### 3.2.3. The combined role of α-and β-asarone

The $IC_{50}$ values for α-asarone, β-asarone, and metformin HCl derived through the colorimetric based MTT assay *(Section 3.2.1.)* are tabulated, and the concentration for the further study was chosen as one half of the $IC_{50}$ value (Table 37).

**Table 37:** The $IC_{50}$ values of α-asarone, β-asarone and metformin HCl based on the MTT assay.

| **Test compounds** | **$IC_{50}$ values** (MTT assay) | **One half of the $IC_{50}$** (Selected for further study) |
|---|---|---|
| α-asarone | 1.22 mM | 0.61 mM |
| β-asarone | 1.46 mM | 0.73 mM |
| Metformin HCl | 25.74 mM | 12.87 mM |

Furthermore, the combined effect of α- and β-asarone (0.61 mM + 0.73 mM) was stronger than the single effect of α- or β-asarone (0.61 mM or 0.73 mM). As shown in figure 43, the cell viability of α- and/or β-asarone was reduced compared to the untreated group. Whereas the combination of α- and β-asarone was stronger than its single effect, as evident with the decreased viability of the cells ($p<0.05$).

The HepG2 cells morphology when cultured under two conditions of glucose (5.5 and 25 mM) and the effect of a combined dose of asarone (0.61 mM α-asarone; 0.73 mM β-asarone) and metformin (12.87 mM) under high glucose condition as observed has been illustrated in figure 44. Following treatment with asarone and metformin for 48-h, poor growth or inhibition of HepG2 cells was observed compared to the untreated group.

Based on the above results, the HepG2 cells were categorized into four groups consisting of:

1) Normoglycemic (NG): The HepG2 cells were grown in normal glucose at a concentration of 5.5 mM.
2) Hyperglycemic (HG): The HepG2 cells were grown in high glucose at a concentration of 25 mM.
3) HG+Asarone: The HepG2 cells treated with a combination dose of 0.61 mM of α-asarone and 0.73 mM of β-asarone in HG condition and,
4) HG+Metformin: The HepG2 cells were treated with 12.87 mM of metformin HCl in HG condition.

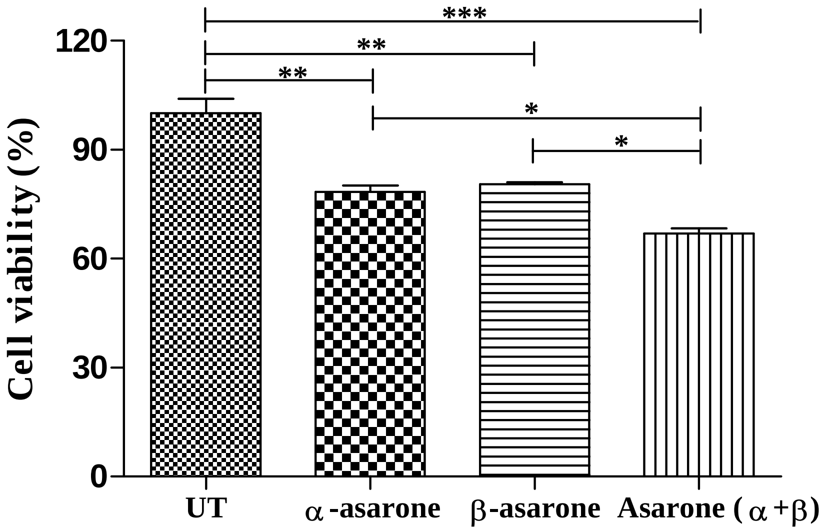


**Figure 43** (Combined effect of Asarone)**:** The MTT test was used to investigate the combined effect of asarone, where the viability of the cells decreased in the α- and/or β-asarone group in comparison to the untreated (UT) group. The mean ± SEM for triplicate experiments was used to calculate the results, where $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared as specified.

