

# Asarone and Metformin Modulates the Oxidant-Antioxidant Imbalance on Experimentally Induced Hepatocellular Carcinoma during Diabetic Condition

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## ABSTRACT

**Aim:** The aim of the present study was planned to investigate the efficacy of asarone and metformin HCl as an antioxidant on experimentally induced diabetic-hepatocellular carcinoma (HCC) condition in male Wistar rats. **Methods:** Diabetes was induced in experimental rats by single intraperitoneal injection of streptozotocin (STZ; 55 mg/kg b.w.). Following two weeks of STZ injection, the diabetic-HCC condition was simulated in the rats with a single intraperitoneal dose of diethylnitrosamine (DEN; 200 mg/kg b.w.). The  $\alpha$ - and  $\beta$ -asarone combination in the ratio of 1:1 (50  $\mu$ g/kg b.w.) and metformin HCl (250 mg/kg b.w.) treatment were orally given to the diabetic-HCC rats up to 18-weeks. At the end of the experimental period, rats were sacrificed after the withdrawal of blood for biochemical and liver samples were isolated for the antioxidant and histopathological analysis. **Results:** The elevated levels of liver function test such as alkaline phosphatase, bilirubin and decreased levels of total protein, albumin and globulin indicated the hepatic damage in the diabetic-HCC rats. Further, the STZ + DEN-induced oxidative stress was confirmed by the elevated levels of lipid peroxidation (LPO) and decreased levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH) and Vitamin-C. Treatment with asarone and metformin significantly ameliorated the STZ + DEN-induced hepatic damage and oxidative stress. Histopathological evidence also showed recovery of the hepatic architecture in diabetic-HCC rats treated with asarone and metformin. **Conclusion:** Asarone and metformin exhibit good hepatoprotective and antioxidant potential against STZ + DEN-induced HCC in rats.

**Key words:** Asarone, Diethylnitrosamine, Metformin, Oxidative stress, Streptozotocin.

## INTRODUCTION

Diabetes is the result of chronic hyperglycaemia, the most prevalent metabolic syndrome categorized by defective secretion and/or action develops due to the imbalance in carbohydrate, protein and fat metabolism.<sup>1</sup> The chronic increase in the glucose level has undesirable effects on numerous tissues or organs, namely kidneys, liver, muscles, adipose tissues and pancreas henceforth promoting the complications of early diabetes.<sup>2</sup> Further, a large number of epidemiological reports from Asia, North America and

Europe strongly associate diabetes with the elevated risk of incidence and growth of hepatocellular carcinoma (HCC).<sup>3,4</sup> Studies have shown that oxidative stress plays a central role and also shared as causal factors thereby correlating with the instigation and development of HCC during diabetic condition.<sup>5,6</sup>

During oxidative stress, the generation of free radical and their elimination disturb the equilibrium along with its cellular integrity and as a result, it leads to cytotoxicity, damage of DNA, protein degradation

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and carcinogenicity.<sup>5-7</sup> Here, the antioxidant defence mechanisms protect the cells against the reactive oxygen species (ROS) and therefore the use of antioxidants would restore or protect against hepatic impairment relating oxidative stress.<sup>5</sup>

There are epidemiological, clinical and experimental reports suggesting that metformin HCl, a biguanide class of antidiabetic drug decreases the risk of incidence of cancer with diabetes.<sup>8,9</sup> This reduction in the severity of cancer might be due to decreased cell proliferation, cell cycle arrest, reduced insulin-like growth factor, apoptosis and inhibition of angiogenesis which are connected with both the regulation of 5' adenosine monophosphate-activated protein kinase (AMPK)-dependent and independent signalling pathway.<sup>8,10</sup> On the other hand,  $\alpha$ - and  $\beta$ -asarone, the two major active constituents in volatile oils of *Acorus calamus* along with its extract showed cytotoxic as well as antidiabetic effects.<sup>11-13</sup>

Our earlier study reported that asarone treatment decreased the development of HCC in a hyperglycaemic condition comparable to metformin.<sup>14</sup> Considering the above fact that the altered cellular oxidative stress is responsible and also shared as a causal factor between both the diseases, however, there is no evidence related to asarone to support their protection against oxidative stress-induced damage in diabetic-HCC condition. Therefore, the present investigation evaluated the efficacy of asarone and metformin HCl in maintaining the balance in the oxidant-antioxidant status during the diabetic-HCC condition in male Wistar rats.

