

Chemopreventive Action of *Garcinia Mangostana* Linn. on Hepatic Carcinoma by Modulating Ornithine Decarboxylase Activity

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History

- Submission Date: 25-05-2020;
- Review completed: 22-07-2020;
- Accepted Date: 03-08-2020.

DOI : 10.5530/pj.2020.12.191

Article Available online

<http://www.phcogj.com/v12/i6>

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ABSTRACT

Ornithine decarboxylase and alpha-L-fucosidase over-expression is associated with advanced hepatocellular carcinoma (HCC) development. The objective of this study was to elucidate the action of *Garcinia Mangostana* fruit extract (GME) on these overexpressed enzymes and apoptotic proteins in diethyl nitrosamine (DEN) induced hepatocellular carcinoma (HCC) rats. The cancer was induced using DEN to the experimental rats and treated with GME (400 mg/kg) to find its anticancer property. The lysosomal enzymes such as alpha-L-fucosidase, beta-D-glucosidase, ornithine decarboxylase activity (ODC), apoptotic and antiapoptotic proteins such as Bcl-2, Bax and Bcl-xL and H³ thymidine incorporation assay were done to prove GME's chemo preventive property. DEN induction caused significant increase in the activities of ornithine decarboxylase; lysosomal enzymes and increased cell proliferation with decreased apoptosis were observed. In contrast, the groups with GME treated rat's elicited significant ($P < 0.001$) reduction in ornithine decarboxylase, lysosomal enzymes, and decreased cell proliferation with increased apoptosis. GME has effective chemo preventive property and can serve as an anticarcinogenic therapeutic drug against hepatocellular carcinoma.

Key words: Diethyl nitrosamine, *G. Mangostana* extract, Ornithine decarboxylase, Hepatocellular carcinoma.

INTRODUCTION

Hepatocellular carcinoma (HCC) holds the second place in cancer mortality cause in the world population.¹ HCC is the most common liver cancer and among the most fatal and prevalent malignancies in humans. Ornithine decarboxylase (ODC) is the first rate-limiting enzyme in the polyamine biosynthesis pathway in mammals and its intracellular concentration is tightly controlled. ODC activity is induced in response to cell growth stimuli and is highly expressed in diseases such as inflammation and cancer.² Apoptotic cell death is documented as a critical event in chemical-induced hepatocyte cell death and development of HCC.³ In hepatocarcinogenesis several Bcl-2 family members have been implicated along with coupling apoptosis regulation and cell proliferation in distinctive ways.^{4,5} Considering the narrow treatment options and prognosis of HCC, chemoprevention has considered being the best approach in lowering the morbidity and death associated with HCC. Epidemiologic evidence has exposed the chemo preventive effect of the many medicinal plants and their phytochemicals in the prevention of the cancer disease.

HCC can be triggered in experimental animal models using a solitary postnatal shot of the chemical carcinogen diethyl nitrosamine (DEN).⁶ DEN induces chromosomal aberrations, micronuclei and chromatid exchanges in the liver

and these mutations are responsible for the gradual events of carcinoma such as lysosomal enzymes changes and ornithine decarboxylase activity.⁷ The rat model of DEN induced carcinoma is the foremost accepted and widely used experimental model to study the chemo preventive and chemotherapeutic potential of many drugs.

The *Garcinia mangostana* Linn. (mangosteen) is a tropical fruit native of Southeast Asia and reported to contain more health promoting properties. It reduces the risk of many human ailments such as dysentery, eczema and skin related problems due to the presence of polyphenols, xanthones and other important bioprotective constituents. They play a very important role in the protection of liver, intestine, bone and cartilage functions.^{8,9} The bioactive compounds of the mangosteen showed anticancer property against colon preneoplastic lesions, human leukemia, hepatoma, breast, lung and gastric carcinomas cells.¹⁰

The previous study in our laboratory proved the anticancer efficacy of *Garcinia mangostana* extract (GME) against DEN induced hepatocellular carcinoma in rats.¹¹ This study served as the background for our current research work. Therefore, the study was to evaluate the potential role of GME on hepatocellular cancer in rats to monitor the lysosomal enzymes, cell proliferation, apoptotic proteins and ornithine decarboxylase activities in liver tissues.

