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Asarone and metformin delays experimentally induced hepatocellular carcinoma in diabetic milieu

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ABSTRACT

Aims: The evidence suggests that the hyperglycemia and hyperinsulinemia of diabetes mellitus (DM) are risk factors for the development of hepatocellular carcinoma (HCC). The aim of the present study was to examine the effect of streptozotocin (STZ)-induced DM on promoting diethylnitrosamine (DEN) induced HCC in male wistar rats. Further, we investigated the administration of (α)-and (β)-asarone and metformin HCl on experimentally induced diabetic-hepatocellular carcinoma.

Materials and methods: Diabetes was induced by single dose of STZ (55 mg/2 ml/kg b.w. *i.p.*) and HCC by single dose of DEN (200 mg/ml/kg b.w. *i.p.*). Another group received the STZ followed by DEN two weeks later to mimic diabetic-HCC. The combined dose of (α)-and (β)-asarone (50 μg/1.5 ml/kg b.w. *p.o.* in the ratio of 1:1) and metformin HCl (250 mg/1.5 ml/kg b.w. *p.o.*) treatment was compared with the STZ + DEN group. The blood and liver samples were collected at the end of 12 and 18-weeks to study biochemical and histopathological changes in liver.

Key findings: The STZ induced diabetes promoted the tumor progression due to administration of DEN. The treatment of asarones and metformin significantly reduced the levels of glucose, glycosylated hemoglobin, liver dysfunction markers and tumor biomarkers along with an increase in level of insulin when compared to diabetic-HCC group. Histopathological examination indicated that asarones and metformin attenuate the inflammation, fibrosis, cirrhosis and development of spontaneous HCC.

Significance: The STZ can be used to promote the DEN induced HCC. Treatment with (α)-and (β)-asarone attenuates the effect of STZ + DEN induced HCC akin to metformin.

1. Introduction

The improper utilization or secretion of insulin leads to defective carbohydrate, protein and fat metabolism in diabetes mellitus (DM) [1–3]. The long-standing DM leads to the development of classical nephro, neuro and vascular complications. Beyond these, the epidemiologic evidence suggest that the DM is correlated with a higher incidence of cancer of various organs, including pancreas, liver, breast, colorectal, urinary tract, stomach and reproductive organs [4]. The increased chance of incidence and development of malignant hepatocellular carcinoma (HCC) in the patients of DM is suggested by various case-control, cohort and retrospective observational studies. Further, it is also suggested that the DM might play a role of an independent risk factor for the progression of HCC [5–7].

Liver undergoes histopathological and biochemical changes during streptozotocin (STZ) induced diabetes. The biochemical changes associated with liver injury during diabetes are increased levels of liver function markers (alanine transaminase, aspartate transaminase, and alkaline phosphatase) as well as lipid risk ratios. Histologically, the STZ leads to accumulation of fat or glycogen into the hepatocytes and causes hypertrophy [8]. At subcellular level, a significant reduction in rough endoplasmic reticulum with decreased densities of pyknotic nuclei, damaged nuclear membrane and an increase in mitochondrial volume fractions are observed [9,10]. These changes accompanied by other pathological conditions impact the collagen spectrum of the liver and lead to the development of fibrosis and cirrhosis [11–13].

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The complex pathophysiological association between HCC and DM is manifold. The hyperglycemia, insulin resistance and hyperinsulinemia act as risk factors either independently or synergistically for the development of HCC in diabetes. Majority of reports elucidate the link between high insulin level and HCC and few epidemiological studies support hyperglycemia as a cause for the progression of HCC [14,15]. Beside these, literature also suggests that the mechanistic link may be due to elevated levels of insulin-like growth factor (IGF)-1, obesity, chronic inflammation (cytokines) and other than metformin anti-diabetic drugs such as insulin analogues, secretagogues (sulfonylureas) and incretins [16,17].

The results based on many cohort and experimental studies suggested that the biguanide class of anti-diabetic drug, metformin HCl has shown to decrease the chances of occurrence of cancer [4,16,18,19]. This could be due reduction in cell proliferation, partial cell cycle arrest and apoptosis which are linked to the activation of 5′ adenosine monophosphate-activated protein kinase (AMPK) signaling pathway [20]. Separately, the two main bioactive phytochemicals of *Acorus calamus*, alpha (α)-and beta (β)-asarone as well as its extract are reported to possess cytotoxic effects as well as hypoglycemic effects [21–23].

Our own previous study showed that (β)-asarone treatment protect the rats from the diethylnitrosamine (DEN)-induced HCC [24]. However, the asarones effect as cytotoxic agents in diabetic condition remain unexplored. In this study, we investigated the role of hyperglycemia in aiding the progression of hepatocarcinogenesis and evaluated the combined effects of alpha (α)-and beta (β)-asarone as well as metformin HCl in controlling the progression of disease.

2. Materials and methods

2.1. Chemicals and drugs

Diethylnitrosamine (DEN) (Lot # BCBP8749V; Purity ≥99% w/w; PubChem CID: 5921), alpha-asarone (Lot # S18779; Purity 98% w/w; PubChem CID: 636822), beta-asarone (Lot # STBF-1179V; Purity 70% w/w; PubChem CID: 5281758) and Direct red 80/Sirius red (dye content 25%; PubChem CID: 124203941) were purchased from Sigma-Aldrich Chemical Company (USA). Streptozotocin (STZ) extra pure (Lot No: 2582459; Purity 98% w/w; PubChem CID: 29327) was procured from Sisco-Research Laboratories (SRL) Pvt. Ltd., Mumbai, India. Metformin HCl (Lot No: METI-1710010; PubChem CID: 14219) was a gift sample from Angels Pharma India Pvt. Ltd. Hyderabad, India. All other chemicals used in this study were of analytical reagent grade obtained from standard commercial suppliers.

2.2. Experimental animal selection and ethical consideration

The study was conducted on five-month-old male albino rats of wistar strain weighing 150–200 g. All rats were housed in polypropylene cages, maintained at 27 ± 2 °C under 12 h dark and light cycle with free access to standard rat feed (VRK Nutritional Solutions, Pune, India) and water. The study was approved (Approval No. 07/KLEU'SCOPH/16) by the Institutional Animal Ethics Committee (IAEC) and was conducted in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

2.3. Induction of diabetes and hepatocellular carcinoma (HCC)

Experimental diabetes was induced in 6 to 8 h fasted rats by single injection of freshly prepared STZ (55 mg/2 ml/kg b.w. *i.p.* in 0.05 M citrate buffer, pH 4.5). The rats were provided with 10% w/v sucrose water solution for first 24 h after the injection of STZ to avoid fatal hypoglycemia [25]. The hepatocarcinogenesis in rats was induced by the administration of single injection of carcinogenic chemical agent DEN (200 mg/ml/kg b.w. *i.p.* in 0.9% w/v NaCl) [26].

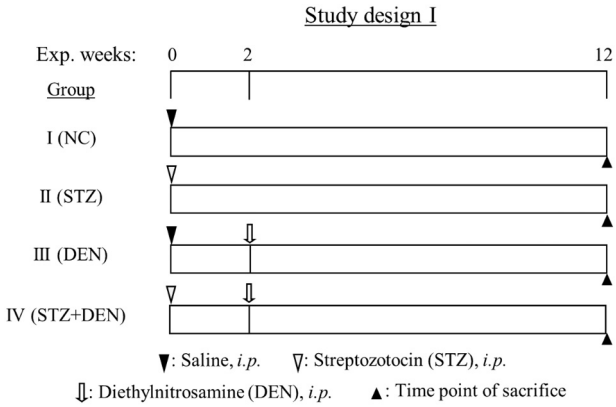

Fig. 1. (Study Design I). The diabetes mellitus was confirmed at second week after the administration of the STZ. The DEN was administered two weeks later to the administration of STZ to induce the HCC in diabetic rats. The total duration of the study was for the period of 12-weeks.