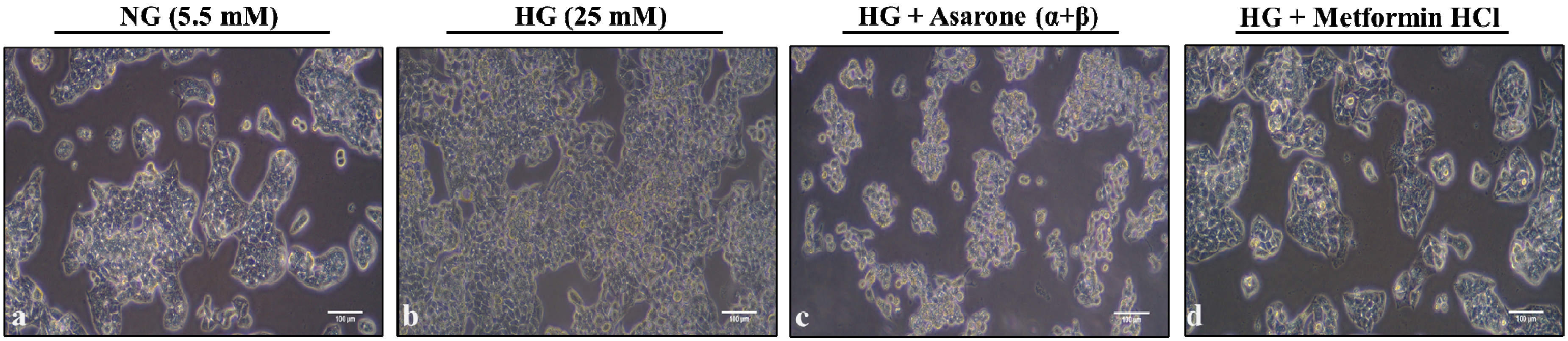


**Figure 44** (The morphology of human HepG2 cells and combined effect of Asarone and Metformin)**:** The changes of HepG2 cell morphology shown as representative images were observed by an inverted microscope (100 µm). (a) In the normoglycemic (NG) group, HepG2 cells exhibited a 50-60% confluency with adherent cell colonies in their morphology. The hyperglycemic (HG) group (b) was 80-90% confluency with epithelial-like morphology compared to the NG group. On the other hand, the cells exposed to combined treatment of asarone (c) and metformin (d) supported the results showing cell cytotoxicity with decreased cell viability compared to the HG group.

### 3.2.4. Cell cycle analysis

The flow cytometry technique with propidium iodide-RNase (PI-RNase) labeling was used to assess the different cell cycle phases for asarone and metformin-mediated growth inhibition of HepG2 cells. The cell cycle of HepG2 cells was halted in the $G_0/G_1$ phase when they were treated with asarone and metformin in high glucose conditions. However, compared to the hyperglycemic condition, the percentage of cells in the S-phase was much lower in the treatment groups. Furthermore, the HepG2 cells in the sub$G_1$ population stage were retained when treated with metformin (Figures 45 and 46). These results indicated that asarone and metformin impair cell growth at the $G_0/G_1$ phase of the cell cycle during the hyperglycemic condition.

