEN-induced HCC rats model. Our finding confirmed the induction of apoptosis and cells will be arrested at phase G_0/G_1 in the HepG2 cell cycle. Studies in rat-model of HCC confirmed its efficacy. It can be studied for radical screening for HCC with the detailed mechanistic approach. Further, phytochemical isolation and standardization of the extract are necessary to have a clear idea for clinical explored or clinical efficacy studies.

Conflict of interest statement

Authors declare that they have no conflict of interest.

Acknowledgements

Authors like to acknowledge the Manipal Academy of Higher Education, Manipal for infrastructure and Central Animal Research Facilities. Authors like to thank Dr Alexander T., Research Associate (Environmental science), Environmental Resource Research Center, Trivandrum, India, for taxonomically identifying, making a botanical specimen and supplying the raw ripened fruits. Authors would like to thank Dr. Richard Lobo, for helping in preparing and preserving the herbarium sample.

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