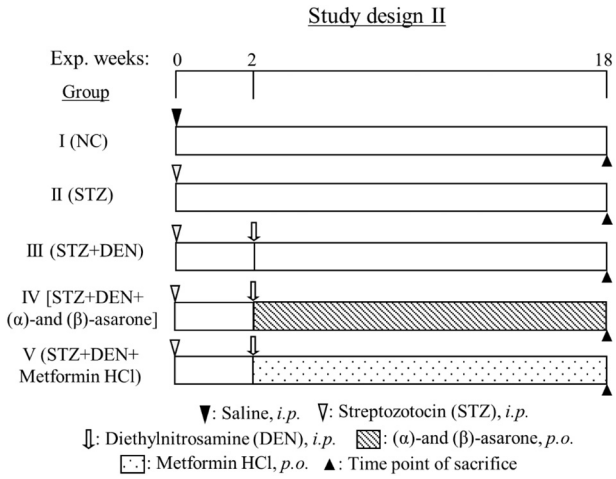

Fig. 2. (Study Design II). The administration of STZ and DEN were similar as in study design I. The asarone and metformin were used as treatment to study their effect on diabetic-HCC for the duration of 18-weeks.

2.4. Experimental design

Study design I (Fig. 1): The role of the STZ-induced hyperglycemia on the progression of DEN administered HCC was investigated for the duration of 12-weeks. The rats were randomly divided into four groups with eight in each (n = 8) except in group IV, where n = 7 due to the death of one rat during the study period. Group I served as normal and received 0.9% w/v NaCl (1 ml/kg b.w. *i.p.*). Group II served as diabetogenic (STZ) and were administered with a single dose of streptozotocin (Section 2.3). Group III served as hepatocellular carcinoma (DEN) and were administered with a single dose of diethylnitrosamine (Section 2.3). Group IV served as diabetic-hepatocellular carcinoma (STZ + DEN) and received the injection of STZ followed by DEN two weeks later after confirming the elevated glucose levels. The blood/serum was collected after anesthetization and liver tissue was collected after sacrificing the animals at the end of 12 weeks for the assessment of different parameters.

Study design II (Fig. 2): The combined effect of (α)-and (β)-asarone and metformin was investigated on diabetic-HCC animals for the duration of 18-weeks. The rats were randomly divided into five groups with eight in each (n = 8) except in group II and IV (n = 7) and in group III (n = 6) due to the death of animals during the study period. Group I and II were similar as in study design I. Group III served as diabetic-hepatocellular carcinoma (STZ + DEN) and was similar to

Table 1
The relative liver weight, insulin, glycosylated hemoglobin (HbA_{1c}), serum liver dysfunction markers and tumor bio-markers (GGT and AFP) in rats of study design I.

Parameters/Group	I (NC)	II (STZ)	III (DEN)	IV (STZ + DEN)
Relative liver weight (g%)	3.94 ± 0.15	5.88 ± 0.37**	5.76 ± 0.08**	6.79 ± 0.66***
Insulin (μU/ml)	16.11 ± 0.26	5.80 ± 0.20***	16.87 ± 0.21	6.52 ± 0.19***, a
HbA_{1c} (%)	5.30 ± 0.13	8.43 ± 0.24***	5.82 ± 0.16	7.67 ± 0.26***, a
ALT (IU/L)	47.17 ± 1.81	58.88 ± 3.13*	69.59 ± 1.41***	82.86 ± 3.97***, c
AST (IU/L)	55.97 ± 4.16	75.38 ± 3.35*	88.10 ± 3.56***	102.00 ± 7.31***
GGT (IU/L)	28.85 ± 1.38	37.38 ± 1.14	48.25 ± 2.19***	62.86 ± 4.41***, b
AFP (ng/ml)	0.33 ± 0.02	0.37 ± 0.03	0.60 ± 0.01*	0.77 ± 0.11***

Relative liver weight and various biochemical parameters of different experimental groups of study design I. Each value represents the mean ± SEM; One-way ANOVA followed by Bonferroni test, where, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to normal group and $^{c}p < 0.05$, $^{b}p < 0.01$, $^{a}p < 0.001$ compared to DEN group.

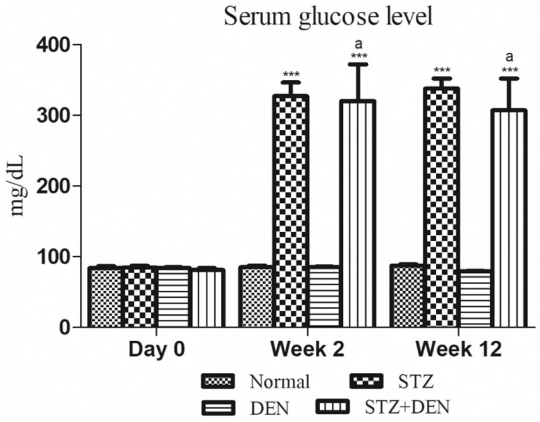

Fig. 3. (Serum glucose level of study design I). Serum glucose level (mg/dL) of different experimental groups of study design I and is represented as the mean ± SEM; One-way ANOVA followed by Bonferroni test, where, $^{***}p < 0.001$ compared to normal group and $^{a}p < 0.001$ compared to DEN group.

Table 2
Morphometric analysis (Nodule incidence, nodule multiplicity and size of nodules).

Group (Treatment)	Nodule incidence (%)	Nodule multiplicity	Relative nodular size (% of total numbers)		
			< 1 mm (%)	1 to 3 mm (%)	> 3 mm (%)
III (DEN)	37.5	1.66 ± 0.33	5 (100)	–	–
IV (STZ + DEN)	87.5	5.28 ± 0.68^{c}	18 (48.7)	12 (32.4)	07 (18.9)

Morphometric analysis of visible hepatocyte nodules in DEN and STZ + DEN treated rats of study design I. Nodule multiplicity is presented as mean ± SEM; Student's t-test, where, $^{c}p < 0.05$ compared to DEN group.

group IV of study design I. Group IV served as treatment group which was administered with a combination dose of (α)-and (β)-asarone. The asarones (50 μg/1.5 ml/kg b.w., *p.o.*) prepared in 0.5% w/v sodium carboxymethyl cellulose were given in the ratio of 1:1 for five days per week after two weeks of STZ + DEN injections [21,24]. Group V was similar to group IV except that the freshly prepared metformin HCl (250 mg/1.5 ml/kg b.w.) replaced the asarones [27]. The blood/serum was collected after anesthetization and liver tissue was collected after sacrificing the animals at the end of 18 weeks for the assessment of different parameters.

2.5. Collection of blood, serum and tissue samples

Blood samples were collected by retro-orbital venous plexus puncture method without anticoagulant under mild anesthesia. The serum sample collected at the end of 2-weeks was to ascertain the hyperglycemia induced by STZ. The blood sample collected at the end of study period was for the estimation of all biochemical parameters. One part of the blood was collected in EDTA tubes for estimating the glycosylated hemoglobin (HbA_1c). Another part of the blood was allowed to stand for 30 min at room temperature, centrifuged at 2500 rpm for 10 min to separate the serum for evaluation of various biochemical parameters. Later, animals were euthanized, and liver tissues were isolated, blotted off blood, washed with ice-cold physiological saline (0.9% w/v NaCl), weighed and stored in 10% v/v neutral buffered formalin for staining procedures.

2.6. General observation

The weekly body weight changes along with average food and water consumption were noted for all the groups. The data was expressed as relative food and water consumption for 100 g of b.w. of rat per week. The liver weight was taken at the end of study for every animal from each group and the relative liver weight (g %) was calculated by taking the percentage of liver weight to body weight of each animal.

2.7. Morphometric analysis (nodule incidence, nodule multiplicity and size of nodules)

The excised liver samples were observed for visible liver tumors and were classified into < 1, 1 to 3, and > 3 mm sized tumors. The nodules bearing liver (nodule incidence) and average number of nodules per nodule-bearing liver (nodule multiplicity) in all the group were recorded [28].