## MATERIALS AND METHODS

### Experimental animals

Healthy male Albino Wistar rats five-month-old weighing from 150-200 g were used for this study. They were housed and maintained at  $27 \pm 2^\circ\text{C}$  with 12 h light and dark cycle. Animals were provided with

standard pelleted diet and water *ad libitum*. All animal experiments were achieved in agreement with the guidelines after needed approval by the Institutional Animal Ethical Committee (Approval no. 07/KLEU'SCOPH/16).

### Chemicals

Streptozotocin (STZ) extra pure was obtained from Sisco-Research Laboratories (SRL) Pvt. Ltd., Mumbai, India. Diethylnitrosamine (DEN),  $\alpha$ - and  $\beta$ -asarone were procured from Sigma-Aldrich Chemical Company (USA). Metformin HCl was obtained as gratis from Angels Pharma India Pvt. Ltd., Hyderabad, India. All the other reagents and chemicals were analytically graded and procured from standard commercial suppliers.

### Experimental induction of diabetes and hepatocellular carcinoma (HCC)

The animals were made diabetic with a single intraperitoneal dose of freshly prepared solution of streptozotocin (STZ; 55 mg/kg b.w.) in overnight fasted rats. The ice-cold citrate buffer solution (0.05 M; pH 4.5) was used for the preparation of STZ. After the injection of STZ, the rats were supplied with sucrose (prepared as 10 % w/v water solution) for the next 24 h to prevent fatal hypoglycaemia. The HCC was induced with the help of a single intraperitoneal dose of carcinogenic agent diethylnitrosamine (DEN; 200 mg/kg b.w.) prepared in NaCl solution (0.9% w/v).<sup>14</sup>

### Experimental protocol

The animals were randomly divided into five (5) groups ( $n=8$ ), except one rat each from group II and IV and two rats from group III died before the end of the study as tabulated in Table 1. Following 18-weeks of study, blood was collected from the overnight fasted rats for serum separation under mild ether anaesthesia. The liver tissues were collected from the euthanized rats for evaluation of different parameters.

**Table 1: Experimental design and treatment protocol (for a duration of 18-weeks).**

Groupings	Treatment
I	Normal control (NC); received intraperitoneal injection of 0.9% w/v NaCl solution (1ml/kg b.w.).
II	Diabetogenic (STZ); received a single dose of streptozotocin (STZ).
III	Diabetic-hepatocellular carcinoma (STZ+DEN); following two weeks of STZ-injection and after confirmation of increased glucose levels, this group received a single injection of diethylnitrosamine (DEN) to simulate diabetic-HCC condition.
IV	Treatment (STZ+DEN+Asarone); following two weeks of STZ +DEN-injections, this group received a mixture dose of alpha-and beta-asarone (50 $\mu\text{g}/\text{kg}$ b.w.) in the ratio of 1:1. The asarone was administered orally for five days per week till the end of the study after preparing in 0.5% w/v sodium carboxymethyl cellulose solution. <sup>14</sup>
V	Treatment (STZ+DEN+Metformin HCl); alike to group IV except that the test compound metformin HCl (250 mg/kg b.w.), which was freshly prepared in distilled water substituted the asarone. <sup>14</sup>

### Preparation of liver tissue homogenates

The excised liver tissues from all the experimental groups were rinsed with physiological NaCl solution (0.9% w/v) and further one portion was used for the preparation of tissue homogenate (10 % w/v) in 0.15 M Tris-HCl buffer solution of pH 7.4. Subsequently, it was centrifuged (10,000×g; 4°C for 10 min) and the clear supernatant obtained was taken for further studies.