Cite this article: Priya VV, Hussain S, Balakrishna JP, Gayathri R, Mohan SK. Chemopreventive Action of *Garcinia Mangostana* Linn. on Hepatic Carcinoma by Modulating Ornithine Decarboxylase Activity. Pharmacogn J. 2020;12(6):1383-8.

MATERIALS AND METHODS

Test drug and chemicals

G. mangostana pericarp extracts powder was obtained from Avasthagen Company, California, USA as a compliment and was applied for the present investigation. All other chemicals used were of analytical grade.

Animals

Specific and pathogen free Wistar strain rats were chosen for this study. They were obtained and acclimatized to laboratory environment. They were housed in polypropylene cages with stainless steel grid covers and bedding material used is paddy or wheat husk. They were groomed in a controlled environment with feed and drinking water provided in polypropylene bottles. Experimental protocols were approved by Institutional Animal Ethics Committee (IAEC), which follows the guidelines of CPSCEA (Committee for the purpose of control and supervision of experiment on Animals) and the Guide for the care and use of laboratory animals. The IAEC approval number for this study was: Biochem: 005/2008 & 01/003/10.

Test compound and dose administration levels

As the toxicity of the plant *Garcinia mangostana* Linn. was determined in earlier studies, the drug dose was chosen accordingly. Four groups of 6 rats each were used for the study. Group I-Normal rats received distilled water (1 ml/kg b.wt); Group II: Rats induced with diethyl nitrosamine (DEN) (0.01% DEN through drinking water upto 16 weeks); Group III: Rats induced with DEN (0.01% DEN through drinking water up to 16 weeks and cotreated with GME orally (400 mg/kg wt).¹¹ Group IV: Rats treated with GME (400 mg/kg wt) alone. Food and water were accessible ad libitum. Animals were sacrificed after 16 weeks and livers were dissected and then excised samples of lobes were used for further estimations. Extraction of the target enzymes from liver tissue: 300 mg of liver tissue was homogenized with 6 ml ice-cold distilled water as described elsewhere.¹² Total protein was determined in each enzyme substrate prior to enzyme assessment, by the method Lowry *et al.*¹³

Biochemical analyses

Lysosomal enzymes such as Alpha-L-fucosidase¹⁴ and beta-D-glucosidase¹⁵ were measured using previously described method. ODC activity was determined using 0.4 ml hepatocellular 105,000×g supernatant fraction per assay tube by measuring release of $^{14}\text{CO}_2$ from DL-[^{14}C] ornithine by the method of Athar *et al.*¹⁶

H^3 thymidine incorporation

H^3 thymidine incorporation study was done using tissue homogenate, 20 μl of the same was taken and 0.5 μCi of H^3 -thymidine per well was added. After 4 hours of incubation, the medium was removed, and cell pellets were washed twice with cold 0.05 M Tris-HCl and 5% trichloroacetic acid, scrapped and transferred to scintillation cocktail. The level of incorporated H^3 -thymidine was assessed using Beckman liquid scintillation counter.