2.8. Biochemical parameters

Serum fasting glucose, liver dysfunction markers [aspartate aminotransferase (AST), alanine aminotransferase (ALT)] and tumor biomarker gamma-glutamyl transferase (GGT) were measured according to the procedure given by commercial kit (Erba Transasia Bio-Medicals Ltd., India) using a semi-auto analyzer. The whole blood HbA_1c was assayed by using NycoCardTM (HbA_1c) test kit and analyzed in NycoCard™ reader according to the instructions supplied. The quantitative estimation of liver tumor bio-marker α-fetoprotein (AFP; LifeSpan BioSciences, USA) and serum insulin concentration (Mercodia, Sweden) was determined by ELISA technique as per instruction given by supplier.

2.9. Histopathological observation of liver (H&E; Sirius red)

The liver tissues fixed in formalin were dehydrated by passing them through an increasing concentration of isopropyl alcohol (60 to 100%), followed by clearing agent (xylene) and were embedded in paraffin wax. The paraffin-embedded sections of the liver (5 μm thick) were

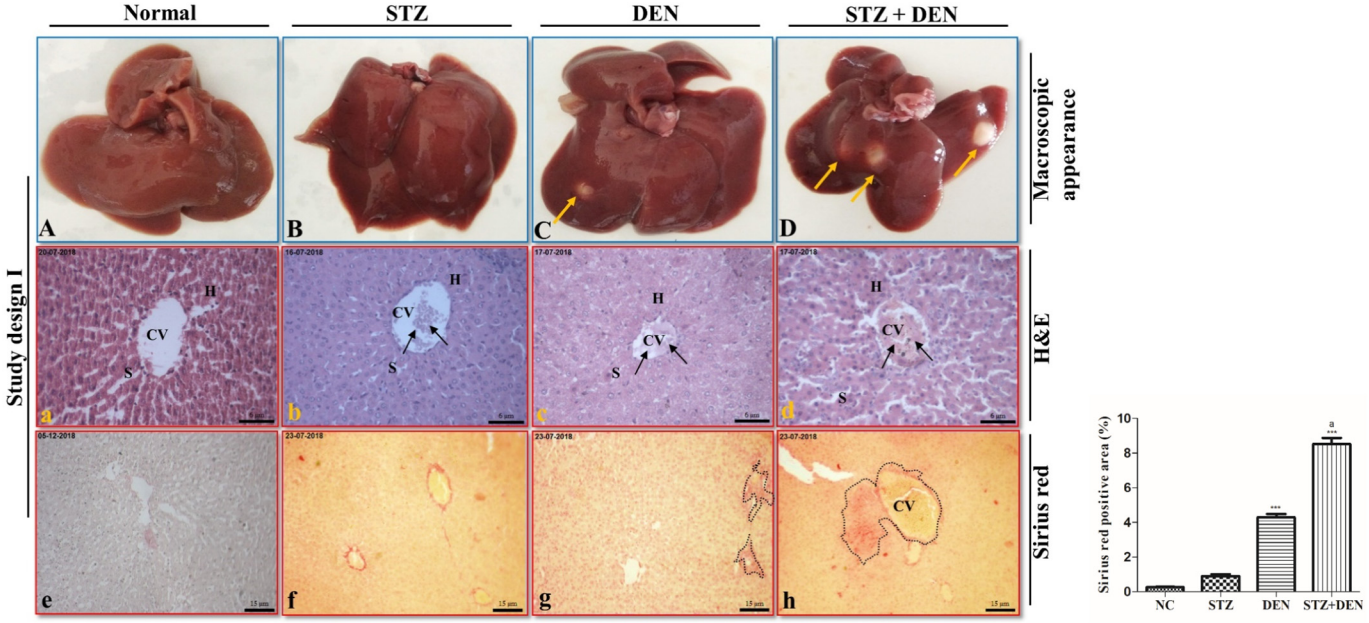

Fig. 4. (Macroscopic and Histological features of study design I). Representative gross macroscopic appearance of livers from normal (A), STZ (B), DEN (C) and STZ + DEN (D) administered groups at the end of 12-weeks study (arrow heads indicate nodules). Representative liver sections stained with hematoxylin-eosin (H&E) (a–d, magnification: 200) depicts central vein and Sirius red (e–h, magnification: 100) depicts the plates of hepatocytes. Black arrow heads indicate congestion in CV; black dotted lines (g–h) indicates collagen deposition. Liver fibrosis observed by Sirius red staining was quantified as percentage of Sirius red-positive area in each group as shown in the bar diagram. Data are expressed as the mean ± SEM; One-way ANOVA followed by Bonferroni test, where, $^{***}p < 0.001$ compared to normal group and $^{a}p < 0.001$ compared to DEN group.
CV, Central vein; H, Hepatocytes; S, Sinusoids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Comparative percentage (%) changes and histopathological characteristics between study designs I and II.

Parameters	STZ + DEN	
	12-weeks	18-weeks
Relative liver weight	89.81	100
Nodule incidence	87.5	100
Nodules < 1 mm in diameter	48.7	18.5
Nodules 1–3 mm in diameter	32.4	34.5
Nodules > 3 mm in diameter	18.9	47.0
Average no. of nodules	45.67	100
GGT	86.31	100
AFP	28.51	100
Histological features/changes		
Metastasis	–	+
Ballooning hepatocytes	–	+ + +
Ground glass change	–	+ +
Kupffer cell hyperplasia	–	+ +

Comparative percentage (%) changes in morphological, biochemical and histological features observed in STZ + DEN treated groups at the end of 12- and 18-week duration. Histological features are scored as mild (+), moderate (+ +), severe (+ + +) and absent (−).

stained with Hematoxylin and Eosin (H&E) and Sirius red to investigate the histological changes in liver. The photomicrographs were captured from the stained slides (CKX41, Olympus Microsystem, Japan). The relative hepatic fibrosis area (expressed as % Sirius red positive area) was quantified using Image J software (Image J 2010, IJ 1.46r). Further, inflammation was graded 0 to 3 on the basis number of inflammatory foci. Score 0 (Normal, < 0.5 foci); score 1 (slight, 0.5–1.0 foci); score 2 (moderate, 1.0–2.0 foci) and score 3 (severe, > 2.0 foci) were assigned. Hepatocyte ballooning was assigned with score 0 to 2 based on the number of balloon cells. Score 0 (no balloon cells); score 1 (scattered balloon cells) and score 2 (panacinar balloon cells) were assigned.

2.10. Data analysis

Statistical significance was determined using statistical software GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± SEM. The results obtained were analyzed using either student's *t*-test or one-way ANOVA (Analysis of variance) followed by Bonferroni multiple comparison test as applicable and *p* values of $^{<}0.05$ were considered as significant.

3. Results

3.1. Study design I

In this study design, we studied the role of streptozotocin (STZ)-induced hyperglycemia on the progression of hepatocellular carcinoma (HCC) due to diethylnitrosamine (DEN) for the duration of twelve weeks. The STZ and/or DEN rats showed significant decrease in body weight and increase in relative liver weight (Table 1) in spite of high food and water consumption compared to normal rats (data not shown). The Fig. 3 illustrates the induction of hyperglycemia by STZ by the end of 2nd week confirmed by significantly elevated levels ($p < 0.001$) of serum glucose. The STZ alone group showed elevated levels of glycosylated hemoglobin (HbA_{1c}), serum liver dysfunction markers and decreased levels of insulin (Table 1) at the end of 12-weeks. The DEN alone group exhibited increased levels of tumor bio-markers (GGT and AFP), liver dysfunction markers and the presence of few carcinoma nodules. The STZ + DEN group animals exhibit all the hallmarks of diabetes and cancer with increased number of hepatic nodules (Tables 1 and 2).These changes were further enunciated by the morphological and histopathological changes such as increased central vein congestion and fibrosis of liver at the end of 12-weeks (Fig. 4).