### Estimation of hepatic damage

The study of liver function parameters such as alkaline phosphatase, bilirubin, total protein and albumin were measured with the help of commercially available kit using Erba Chem 7 semi-automated analyser obtained from Erba Transasia Bio-Medicals Ltd., India.

### Measurement of STZ+DEN-mediated oxidative stress markers

The enzymatic antioxidants namely superoxide dismutase (SOD) were examined according to the method of Kakkar *et al.*<sup>15</sup> catalase (CAT) according to the method of Sinha AK,<sup>16</sup> lipid peroxidation (LPO) according to the methods of Niehaus *et al.* Jiang *et al.* and Ohkawa *et al.*<sup>17-19</sup> and glutathione peroxidase (GPx) as described by Rotruck *et al.*<sup>20</sup> in the liver tissue homogenate for all the experimental groups. The non-enzymatic antioxidants namely reduced glutathione (GSH) were carried out as described by Ellman GL<sup>21</sup> and Vitamin-C according to the method of Omaye *et al.*<sup>22</sup>

### Histological study of liver tissue (H and E)

The formalin-fixed liver tissues were dehydrated with 60 to 100% isopropyl alcohol solution and fixed in paraffin wax. For the histology study, liver section slides stained with haematoxylin-eosin (H and E) were captured with a light microscope (Olympus Microsystem, Model-CKX41, Japan).

### Statistical analysis

The obtained data from the various experimental groups were expressed as mean ± SEM using statistical software package 'GraphPad Prism version 6.0'. The results were analysed using a one-way ANOVA test. Further, Bonferroni multiple comparison test was followed considering *p* values of < 0.05 as significant.

## RESULTS

### Effect of asarone and metformin against STZ+DEN-induced hepatic damage

The effect of asarone and metformin on the levels of liver function parameters are illustrated in Table 2. The pathophysiological indices in the STZ alone and STZ+DEN administered rats showed a significant increase (*P* < 0.001) in the levels of ALP, TB and DB along with a decrease in total protein, albumin and globulin levels compared to the normal group. Further, the treated groups significantly overcame the deleterious effects in comparison to STZ+DEN group.

### Effect of asarone and metformin on oxidative stress markers

The alterations in the levels of enzymatic and non-enzymatic antioxidants in the hepatic tissues for all the experimental groups are shown in Table 3. Animals administered with STZ alone and STZ+DEN rats showed a significant increase in the levels of LPO along with a decrease in the levels of SOD, CAT, GPx, GSH and Vitamin-C compared to the normal group. The treatment groups (asarone and metformin) significantly restored the levels of enzymic and non-enzymic antioxidants to normal compared to STZ+DEN group.

### Histopathological features of liver tissue

The typical histopathological features of rat livers for all the groups by haematoxylin-eosin (H&E) staining are represented in Figure 1. The STZ-treated livers at the

**Table 2: Effect of asarone and metformin on hepatic serum markers of different experimental groups.**

Parameters/Group	I (NC)	II (STZ)	III (STZ+DEN)	IV (STZ+DEN+Asarone)	V (STZ+DEN+Metformin)
ALP (IU/L)	93.09±0.91	164.80±2.24***	263.70±3.33***	128.70±2.11***.a	110.20±5.33***.a
TB (mg/dl)	0.33±0.01	0.58±0.01***	1.07±0.04***	0.39±0.04 <sup>a</sup>	0.35±0.02 <sup>a</sup>
DB (mg/dl)	0.07±0.006	0.27±0.01***	0.97±0.02***	0.35±0.01***.a	0.14±0.01 <sup>a</sup> .a
Total protein (mg/dl)	8.84±0.19	7.18±0.06***	4.76±0.17***	6.32±0.16***.a	7.20±0.08***.a
Albumin (mg/dl)	5.63±0.16	4.22±0.27***	2.14±0.14***	4.09±0.04***.a	4.57±0.12***.a
Globulin (mg/dl)	3.33±0.06	2.87±0.11	2.27±0.14***	2.86±0.11 <sup>c</sup>	3.00±0.13 <sup>b</sup>