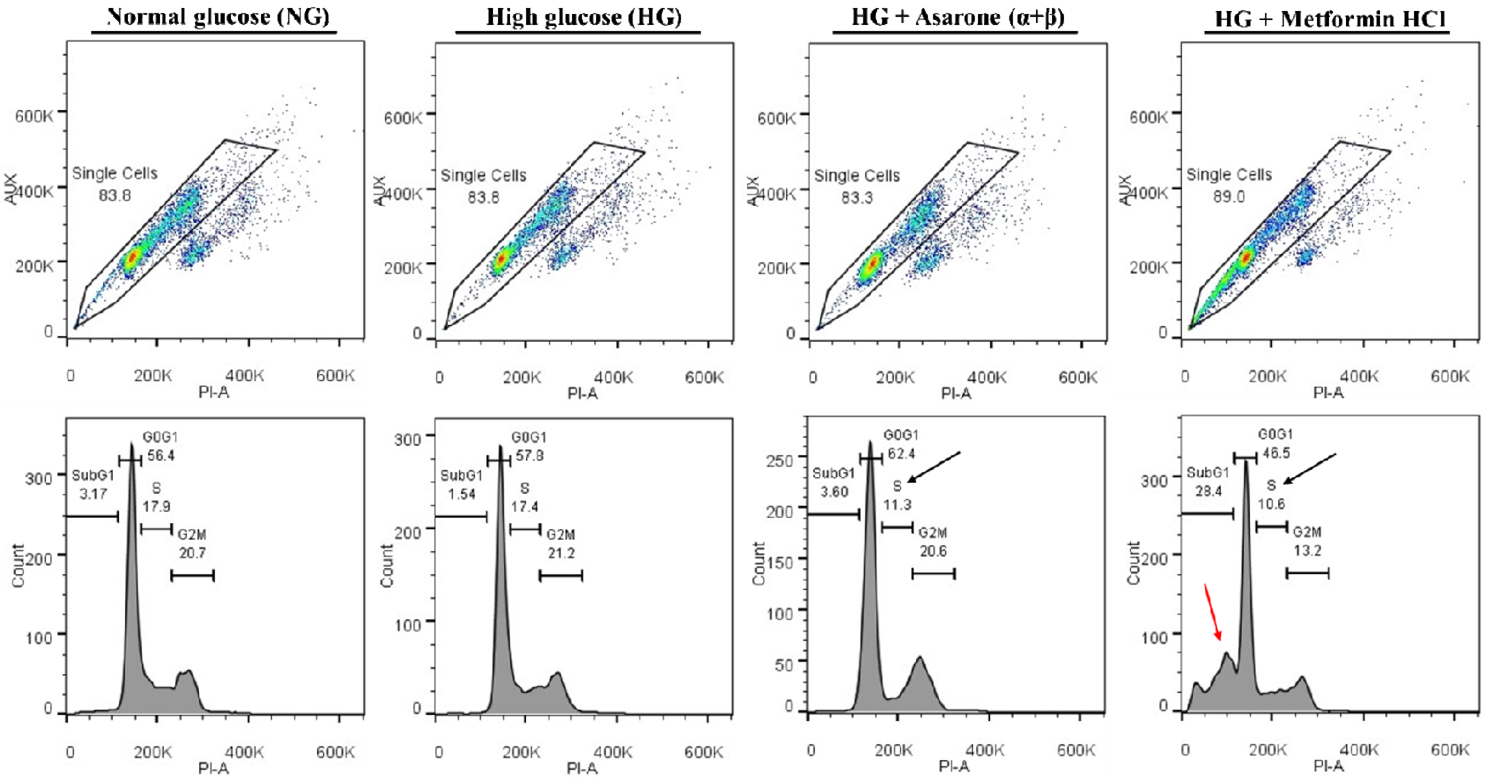


**Figure 45** (Asarone and Metformin arrest HepG2 cells in $G_0/G_1$ phase)**:** Upper panel: In this, the samples were first gated for singlets stained with propidium iodide-RNase (PI-RNase) and then the cell cycle profile was determined from this gated population. Lower panel: Representative flow cytometric histograms of HepG2 cells grown in normal glucose (NG), high glucose (HG), HG+Asarone (α+β) and HG+Metformin. Here, the black arrowhead indicates the percentage of the reduced cells in the S-phase, and the red arrowhead indicates the percentage of the cells in the subG1 stage.