Table 4
The relative liver weight, insulin, glycosylated hemoglobin (HbA_{1c}), serum liver dysfunction markers and tumor bio-markers (GGT and AFP) of study design II.

Parameters/Group	I (NC)	II (STZ)	III (STZ + DEN)	IV [STZ + DEN + (α)-and (β)-asarone]	V [STZ + DEN + Metformin HCl]
Relative liver weight (g%)	3.35 ± 0.08	6.56 ± 0.24***	7.56 ± 0.49***	6.06 ± 0.08***, b	4.90 ± 0.12***, a
Insulin (μU/ml)	16.57 ± 0.26	4.63 ± 0.18***	5.73 ± 0.27***	10.03 ± 0.49***, a	12.60 ± 0.33***, a
HbA_{1c} (%)	5.50 ± 0.09	8.90 ± 0.15***	8.10 ± 0.15***	6.89 ± 0.28***, b	5.88 ± 0.24[a]
ALT (IU/L)	46.62 ± 1.84	64.88 ± 1.55***	176.20 ± 2.41***	65.16 ± 2.05***, a	56.01 ± 3.20[a]
AST (IU/L)	55.51 ± 0.93	78.09 ± 0.72***	166.50 ± 1.80***	71.40 ± 1.91***, a	68.78 ± 1.66***, a
GGT (IU/L)	29.50 ± 1.41	35.86 ± 1.59	72.83 ± 2.52***	44.60 ± 3.08***, a	38.88 ± 2.72[a]
AFP (ng/ml)	0.34 ± 0.02	0.42 ± 0.02	2.70 ± 0.10***	1.31 ± 0.02***, a	0.99 ± 0.03***, a

Effect of (α)-and (β)-asarone and metformin HCl on relative liver weight and various biochemical parameters. All the values are presented as mean ± SEM; One-way ANOVA followed by Bonferroni test, where, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to normal group and $^{b}p < 0.01$, $^{a}p < 0.001$ compared to STZ + DEN group.

Table 5
Morphometric analysis (Nodule incidence, nodule multiplicity and size of nodules).

Group (treatment)	Nodule incidence (%)	Nodule multiplicity	Relative nodular size (% of total numbers)		
			< 1 mm (%)	1 to 3 mm (%)	> 3 mm (%)
III (STZ + DEN)	100	10.13 ± 0.71	15 (18.5)	28 (34.5)	38 (47.0)
IV [STZ + DEN + (α)-and (β)-asarone]	62.5	7.00 ± 0.31[c]	7 (20.0)	12 (34.2)	16 (45.8)
V (STZ + DEN + Metformin HCl)	50	5.75 ± 0.75[b]	4 (17.3)	8 (34.8)	11 (47.9)

Morphometric analysis of visible hepatocyte nodules in STZ + DEN treated rats and effect of (α)-and (β)-asarone and metformin HCl of study design II. Nodule multiplicity is presented as mean ± SEM; One-way ANOVA followed by Bonferroni test, where, $^{c}p < 0.05$, $^{b}p < 0.01$ compared to STZ + DEN group.

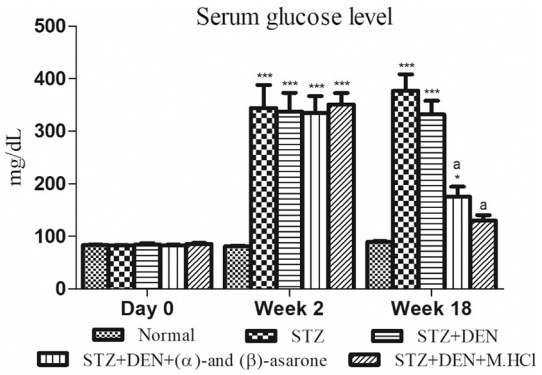

Fig. 5. (Serum glucose level). Serum glucose level (mg/dL) of different experimental groups and effect of (α)-and (β)-asarone and metformin HCl of study design II and is represented as the mean ± SEM; One-way ANOVA followed by Bonferroni test, where, $^{*}p < 0.05$, $^{***}p < 0.001$ compared to normal group and $^{a}p < 0.001$ compared to STZ + DEN group.

3.2. Study design II

In this study design, the chemo-preventive role of (α)-and (β)-asarone was investigated and compared with Metformin HCl for duration of 18-weeks against STZ + DEN induced diabetic-hepatocellular carcinoma (HCC). The 18-week duration was preferred over 12-week duration of diabetic-HCC due to robustness of the 18-week study design as compared in Table 3. Histopathologically, the metastasis, ground glass change and kupffer cell hyperplasia were observed only in 18-week study design. Further, all morphological features as well as cancer biomarkers were also found to be high in 18-week study design compared to 12-week one (Table 3).

3.2.1. General observation

The body weight as well as food and water consumption of STZ and STZ + DEN groups of this study design was similar to 12-week study. The water consumption in asarone and metformin treated animals was significantly lesser compared to STZ + DEN group but was higher than normal animals. On the contrary, neither asarone nor metformin statistically altered the food consumption pattern (data not shown). The treatment groups showed significant reduction ($p < 0.01$) in the relative liver weight compared to STZ + DEN group which in turn was significantly high ($p < 0.001$) compared to normal animals (Table 4).

3.2.2. Morphometric analysis

Table 5 summarizes the gross examination about nodule incidence, nodule multiplicity and size of nodules. The STZ group rats did not show the occurrence of hepatocyte nodules even up to the end of 18-weeks. These macroscopic nodules were clearly visible in the STZ + DEN as well as asarone and metformin treated groups. However, the treatment with asarone ($p < 0.05$) and metformin ($p < 0.01$) decreased the nodule multiplicity, number and size of nodules compared to STZ + DEN group.

3.2.3. Biochemical analysis

As mentioned previously in the Section 3.1, the STZ could induce the hyperglycemia at the end of 2-weeks after its administration. The high serum glucose level was maintained up to the end of 18-weeks in STZ and STZ + DEN group. As expected, the metformin ($p < 0.001$) was successful in controlling the levels of serum glucose. The asarone ($p < 0.001$) was also able to control the serum glucose level (Fig. 5).

Table 4 shows the effect of (α)-and (β)-asarone and metformin on insulin, glycosylated hemoglobin (HbA_{1c}), liver dysfunction marker (ALT, AST) levels and HCC biomarkers (GGT and AFP). The STZ and STZ + DEN group showed significant reduction ($p < 0.001$) in the levels of insulin and an increase ($p < 0.001$) in the levels of HbA_{1c} and liver dysfunction markers ($p < 0.001$) compared with the normal group. However, only in STZ + DEN group, the levels of GGT and AFP were found to be significantly ($p < 0.001$) higher compared to normal animals. Oral administration of asarone and metformin caused significant correction in all the above-mentioned biochemical markers when compared to STZ + DEN group with varying degree.

3.2.4. Histopathological assessment

Macroscopically, the livers of STZ receiving rats appeared darker in color without any visible subcapsular nodules compared to the livers from the normal group, which did not show discoloration. The STZ + DEN group showed enlargement of liver and appearance of foci