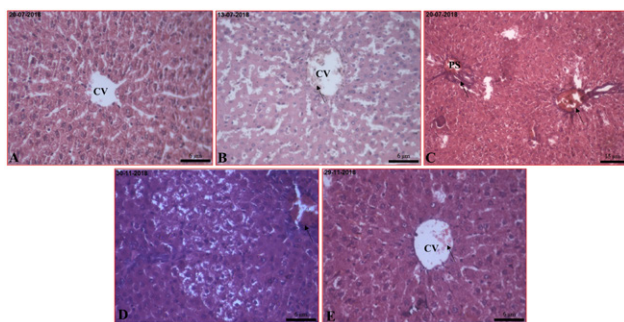
Values expressed as mean ± SEM. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 Vs normal group and <sup>c</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>a</sup>*P* < 0.001 Vs STZ+DEN group. NC=Normal control, STZ=Streptozotocin, DEN=Diethylnitrosamine, ALP=Alkaline phosphatase, TB=Total bilirubin, DB=Direct bilirubin.

**Table 3: Effect of asarone and metformin on antioxidant enzymes in hepatic tissues of different experimental groups.**

Parameters/Group	I (NC)	II (STZ)	III (STZ+DEN)	IV (STZ+DEN+Asarone)	V (STZ+DEN+Metformin)
SOD (units/min/mg protein)	8.11±0.05	4.05±0.07***	3.85±0.24***	6.21±0.14***.a	6.74±0.29***.a
CAT (µmol/min/mg protein)	47.38±1.56	28.79±1.09***	20.39±0.74***	37.71±1.63***.a	39.55±0.84***.a
LPO (µmol/min/mg protein)	2.49±0.16	6.22±0.19***	7.55±0.16***	4.70±0.13***.a	3.93±0.16***.a
GPx (µmol/min/mg protein)	2.55±0.12	1.94±0.10**	0.99±0.09***	2.03±0.05*.a	2.17±0.11 <sup>a</sup>
GSH (µg/g wet tissue)	21.17±0.39	14.17±0.93***	11.19±0.43***	13.89±0.48***.c	15.67±0.19***.a
Vitamin-C (mg/g wet tissue)	0.77±0.02	0.62±0.03**	0.33±0.02***	0.50±0.03***.b	0.60±0.02***.a

Values expressed as mean ± SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  Vs normal group and <sup>c</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>a</sup> $P < 0.001$  Vs STZ+DEN group. Units are as follows: SOD=1 unit of activity equals the enzyme reaction that gave 50% inhibition of nitro-blue tetrazolium (NBT) reduction in 1 min, CAT=µmoles of H<sub>2</sub>O<sub>2</sub> utilized in 1 min, LPO=µmoles of malondialdehyde (MDA) formed in 1 min, GPx=µmoles of glutathione (GSH) oxidized in 1 min.

NC=Normal control, STZ=Streptozotocin, DEN=Diethylnitrosamine, SOD=Superoxide dismutase, CAT=Catalase, LPO=Lipid peroxidation, GPx=Glutathione peroxidase, GSH=Reduced glutathione.



**Figure 1: Photomicrographs of haematoxylin-eosin (H and E) stained histological features of liver sections obtained from normal rats (A), STZ-induced diabetic rats (B), STZ+DEN-induced diabetic-HCC rats (C), STZ+DEN+Asarone (D) and STZ+DEN+Metformin (E) respectively. Black arrowheads indicate congestion in the central vein (CV) and the portal system (PS). (A, B, D, E, magnification: 200; C, magnification:100).**

STZ=Streptozotocin, DEN=Diethylnitrosamine, CV=Central vein, PS=Portal system.

end of 18-weeks showed the features of inflammation accompanied by congestion in the sinusoids, central vein (CV) and portal system (PS) in between the hepatocytes compared to normal livers where the hepatic lobules remain intact with well-defined architecture. Whereas, the STZ+DEN-induced diabetic-HCC rats showed structural disorganization of the livers with the development of all the typical features of HCC. Furthermore, following treatment with asarone and metformin, the degree of the toxic effect was reduced through the restoration of the hepatic architecture compared to STZ+DEN group.