Estimation of Bcl-xl, Bax &Bcl-2

The quantification of Bcl-xl, Bax and Bcl-2 protein in liver tissue was done by ELISA-based method, as previously validated by Barrow *et al.*¹⁷ First, 96-well microplates were coated with Bcl-2 mouse antibody (diluted 1:200 in pH 9.6 bicarbonate buffers, Santa Cruz) and incubated at 4°C overnight. Plates were then blocked with 1% Blotto non-fat dry milk in pH 7.2 PBS at RT for 1 hr and rinsed in PBS 3 times. The normalized mitochondrial samples were then added to the wells, and the microplates were incubated with Bcl-xl antibody at 4°C for a second night. The plates were again rinsed 3 times in PBS, Bax rabbit IgG antibody (diluted 1:200 in PBS, Santa Cruz) was added to the wells,

and the plates were again incubated overnight at 4°C. After washing again with PBS 3 times, the anti-rabbit secondary antibody conjugated to alkaline phosphatase (diluted 1:1000 in PBS) was added to the wells, which were incubated at 4°C for the fourth and final night. The wells were then rinsed with TBS to ensure inorganic phosphate could not inhibit alkaline phosphatase. Then, p-nitrophenyl phosphate, a phosphatase substrate, was dissolved in pH 10.4 glycine buffer (2 mg/ml) with 1mM ZnCl_2 and was added to the wells. The microplates were then incubated in the dark for 30 min at RT and absorbance was read at 415 nm using a BioRad microplate reader.

Statistical analysis

Results were expressed as mean \pm S.E.M. Statistical significance was determined by one-way analysis of variance (ANOVA) and post hoc least-significant difference test. P values less than 0.05 was considered significant.

RESULTS

Effect of GME on lysosomal enzymes

The alpha-L-fucosidase and beta-D-glucosidase activities were increased in DEN induced liver tissue. GME treatment markedly decreased its level when compared with the DEN induced hepatocellular tissue. DEN and GME (400mg/kg b.wt) co-treated liver tissue decreased the lysosomal enzyme levels when compared with the respective DEN induced group (Figure 1).

Effect of GME on Ornithine decarboxylase and H^3 thymidine assay

A significant ($***P<0.001$) increase in ornithine decarboxylase level and H^3 thymidine incorporation were evaluated in DEN administered rats (Group II) which indicates cell proliferation, inflammation and cancer when compared with those in control rats (Group I) in which it was not detected. The DEN induced alterations in the enzyme levels DEN were significantly ($^*P<0.001$) suppressed in GME treated rats (Figures 2 & 3).

Effect of GME on apoptotic proteins

The levels of pro apoptotic and antiapoptotic proteins were analysed in control and experimental rats (Figure 4). Upon DEN induction the apoptotic protein Bax was downregulated and antiapoptotic proteins Bcl-2 and Bcl-xl was significantly ($^*P<0.001$) upregulated. Administration with GME (400 mg/kg b.wt) induced apoptosis yet after the induction of DEN with increasing Bax and decreasing Bcl-2 and Bcl-xl expressions.

DISCUSSION

Dysregulation of apoptosis is a vital reason for HCC progression, initiation of tumour; metastasis and maintenance normally are mediated by changes in apoptosis-related proteins.¹⁸ Some herbal extracts could ameliorate anticancer-induced lysosomal abnormalities, conserving lysosomal integrity, probably, through an antioxidant mechanism. The synthesis of proteins by the tumour with consequent increased fucose turn over.¹⁹ Alpha-L-fucoidase, lysosomal enzyme remains a gold standard hepatoma marker. The autophagy-lysosomal pathway is involved in the turnover of damaged organelles and misfolded proteins.²⁰ Because of this role of autophagy in cell homeostasis and its relationship with apoptosis, we assessed the levels of lysosomal enzymes such as beta-D-glucosidase during DEN induction and upon GME treatment. In this study the levels of lysosomal enzymes was increased during DEN induction, indicating the autophagy mechanism favouring tumour promotion and oncogenesis. On treatment with GME the levels of lysosomal enzymes decreased indicating protective effect of GME on liver cancer. Even though the crosstalk between