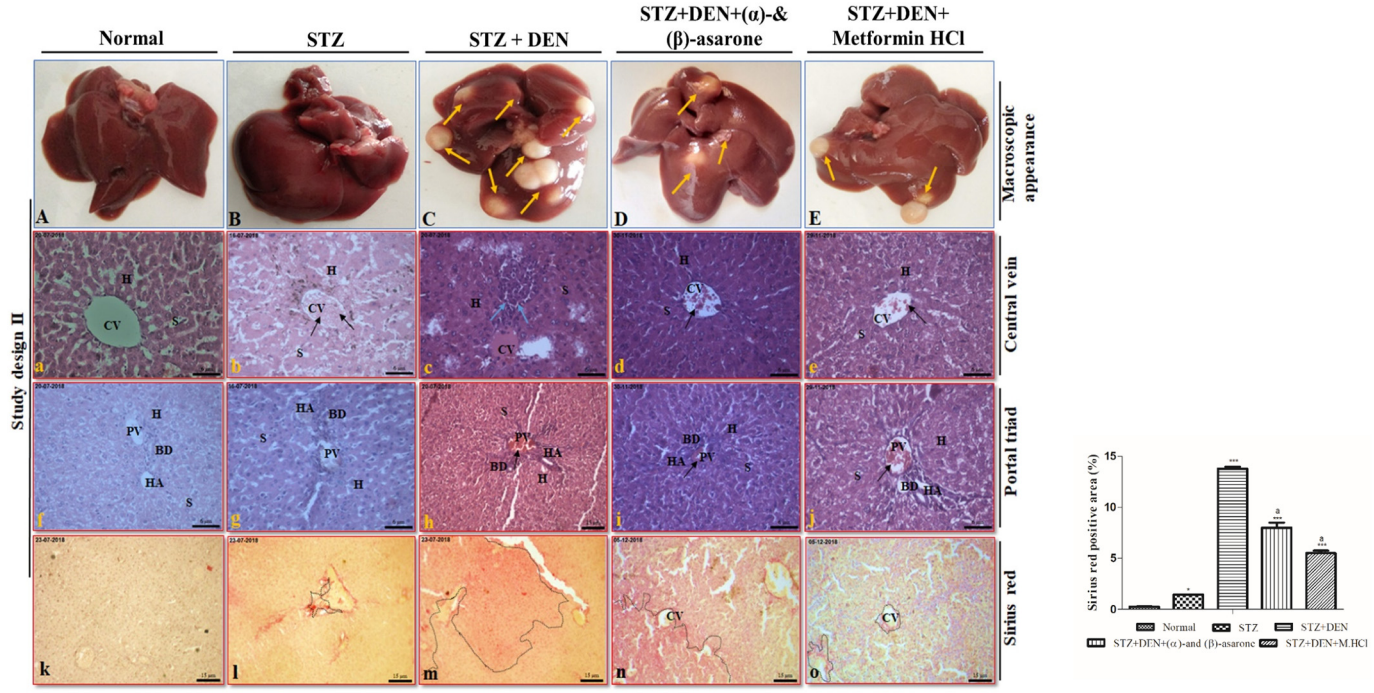

Fig. 6. (Macroscopic and histological features of study design II). Top panel is representative gross macroscopic appearance of livers from normal (A), STZ (B), STZ + DEN (C), STZ + DEN + asarone (D) and STZ + DEN + metformin HCl (E) administered groups at the end of 18-weeks study wherein the arrow heads indicates nodules. The middle two horizontal panels are representative liver sections stained with hematoxylin-eosin (H&E) (a–j) which depict the CV and PT. Black and blue arrow heads indicate congestion in CV and PT and necrosis, aggregation and infiltration of lymphocytes. The lower horizontal panel images of liver tissue stained with Sirius red (k–o) depict plates of hepatocytes. The black dotted lines (l–o) indicates collagen deposition (a–g, i & j, magnification: 200; h, k–o, magnification: 100). The fibrosis was quantified as percentage of Sirius red-positive area in the bar diagram and data is expressed as the mean ± SEM; One-way ANOVA followed by Bonferroni test, where, $^{*}p < 0.05$ $^{***}p < 0.001$ compared to normal group and $^{a}p < 0.001$ compared to STZ + DEN group.
CV, Central vein; H, Hepatocytes; S, Sinusoids; BD, Bile duct; HA, Hepatic artery; PV, Portal vein; PT, Portal triad. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

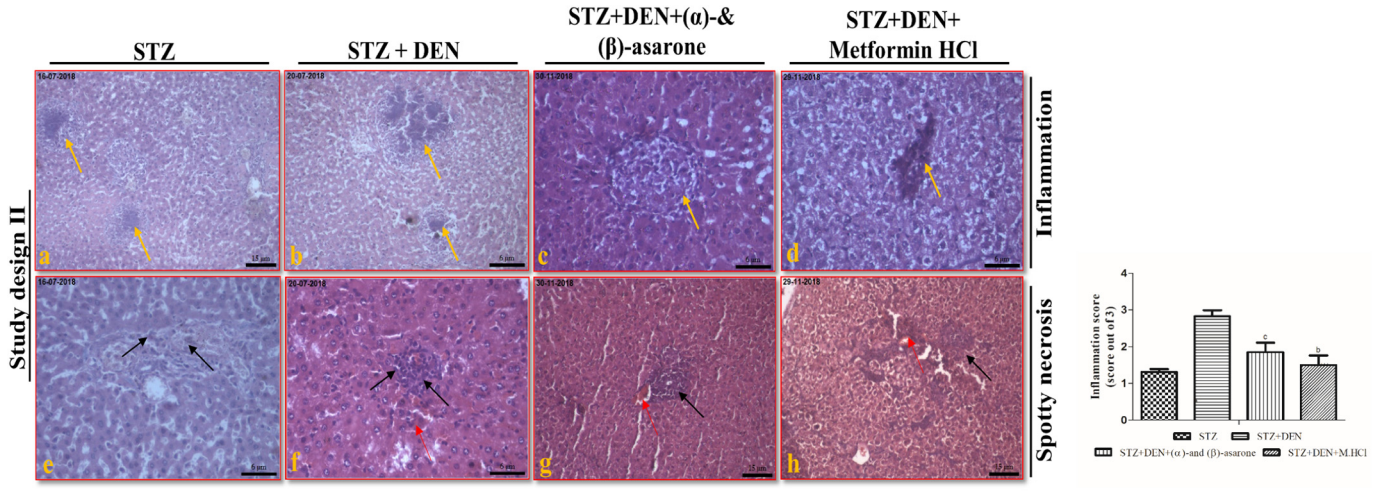

Fig. 7. (Histology of non-tumor region). Representative H&E stained images of sections from non-tumor regions of study design II depict inflammation and spotty necrosis (a, g & h, magnification: 100; b–f, magnification: 200). Yellow arrow heads indicate cellular infiltration depicting inflammation; black arrow heads indicate spotty necrosis; red arrow heads indicate focal hemorrhage. Histology score for inflammation was quantified and expressed as the mean ± SEM; One-way ANOVA followed by Bonferroni test, where, $^{c}p < 0.05$, $^{b}p < 0.01$ compared to STZ + DEN group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

on the peripheral surface followed by several greyish white nodules, which are characteristic features of HCC. We further observed that the groups which were treated with (α)-and (β)-asarone and metformin exhibited a decreased incidence of nodules as compared to STZ + DEN rats (Fig. 6A–E).

Liver histology indicated that the normal group showed normal hepatic architecture described by the presence of intact spherical nucleus, well preserved hepatocellular symmetry, marked lobule boundaries and clear cytoplasm (Fig. 6a, f). The injection of STZ revealed a moderately disturbed liver architecture showing central vein, portal triad and sinusoidal congestion with severe inflammation. The initiation of necrosis following aggregation and infiltration of lymphocytes was observed between the hepatocytes (Figs. 6b, g, 7a, e). Further, STZ group exhibited stage 1 fibrosis confirmed by Sirius red