## DISCUSSION

This study demonstrates the prophylactic role of asarone and metformin against STZ+DEN-induced hepatic damage and oxidative stress. The effect of STZ-instigating hyperglycaemia on the development of HCC following a single injection of DEN in experimental

rats unveiled all the characteristic features of diabetes and hepatocarcinogenesis as confirmed in our previous study.<sup>14</sup>

The diabetic liver is strongly correlated with the increased possibility of HCC by 2.5 folds as observed by some studies in contrast with their non-diabetic counterparts.<sup>23,24</sup> The involvement of the free radicals-mediated oxidative stress during hyperglycaemic condition has been involved in the pathological process and progress of diabetes and it contributes to damage to various tissues and organs, including the liver.<sup>25,26</sup> The STZ and DEN-induced oxidative stress are responsible for its carcinogenic effects due to the production of reactive oxygen species (ROS) in the liver. In this study, the STZ alone and STZ+DEN treated rats showed significantly elevated levels of ALP, TB and DB in the serum, which is indicative of hepatotoxic damage due to both the inducing agents. The increase in the level of ALP could be due to variation in the membrane permeability of the liver leading to defective transport of metabolites. Further, significant elevation of bilirubin level in the STZ+DEN treated group may be due to leakage of bilirubin into the circulatory system resulting from hepatocellular damage. This leads to the permeability of the liver membrane, altering the build-up of unconjugated bilirubin in the blood, as reported in earlier studies.<sup>27,28</sup> The two serum protein levels, albumin and globulin, are routinely measured and are recognized as a predictive index in various types of cancer, including liver cancer.<sup>29</sup> In the present study, administration of STZ+DEN altered the levels of serum albumin and globulin, which is an indication of impairment in the protein biosynthesis of the liver.<sup>30</sup> The production of ROS decreases the total serum protein content in the STZ+DEN-treated rats. This variation could be resultant of DEN toxicification, which causes disruption

and dissociation of polyribosomes on endoplasmic reticulum (ER) thus reducing the biosynthesis of protein.<sup>31,32</sup> Treatment with asarone and metformin significantly altered the activities of the above markers in STZ+DEN-induced diabetic-HCC rats. This indicates the maintenance of plasma-membrane stabilizing activity, thus suppressing the release of marker enzymes through membranes and restoration of the proteins synthesis by protecting the polyribosomes, exhibiting hepatoprotective action and inhibiting carcinogenesis.

The SOD and CAT act as scavengers enzyme and preserve the cellular integrity from oxidative mediated stress by maintaining the homeostasis of the biological system.<sup>33</sup> The SOD particularly scavenges and converts the superoxide anion ( $O_2^-$ ) to oxygen ( $O_2$ ) and hydrogen peroxide ( $H_2O_2$ ) and CAT, a ubiquitous enzyme consisting iron helps in the formation of water and oxygen by detoxifying hydrogen peroxide.<sup>34,35</sup> The GPx also plays a role in detoxifying  $H_2O_2$  similar to CAT.<sup>36</sup> The reduced activities of these enzymic antioxidants in diabetic-HCC-induced rats indicate the lack of ability and uncontrolled utilization in deactivating the ROS produced through the chemical modification of STZ+DEN. Further, the status of LPO of unsaturated fatty acids is also taken as straight validation for oxidative related stress as a result of the direct reaction of oxygen and lipids. This, in turn, produces radical intermediates and semi-stable peroxides such as malondialdehyde (MDA).<sup>37</sup> An elevated level of MDA in the STZ+DEN treated rats indicates enhanced LPO, thereby leading to hepatic injury and failure of the antioxidant defence mechanisms. This increase in the LPO levels is in accordance with earlier reports, as observed in the experimental liver tissues of diabetes or cancer models.<sup>38,39</sup> However, following oral administration of asarone and metformin, the activities of these enzymic antioxidants are restored which is an indicator for its ability to scavenge the ROS, thus preventing the further damage from oxidative stress due to STZ+DEN.