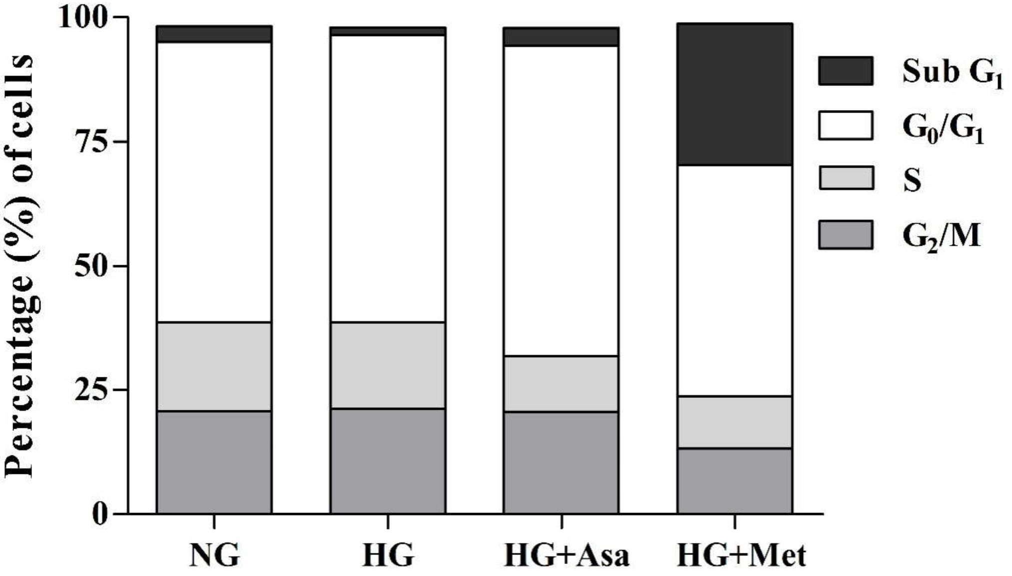


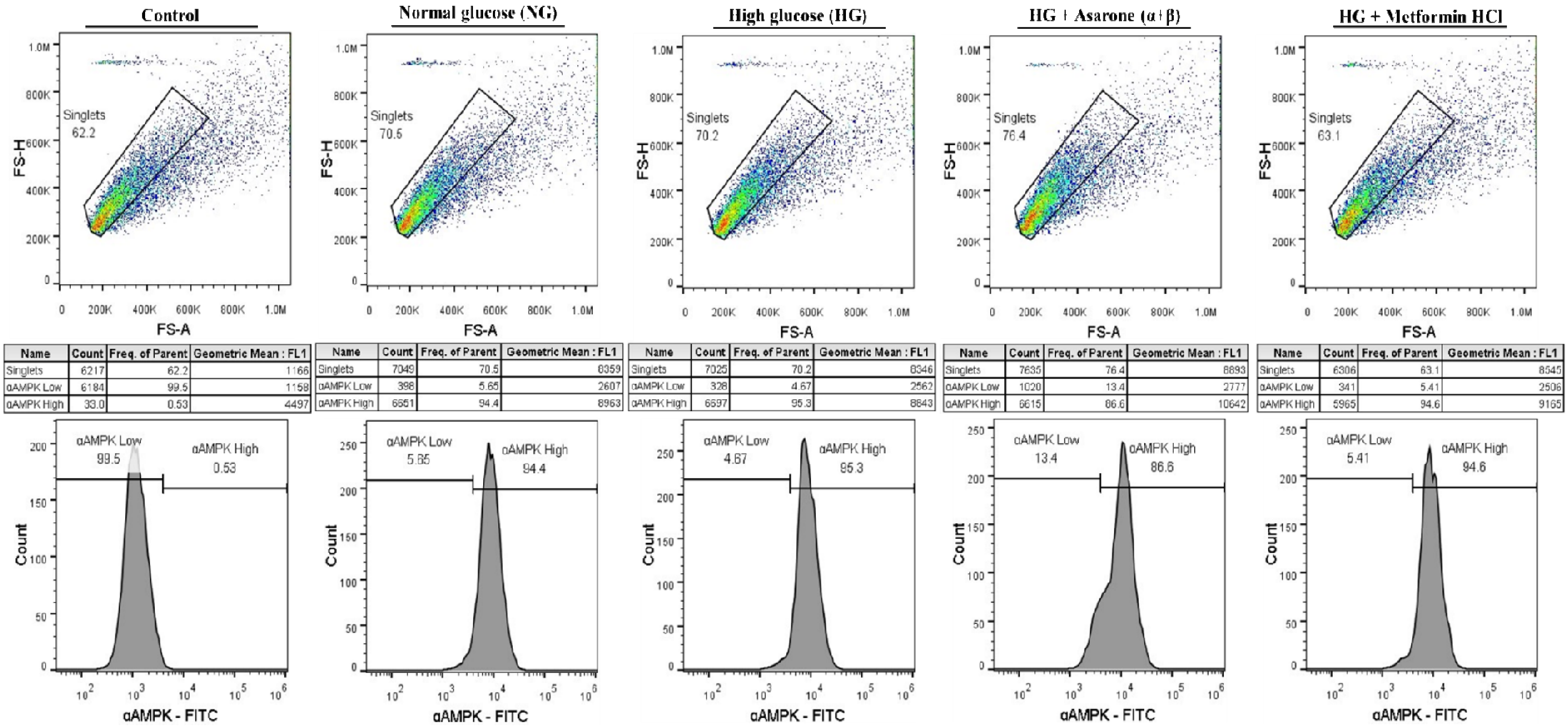
**Figure 46** (HepG2 cells are arrested in the $G_0/G_1$ stage by asarone and metformin)**:** Bar diagram illustrates the proportion (%) of cells measured by flow cytometry at different cell cycle phases. All the results are illustrative of an experiment done in triplicate.