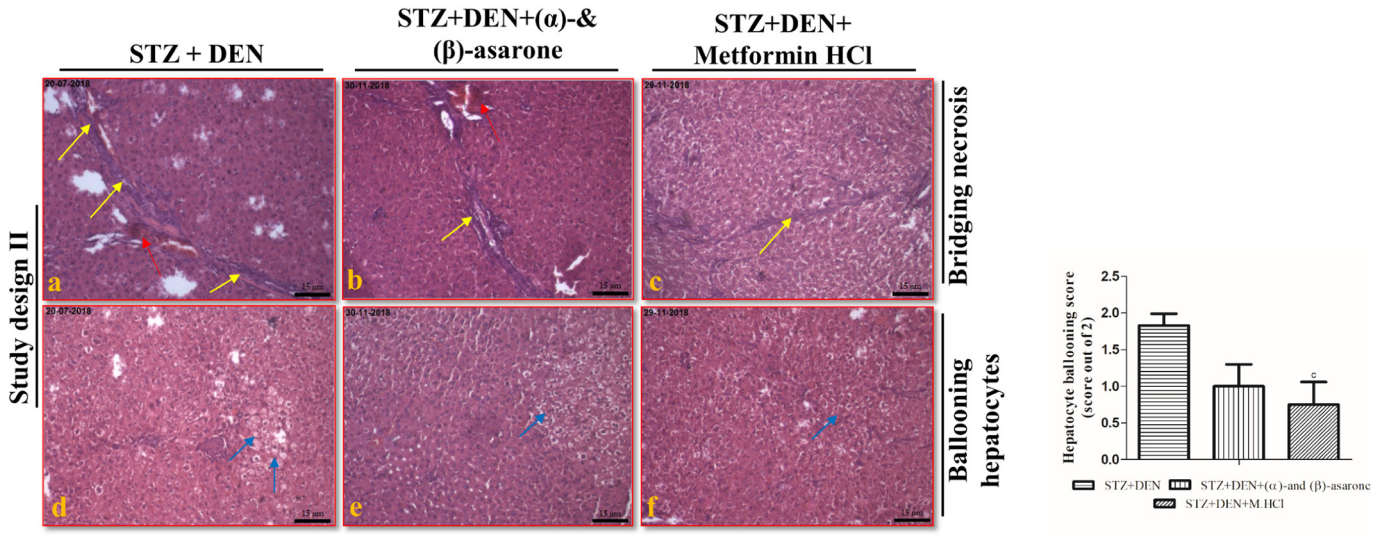

Fig. 8. (Histology of tumor region). Representative H&E stained images of sections from tumor regions of study design II depict bridging necrosis and ballooning hepatocytes (a–f, magnification: 100). Yellow arrow heads indicate bridging necrosis; red arrow heads indicate focal hemorrhage; blue arrow heads indicate enlarged and rounded hepatocytes with clear cytoplasm showing ballooning hepatocytes. Histology score for ballooning hepatocytes was quantified and expressed as the mean ± SEM; One-way ANOVA followed by Bonferroni test, where, $^{c}p < 0.05$ compared to STZ + DEN group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

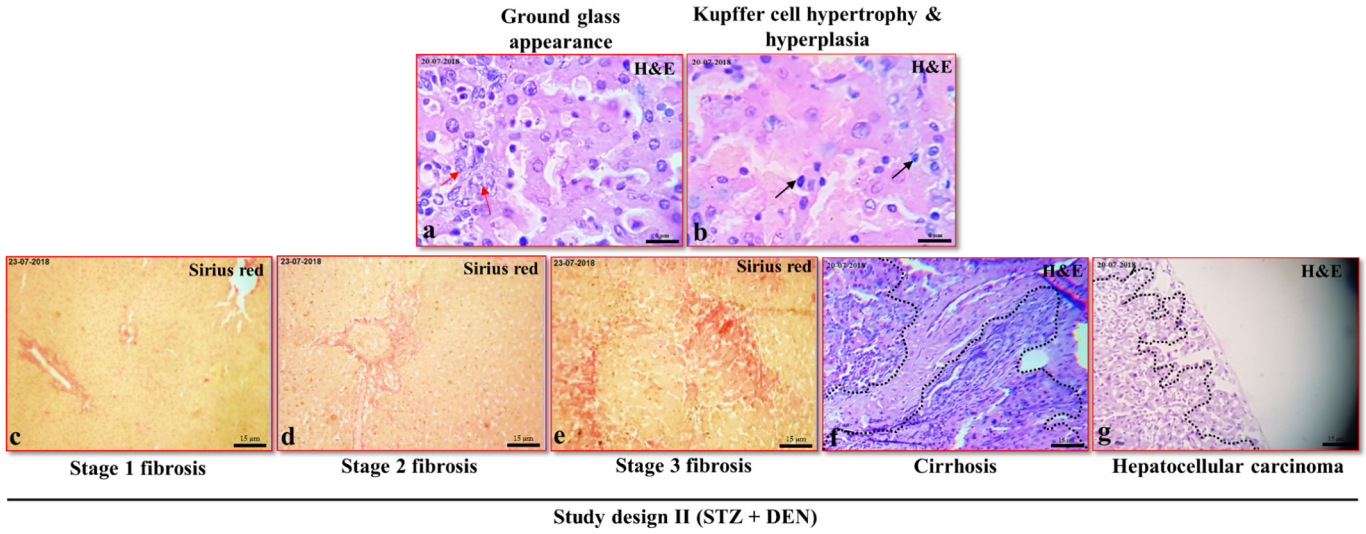

Fig. 9. (Fibrosis, cirrhosis and HCC features). Representative H&E and Sirius red stained images of sections from tumor and non-tumor regions of STZ + DEN rats of study design II sequentially showing different stages of fibrosis, cirrhosis and HCC (a & b, magnification: 200; c–g, magnification: 100). Red arrow heads indicate ground glass appearance; black arrow heads indicate hypertrophy and hyperplasia of kupffer cells; black dotted lines showed well-demarcated cirrhosis (f) and satellite nodules made of clusters of tumoral cells with irregular hepatocytes (g). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

staining (Fig. 6l).

The STZ + DEN group showed central vein and sinusoidal congestion, signs of inflammation noted by the infiltration of leukocytes and portal triaditis followed by focal hemorrhage, spotty necrosis and fibrosis similar to STZ group with greater severity (Figs. 6c, h, m, 7b, f). However, presence of numerous neoplastic cells (HCC), cellular hyperplasia along with extensive loss of architecture (deformed cellular margins), bridging necrosis, ballooning hepatocytes, cholestasis, centrilobular degeneration, ground glass change and kupffer cell hyperplasia was observed (Figs. 8a, d, 9a, b). Further, confirmation of all stages of fibrosis, cirrhosis and finally HCC was observed in STZ + DEN group (Fig. 9c–g).

The magnitude of the deleterious effect of histological structure in STZ + DEN was reduced in asarone and metformin treated groups (Figs. 6, 7 and 8). Few features such as ground glass change, kupffer cell hyperplasia and metastasis were reversed by metformin and asarone. All other features of HCC were greatly reduced by metformin and to lesser extent by asarone treatment (Table 6).

4. Discussion

Many epidemiological and other studies suggest that the Metformin delays the occurrence of various forms of cancer including hepatocellular carcinoma (HCC). The repository of clinical trials, clinicaltrials.gov website suggests that there are 330 studies completed/ongoing/ceased clinical trials when “metformin and cancer” words were used. This number comes down to four, if the cancer word is replaced by “HCC”. This indicates that the metformin is being studied extensively for its usefulness in preventing/curing the cancers [29]. In our study too, metformin delayed the occurrence of HCC at least by few weeks.

Table 6
Histopathological characteristics of liver of animals of study design II.

Histological features/changes	II (STZ) (n = 7)	III (STZ + DEN) (n = 6)	IV [STZ + DEN + (α)-and (β)-asarone] (n = 7)	V [STZ + DEN + Metformin HCl] (n = 8)
Stage (F1/F2/F3)	6/1/0	1/1/4	2/2/3	4/2/2
Metastasis	−	+	−	−
Central vein & sinusoidal congestion	+ +	+ + +	+	+
Inflammation	+ +	+ + +	+ +	+ +
Focal hemorrhage	+ +	+ + +	+	+
Ballooning hepatocytes	−	+ + +	+	+
Altered hepatic foci	−	+ + +	+	+
Spotty necrosis	+	+ + +	+ +	+
Portal triaditis	+	+ + +	+	+
Ground glass change	−	+ +	−	−
Kupffer cell hyperplasia	−	+ +	−	−
Cirrhosis	−	+ +	+	+
Hepatocellular dysplasia	−	+ + +	+	+
Hepatocellular carcinoma	−	+ + +	+ +	+

Severity for different histological features are scored as mild (+), moderate (+ +), severe (+ + +) and absent (−). The scoring/stage of liver fibrosis: F1, expansion of fibrosis into fewer portal areas without septa; F2, expansion of fibrosis in most of the portal areas with occasional portal to portal (P-P) bridging; F3: expansion of fibrosis in almost all of the portal areas with marked portal to portal (P-P) and portal to central (P-C) bridging.