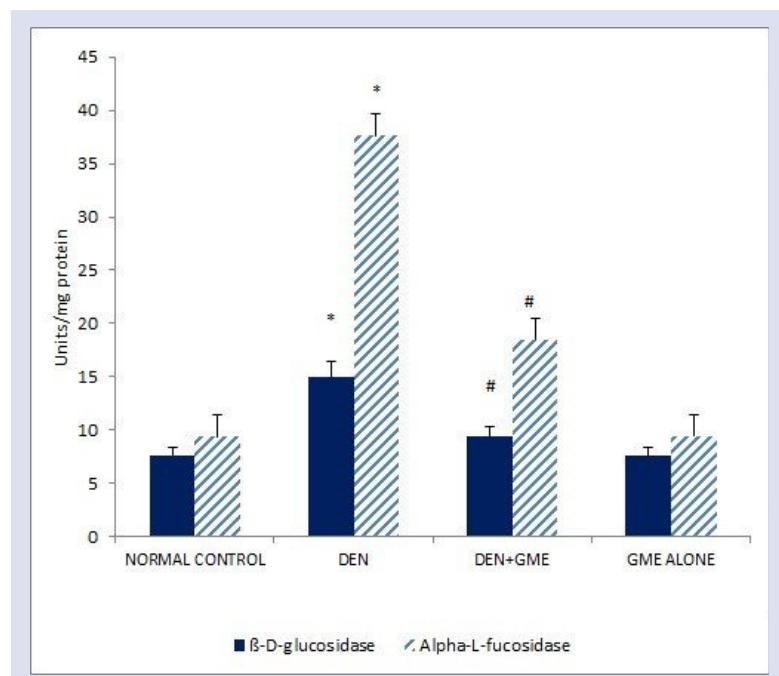


Figure 1: Effect of *G. mangostana* extract on lysosomal enzymes activities in hepatocellular carcinoma tissues.

Results are expressed as mean \pm S.E.M, n = 6. *P < 0.001, statistically significant as compared with control rats and #P < 0.001 statistically significant as compared with DEN control group.

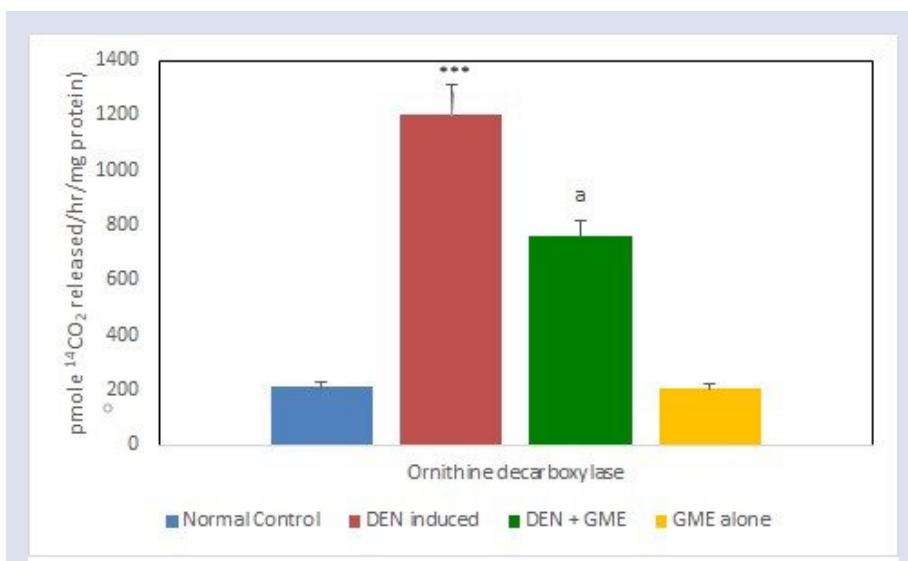


Figure 2: Effect of *G. mangostana* extract on ornithine decarboxylase activity in hepatocellular carcinoma induced rats.

Results are expressed as mean \pm S.E.M, n = 6. ***P < 0.001, statistically significant as compared with control rats and ^aP < 0.001 statistically significant as compared with DEN treated rats.