The STZ+DEN-induced liver damage and ROS-mediated oxidative stress decreased the non-enzymic antioxidants levels, namely the GSH and Vitamin-C. The GSH is mainly involved in inhibiting the oxidative stress and acts against LPO.<sup>40</sup> Similarly, Vitamin-C, a hydrophilic antioxidant in unification with glutathione removes or deactivates the generated free radicals.<sup>41</sup> However, following oral administration of asarone and metformin, the levels of these non-enzymic antioxidants are significantly increased when compared to STZ+DEN group. This elevation to the near-normal level by the test compounds confirms the protection and subsequent prevention form STZ+DEN-mediated oxidative stress.

In confirmation to our experiment, treatment with asarone and metformin to STZ+DEN rats either decreased or altered the severity in hepatocellular damage which is evidenced by the hepatic marker enzymes, antioxidants enzymes and histopathological observation.

In conclusion, reactive oxygen-mediated oxidative stress is predominant in the initiation and growth of hepatocarcinogenesis during the diabetic condition. The administration of asarone and metformin significantly ameliorates the changes in both the hepatic marker enzymes and antioxidant enzymes in diabetic-HCC rats, hence protecting the liver from oxidative stress-induced damage. However, the investigation about the possibility of involvement of the exact mechanism of action through cancer-signalling pathways is essential in controlling the progression of HCC.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## ABBREVIATIONS

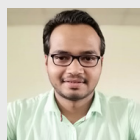
**HCC:** Hepatocellular carcinoma; **STZ:** Streptozotocin; **DEN:** Diethylnitrosamine; **ROS:** Reactive oxygen species; **SOD:** Superoxide; **CAT:** Catalase; **LPO:** Lipid peroxidation; **GPx:** Glutathione peroxidase; **GSH:** Reduced glutathione.

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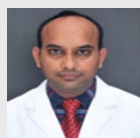
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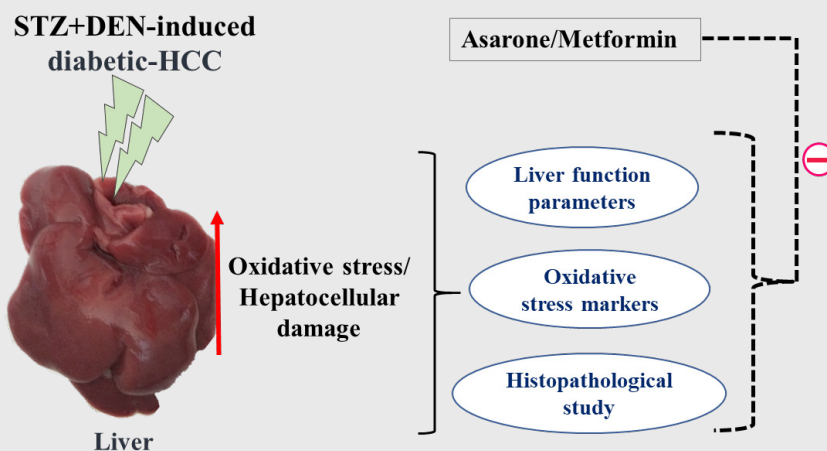


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### PICTORIAL ABSTRACT



STZ: Streptozotocin; DEN: Diethylnitrosamine; HCC: Hepatocellular carcinoma

### SUMMARY

In the present study, the diabetic-hepatocellular carcinoma (HCC) rats were supplemented with asarone and metformin for 18 weeks. Supplementation of asarone and metformin reduced the burden of streptozotocin (STZ) and diethylnitrosamine (DEN) mediated reactive oxygen species (ROS) and restored the balance of oxidation-antioxidant status along with the liver function parameters. In addition, asarone and metformin-treated rats also showed recovery of the hepatic architecture compared to STZ + DEN. Henceforth, asarone and metformin exhibited good hepatoprotective and antioxidant potential against STZ + DEN-induced HCC in rats.

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