NG, Normal glucose; HG, High glucose; HG+Asa, High glucose+Asarone (α+β); HG+Met, High glucose+Metformin.

### 3.2.5. Expression of AMPKα1, PCK-2 and SREBP-1

The asarone and metformin mediated HepG2 cells cytotoxicity might result from altered cellular energy homeostasis during the hyperglycemic condition. In response to the changed energy homeostasis involving in the progression of HCC, the AMPK signaling pathway plays a significant role. The downstream regulators in the AMPK pathway, namely the PCK-2 and SREBP-1, play a role in regulating gluconeogenesis, glucose uptake to glycolysis, and *de novo* lipogenesis. The expression of AMPKα1, PCK-2 and SREBP-1 on HepG2 cells during the hyperglycemic condition in each group was assessed by flow cytometry as indicated by the histogram events and geometric mean fluorescence intensity (GMFI). As observed in figures 47 and 48, the flow cytometry study revealed that asarone and metformin enhanced the expression of AMPKα1. The expression of AMPKα1 (represented as GMFI) was significantly increased for the asarone ($p<0.001$) and metformin ($p<0.01$) in comparison to the high glucose as the negative control.

Furthermore, the results from figures 49 to 52 indicated that asarone and metformin reduced the expression of PCK-2 ($p<0.001$) and SREBP-1 ($p<0.001$; $p<0.01$) when compared to the cells treated with high glucose as the negative control. The expression of PCK-2 was significantly enhanced ($p<0.001$) during hyperglycemic conditions compared to normal glucose conditions. There were no such changes observed with the expression of AMPKα1 and SREBP-1 levels during high glucose conditions. Hence, these data indicate that the HepG2 cell cytotoxicity induced by asarone and metformin treatment results in arresting in the cell cycle is mediated by the upregulation of AMPKα1 and downregulation of PCK-2 and SREBP-1 during the hyperglycemic condition.



| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 6217 | 62.2 | 1166 |
| αAMPK Low | 6184 | 99.5 | 1158 |
| αAMPK High | 33.0 | 0.53 | 4497 |

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 7049 | 70.5 | 8359 |
| αAMPK Low | 398 | 5.65 | 2607 |
| αAMPK High | 6651 | 94.4 | 8963 |