Further, our study test compound asarone too exhibited the effects similar to metformin with a lesser efficacy.

It is a well-known fact that the STZ gains entry into beta cells via glucose transporter 2 (GLUT 2) and causes necrosis due to DNA alkylation preceded with impairment of mitochondrial function, swelling of cells and generation of free radicals and nitric oxide [30,31]. The elevated levels of glycosylated hemoglobin observed in study design II are a consequence of long-standing hyperglycemia. It is interesting to note that, although the STZ was initially developed as anticancer agent, many reports suggest that it is a carcinogenic agent [32]. Our findings too suggest that the STZ on its own leads up to the stage of fibrosis but fails to develop HCC at least until the end of the study duration. The major histopathological sequence of events observed in STZ rats for a period of 18-weeks are inflammation, congestion in central vein, sinusoids and portal triad, followed by initiation of necrosis, infiltration of lymphocytes and stage 1 fibrosis as confirmed by Sirius red staining. All these findings are similar to earlier reports [9,10]. The observed changes could be resultant of release of pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α due to initiation of the oxidative-stress (ROS) and inflammatory mediated damage by the STZ [33]. All these, together with the loss of cellular function via activation of pro-fibrogenic factors leads to hepatic fibrosis.

Many different chemicals are used to mimic the HCC in animals, including the DEN. The DEN is reported to take longer duration to develop cancer, if it is used alone. However, combining the DEN with a promoting chemical can reduce the duration for the occurrence of HCC many folds [34]. The administration of DEN is known to result into the uncontrolled proliferation of cells due to tumor-associated proteins causing the development of subcapsular nodules as evidenced by increased liver and relative liver weight [6,30,35]. The increase in the number, size and focal growth of the hepatocyte nodules observed may be due to the increase in tumor volume, which are reported as a possible precursor for HCC [36–38]. The γ-glutamyl transferase (GGT), a membrane-bound enzyme increases linearly with the increase in tumor mass. It also increases with higher nodule incidence, larger spread of nodules as well as foci in hepatic tissue indicating a response of toxic cellular injury [39,40]. Alpha-fetoprotein (AFP), a common serum protein diagnostic bio-marker also gets elevated in HCC and germ cell cancers [41].

The present scientific literature has a scanty evidence about replicating the diabetes promoted cancer in rodents/animals. Hence, one of the aims of our study was to develop a rodent model to replicate the clinical evidence for diabetic-HCC. We found that the pre-administration of STZ leads to the occurrence of cancer at least by few weeks early. The intra- and inter-animal livers exhibited variations in histomorphological patterns. In our study, non-tumoral liver sections of the STZ + DEN rats confirmed the incidence of the progression of different events of pre-HCC stages (fibrosis, cirrhosis). The development of liver fibrosis may be due to the proliferation, differentiation and activation of hepatic stellate cells (HSC) associated with the accumulation of extra-cellular matrix components including laminin and collagen. The increased deposition of the collagen around central vein, portal triad and fibrous septae further accentuates the fibrosis [42]. The liver damage continues into advanced cirrhosis and finally into HCC due to neovascularization and infiltration of immune cells followed by its encapsulation. Furthermore, a few cases of STZ + DEN rat livers exhibited metastasis towards the lung indicating the irreversibility of the disease. However, our study did not indicate the precipitation of HCC was due to STZ itself or due to long standing higher glucose levels.

There are many studies suggesting that the metformin halts the progress of HCC in diabetic patients [19]. In our study too, the metformin either reversed or reduced the severity of HCC in experimental rats as indicated by all biochemical and pathological evidence. The test compounds, asarones also showed chemo-preventive action similar to the metformin. Further study is ongoing in our lab to confirm the cellular and molecular mechanism of chemo-preventive effect of asarone is due to the activation of AMPK as in the case of metformin [20].

5. Conclusion

Our findings suggest that the diabetogenic agent (STZ) could induce the hepatic fibrosis but not the HCC. The STZ was able to precipitate HCC at least by four weeks when combined with DEN. This action could be due to STZ itself or it could be due to long standing hyperglycemia caused by STZ. Asarone and metformin both successfully either reversed or delayed the deleterious effect of STZ and DEN indicating their chemo-preventive effect.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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References

[1] International Diabetes Federation, IDF Diabetes Atlas [Article Online], 7th ed., International Diabetes Federation, Brussels, Belgium, 2015, pp. B–1170. Available from URL: http://www.idf.org/diabetesatlas.

[2] American Diabetes Association (ADA), Diagnosis and classification of diabetes mellitus, Diabetes Care 37 (2014) S81–S90.

[3] N.H. Cho, J.E. Shaw, S. Karuranga, Y. Huang, J.D. da Rocha Fernandes, A.W. Ohlrogge, B. Malanda, IDF diabetes atlas: global estimates of diabetes prevalence for 2017 and projections for 2045, Diabetes Res. Clin. Pract. 138 (2018) 271–281.

[4] E. Giovannucci, D.M. Harlan, M.C. Archer, R.M. Bergenstal, S.M. Gapstur, L.A. Habel, M. Pollak, J.G. Regensteiner, D. Yee, Diabetes and cancer: a consensus report, CA Cancer J. Clin. 60 (4) (2010) 207–221.

[5] C.A. Vecchia, E. Negri, A. Decarli, S. Franceschi, Diabetes mellitus and the risk of primary liver cancer, Int. J. Cancer 73 (2) (1997) 204–207.

[6] J.A. Davila, R.O. Morgan, Y. Shaib, K.A. McGlynn, H.B. El-Serag, Diabetes increases the risk of hepatocellular carcinoma in the United States: a population-based case control study, Gut 54 (4) (2005) 533–539.

[7] J.M. Yuan, S. Govindarajan, K. Arakawa, M.C. Yu, Synergism of alcohol, diabetes, and viral hepatitis on the risk of hepatocellular carcinoma in blacks and whites in the U.S, Cancer 101 (5) (2004) 1009–1017.

[8] E. Kume, Y. Ohmachi, S. Itagaki, K. Tamura, K. Doi, Hepatic changes of mice in the subacute phase of streptozotocin (SZ)-induced diabetes, Exp. Toxicol. Pathol. 46 (4–5) (1994) 368–374.

[9] K. Doi, J. Yamanouchi, E. Kume, A. Yasoshima, Morphologic changes in hepatocyte nuclei of streptozotocin (SZ)-induced diabetic mice, Exp. Toxicol. Pathol. 49 (3–4) (1997) 295–299.

[10] V.G. Kozyritskii, A.G. Minchenko, Ultrastructural changes in rat hepatocytes with diabetes and insulin administration, Tsitol. Genet. 12 (5) (1978) 397–401.

[11] J.A. Grimaud, M. Druguet, S. Peyrol, O. Chevalier, D. Herbage, N. El Badrawy, Collagen immunotyping in human liver: light and electron microscope study, J. Histochem. Cytochem. 28 (11) (1980) 1145–1156.

[12] T. Kawase, Y. Shiratori, T. Sugimoto, Collagen production by rat liver fat-storing cells in primary culture, Exp. Cell Biol. 54 (4) (1986) 183–192.

[13] M.M. Hassan, S.A. Curley, D. Li, A. Kaseb, M. Davila, E.K. Abdalla, M. Javle, D.M. Moghazy, R.D. Lozano, J.L. Abbruzzese, J.N. Vauthey, Association of diabetes duration and diabetes treatment with the risk of hepatocellular carcinoma, Cancer 116 (8) (2010) 1938–1946.