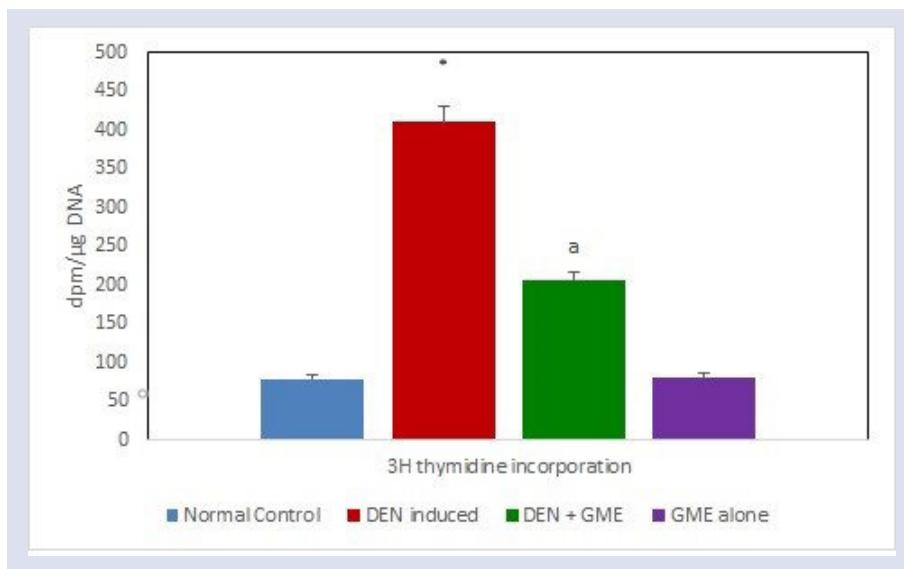


Figure 3: Effect of *G. mangostana* extract on ^3H thymidine incorporation in hepatocellular carcinoma tissues.

Results are expressed as mean \pm S.E.M, n = 6.*P<0.001, statistically significant as compared with control rats and ^aP< 0.001 statistically significant as compared with DEN treated rats.

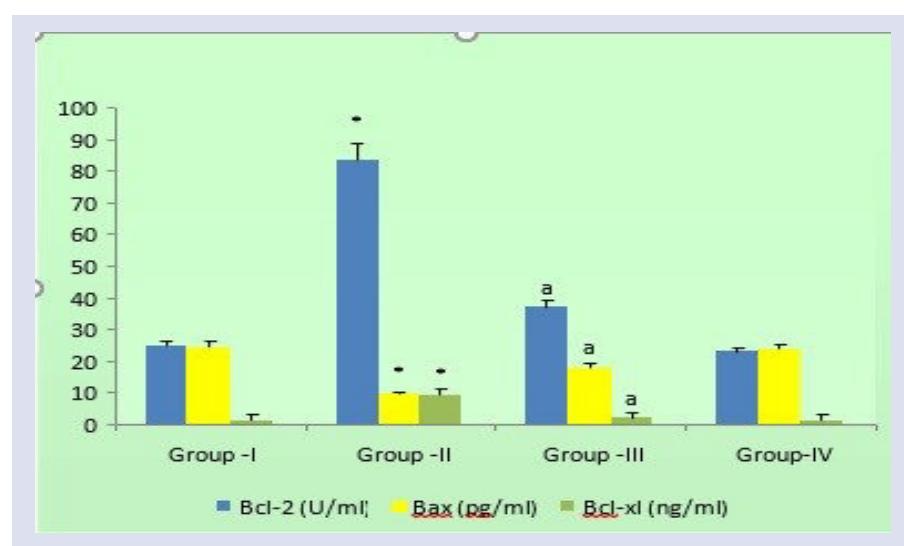


Figure 4: Effect of *G. mangostana* extract on apoptotic and anti- apoptotic proteins.