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 7025 | 70.2 | 8346 |
| αAMPK Low | 328 | 4.67 | 2562 |
| αAMPK High | 6697 | 95.3 | 8843 |

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 7635 | 76.4 | 8893 |
| αAMPK Low | 1020 | 13.4 | 2777 |
| αAMPK High | 6615 | 86.6 | 10642 |

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 6306 | 63.1 | 8545 |
| αAMPK Low | 341 | 5.41 | 2506 |
| αAMPK High | 5965 | 94.6 | 9165 |

**Figure 47** (The expression of AMPKα1)**:** Upper panel: In this, the samples were first gated for singlets stained with FITC (Fluorescein isothiocyanate) dye expression of AMPKα1 was determined from this gated population. Lower panel: Representative flow cytometric peak of HepG2 cells showing the percentage of AMPKα1 grown in normal glucose (NG), high glucose (HG), HG+Asarone (α+β) and HG+Metformin. The middle table represents the details of singlet cells in each group.

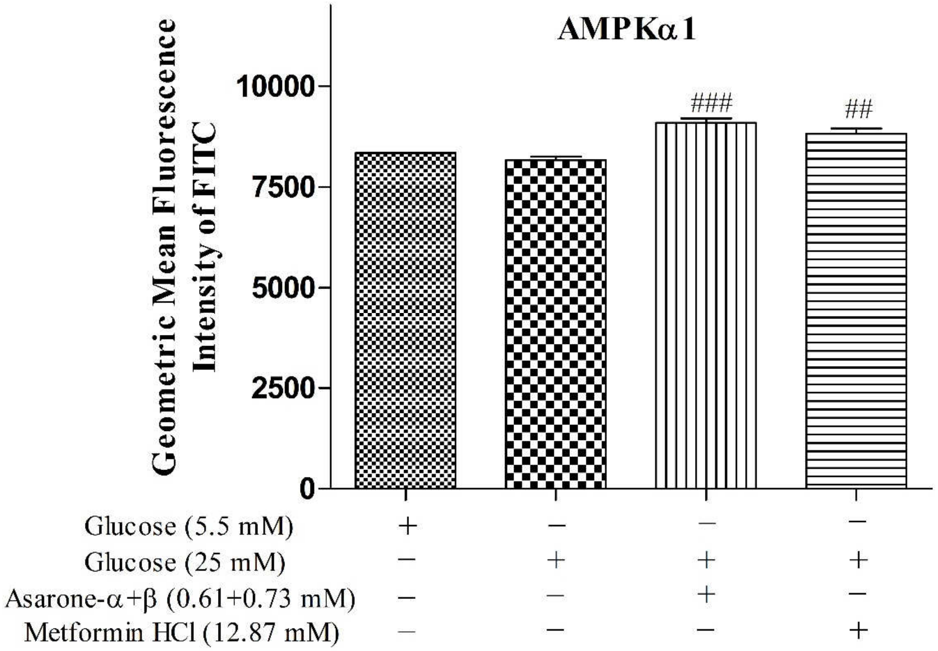
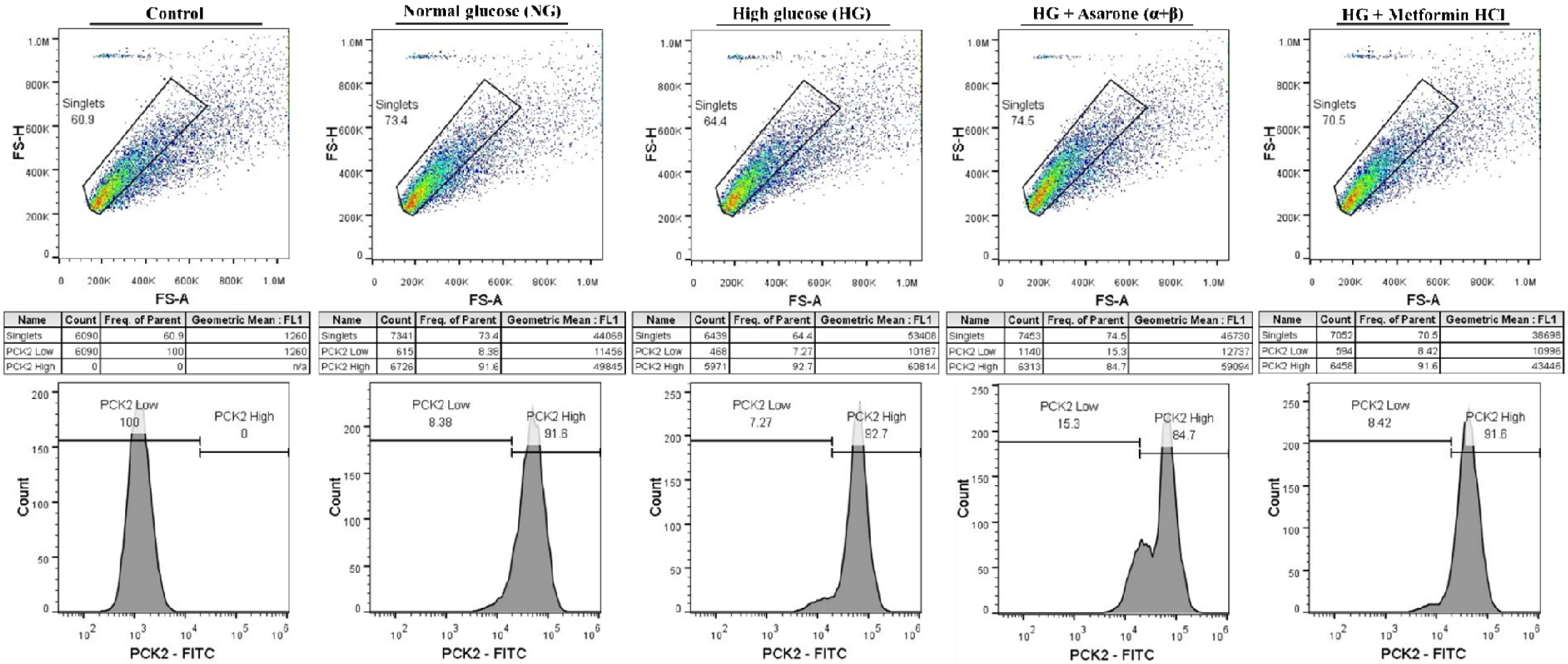