[14] L.T. Chao, C.F. Wu, F.Y. Sung, C.L. Lin, C.J. Liu, C.J. Huang, K.S. Tsai, M.W. Yu, Insulin, glucose, and hepatocellular carcinoma risk in male hepatitis B carriers: results from 17-year follow-up of a population-based cohort, Carcinogenesis 32 (6) (2011) 876–881.

[15] T. Hosokawa, M. Kurosaki, K. Tsuchiya, S. Matsuda, M. Muraoka, Y. Suzuki, N. Tamaki, Y. Yasui, T. Nakata, T. Nishimura, S. Suzuki, K. Ueda, H. Nakanishi, J. Itakura, Y. Takahashi, N. Izumi, Hyperglycemia is a significant prognostic factor of hepatocellular carcinoma after curative therapy, World J. Gastroenterol. 19 (2) (2013) 249–257.

[16] L. Sciacca, R. Vigneri, A. Tumminia, F. Frasca, S. Squatrito, L. Frittitta, P. Vigneri, Clinical and molecular mechanisms favoring cancer initiation and progression in diabetic patients, Nutr. Metab. Cardiovasc. Dis. 23 (9) (2013) 808–815.

[17] R. Novosyadlyy, D.E. Lann, A. Vijayakumar, A. Rowzee, D.A. Lazzarino, Y. Fierz, J.M. Carboni, M.M. Gottardis, P.A. Pennisi, A.A. Molinolo, N. Kurshan, W. Mejia, S. Santopietro, S. Yakar, T.L. Wood, D. LeRoith, Insulin-mediated acceleration of breast cancer development and progression in a nonobese model of type 2 diabetes, Cancer Res. 70 (2) (2010) 741–751.

[18] H. Noto, A. Goto, T. Tsujimoto, M. Noda, Cancer risk in diabetic patients treated with metformin: a systematic review and meta-analysis, PLoS One 7 (3) (2012) e33411.

[19] H. Zhang, C. Gao, L. Fang, H.C. Zhao, S.K. Yao, Metformin and reduced risk of hepatocellular carcinoma in diabetic patients: a meta-analysis, Scand. J. Gastroenterol. 48 (1) (2013) 78–87.

[20] G. Zhou, R. Myers, Y. Li, Y. Chen, X. Shen, J. Fenyk-Melody, M. Wu, J. Ventre, T. Doebber, N. Fujii, N. Musi, M.F. Hirshman, L.J. Goodyear, D.E. Moller, Role of AMP-activated protein kinase in mechanism of metformin action, J. Clin. Invest. 108 (8) (2001) 1167–1174.

[21] R. Chellian, V. Pandy, Z. Mohamed, Pharmacology and toxicology of α- and β-Asarone: a review of preclinical evidence, Phytomedicine 32 (2017) 41–58.

[22] J.R. Nakkala, R. Mata, A.K. Gupta, S.R. Sadras, Biological activities of green silver nanoparticles synthesized with *Acorous calamus* rhizome extract, Eur. J. Med. Chem. 85 (2014) 784–794.

[23] Y.X. Liu, M.M. Si, W. Lu, L.X. Zhang, C.X. Zhou, S.L. Deng, H.S. Wu, Effects and molecular mechanisms of the antidiabetic fraction of *Acorus calamus* L. on GLP-1 expression and secretion *in vivo* and *in vitro*, J. Ethnopharmacol. 166 (2015) 168–175.

[24] S.R. Roy, P.C. Gadad, Effect of β-asarone on diethylnitrosamine-induced hepatocellular carcinoma in rats, Indian J. Health Sci. Biomed. Res. 9 (1) (2016) 82–88.

[25] B.L. Furman, Streptozotocin-induced diabetic models in mice and rats, Curr. Protoc. Pharmacol. 70 (2015) (5.47.1-5.47.20).

[26] B.K. Das, B.C. Koti, P.C. Gadad, Role of *Lycopersicon esculentum* in diethylnitrosamine-induced and phenobarbital-promoted hepatocellular carcinoma, Indian J. Health Sci. Biomed. Res. 9 (2) (2016) 147–152.

[27] D.K. DePeralta, L. Wei, S. Ghoshal, B. Schmidt, G.Y. Lauwers, M. Lanuti, R.T. Chung, K.K. Tanabe, B.C. Fuchs, Metformin prevents hepatocellular carcinoma development by suppressing hepatic progenitor cell activation in a rat model of cirrhosis, Cancer 122 (8) (2016) 1216–1227.

[28] F.S. Moreno, M.B.S. Rizzi, M.L.Z. Dagli, M.V.C. Penteado, Inhibitory effects of ß-carotene on preneoplastic lesions induced in Wistar rats by the resistance hepatocyte model, Carcinogenesis 12 (10) (1991) 1817–1822.

[29] NIH: U.S. National Library of Medicine, ClinicalTrials.gov, https://clinicaltrials.gov/ct2/results?cond=Cancer&term=Metformin&cntry=&state=&city=&dist=, Accessed date: 22 April 2019.

[30] A. Akbarzadeh, D. Norouzian, M.R. Mehrabi, S. Jamshidi, A. Farhangi, A.A. Verdi, S.M.A. Mofidian, B. Lame Rad, Induction of diabetes by streptozotocin in rats, Indian J. Clin. Biochem. 22 (2) (2007) 60–64.

[31] N. Rakieten, M.N. Rakieten, M.V. Nadkarni, Studies on the diabetogenic action of streptozotocin (NSC-37917), Cancer Chemother. Rep. 29 (1963) 91–98.

[32] M. Marselos, H. Vainio, Carcinogenic properties of pharmaceutical agents evaluated in the IARC monographs programme, Carcinogenesis 12 (10) (1991) 1751–1766.

[33] J. Mohamed, A.H. Nazratun Nafizah, A.H. Zariyantey, S.B. Budin, Mechanisms of diabetes-induced liver damage: the role of oxidative stress and inflammation, Sultan Qaboos Univ. Med. J. 16 (2) (2016) 132–141.

[34] N.P. Santos, A.A. Colaço, P.A. Oliveira, Animal models as a tool in hepatocellular carcinoma research: a review, Tumour Biol. 39 (3) (2017) 1010428317695923.

[35] K.C. Naveen, K.S. Asok, S. Raghunandhakumar, S. Jagan, P. Anandakumar, T.A. Augustine, S. Kamaraj, T. Devaki, Potent antitumor and antineoplastic efficacy of baicalein on benzo (a) pyrene-induced experimental pulmonary tumorigenesis, Fundam. Clin. Pharmacol. 26 (2) (2012) 259–270.

[36] E. Farber, R. Cameron, The sequential analysis of cancer development, Adv. Cancer Res. 31 (1980) 125–226.

[37] G.M. Williams, The pathogenesis of rat liver cancer caused by chemical carcinogenesis, Biochim. Biophys. Acta 605 (2) (1980) 167–189.

[38] E. Farber, Clonal adaptation during carcinogenesis, Biochem. Pharmacol. 39 (12) (1990) 1837–1846.

[39] S. Fiala, A. Mohindru, W.G. Kettering, A.E. Fiala, H.P. Morris, Glutathione and gamma glutamyl transpeptidase in rat liver during chemical carcinogenesis, J. Natl. Cancer Inst. 57 (3) (1976) 591–598.

[40] M.O. Elhkim, F. Decloitre, M. Martin, B. Loridon-Rosa, C. Frayssinet, Role of diethylnitrosamine, 2-acetylaminofluorene and partial hepatectomy in the expression of glutathione-S-transferase-P and gammaglutamyltranspeptidase in the early steps of rat liver carcinogenesis, Tumor Biol. 13 (3) (1992) 152–161.

[41] L.O. Kashala, M.R.K. Mbowa, M. Essex, Alpha-fetoprotein synthesis in human hepatocellular carcinoma: correlation with hepatitis B surface antigen expression, Cancer Investig. 10 (6) (1992) 513–522.

[42] S.L. Friedman, Liver fibrosis-from bench to bedside, J. Hepatol. 38 (1) (2003) S38–S53.