Results are expressed as mean \pm S.E.M, n = 6.*P<0.001, statistically significant as compared with control rats and ^aP< 0.001 statistically significant as compared with DEN treated rats.

autophagy and apoptosis is not well defined, a relationship has been established due to the interaction of different autophagy and apoptosis-related proteins.^{21,22}

ODC is considered to be a possible oncogene because over-expression of ODC can transform mammalian cell lines²³, indicating that ODC is a biomarker and a potential target for cancer therapy. In the present study, we investigated the functions of ODC on GME treatment during HCC development. Our data showed that ODC levels was higher in HCC tissues and on treatment with GME decreased ODC activity was seen, indicating that GME might be a promising target for HCC therapy by modulating the ODC activity.

To explain the basis of ODC as a tumor promoter in HCC progression, we examined apoptosis in HCC. Our results showed that down regulation of ODC expression with GME treatment significantly increased total apoptosis in HCC tissues. Impairment of apoptosis

plays a central role in development of cancer and limits the efficacy of conventional cytotoxic therapies. The Bcl-2 family members are a group of proteins playing a central role in apoptosis, because they form the interface between two events. The Bcl-2 family is divided into 2 sub-families: anti-apoptotic proteins (e.g.Bcl-2, Bcl-xL) and pro-apoptotic proteins (Bax, Bak, and Bok).The early signalling event engages the cells towards apoptotic process, and the later event conferring their apoptotic characteristics to the cells and elimination by the immune system.²⁴

A substantial number of cells escape from apoptosis through up-regulation of these antiapoptotic proteins Bcl-2 and Bcl-xL expression.²⁵ The balances between Bcl-2 to Bax as opposed to the levels of the individual proteins are thought to be basic in deciding cell survival or termination.²⁶ A reduced Bcl-2/Bax proportion will promote apoptosis.²⁷ In the present study, polyamine depletion by DEN

induction downregulated the Bax along with upregulation of Bcl-2 and Bcl-xL. On treatment with GME enhanced the expression levels of Bax and at the same time, the expression of Bcl-2 and Bcl-xL was decreased with GME treatment. Our results confirmed that GME by inhibiting polyamine synthesis in HCC resulted in cell death due to apoptosis.

The rate of synthesis of DNA was measured using H^3 -thymidine incorporation assay. GME decreases H^3 -thymidine incorporation in DEN induced group which revealed that GME significantly inhibited cell proliferation and promoted apoptosis. The overall mechanism in this study was, in DEN induction there was an increased inflammation and ROS release which had increased ODC levels initiating cellular proliferation which was proved by thymidine incorporation assay, increased antiapoptotic molecules and increased lysosomal enzymes. Previous study was conducted using the DEN carcinogen to enlighten the hepatoprotective effect of pericarp extract of *Garcinia mangostana* Linn in combating the toxins mediated in the biomedical alterations in rats by carcinogen.^{11,28} GME effectively decreased the proliferation by creating apoptotic environment which was supported by increased apoptotic proteins with decreased ODC, thymidine incorporation and lysosomal enzymes proving GME have an effective apoptotic inducing property confirming GME is an anticarcinogenic drug which could be using in promising treatment of HCC.

CONCLUSIONS

The improvement of ODC, lysosomal enzyme activity and the enlistment of apoptosis were fundamental to the chemotherapeutic impact of GME. Understanding that there are no successful treatment measures for HCC, our outcomes recommend the potential utilization of mangosteen in chemoprevention of HCC.

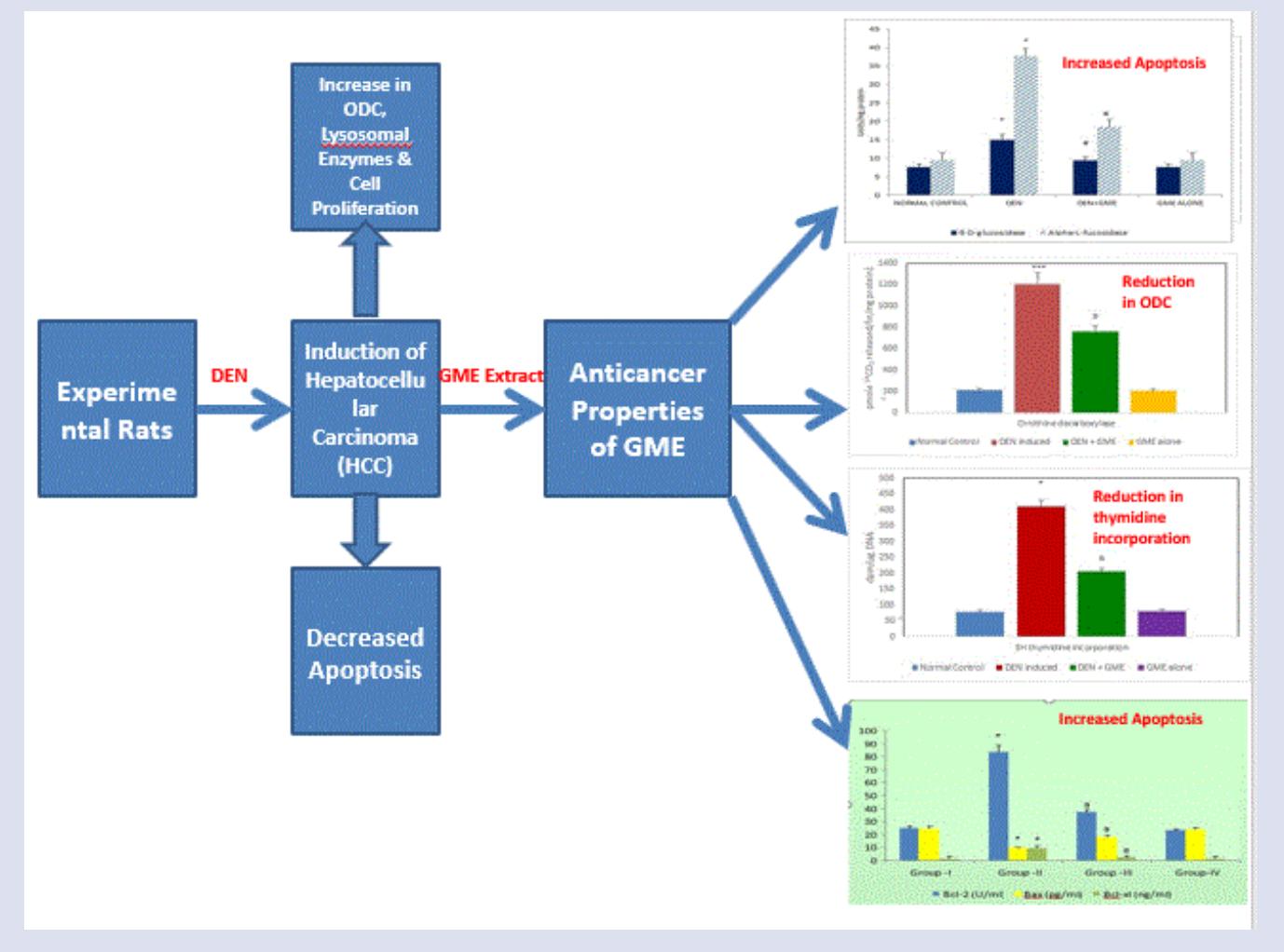
CONFLICTS OF INTEREST

The author's declare that no conflicts of interest.

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GRAPHICAL ABSTRACT



Cite this article: Priya VV, Hussain S, Balakrishna JP, Gayathri R, Mohan SK. Chemopreventive Action of *Garcinia Mangostana* Linn. on Hepatic Carcinoma by Modulating Ornithine Decarboxylase Activity. *Pharmacogn J.* 2020;12(6):1383-8.