**Figure 48** (The treatment with asarone and metformin up-regulates the expression of AMPKα1 during hyperglycemic condition)**:** Here, the bar figures represent the GMFI of AMPKα1 in HepG2 cells grown in normal glucose (NG), high glucose (HG), HG+Asarone and HG+Metformin. The results were calculated and expressed as mean ± SEM for triplicate experiments, where $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ in comparison to the high glucose group.



| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 6090 | 60.9 | 1260 |
| PCK2 Low | 6090 | 100 | 1260 |
| PCK2 High | 0 | 0 | n/a |

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 7341 | 73.4 | 44068 |
| PCK2 Low | 615 | 8.38 | 11456 |
| PCK2 High | 6726 | 91.6 | 49845 |

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 6439 | 64.4 | 53408 |
| PCK2 Low | 468 | 7.27 | 10187 |
| PCK2 High | 5971 | 92.7 | 60814 |

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 7453 | 74.5 | 46730 |
| PCK2 Low | 1140 | 15.3 | 12737 |
| PCK2 High | 6313 | 84.7 | 59094 |

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 7052 | 70.5 | 38698 |
| PCK2 Low | 594 | 8.42 | 10996 |
| PCK2 High | 6458 | 91.6 | 43446 |

**Figure 49** (The expression of PCK-2)**:** Upper panel: In this, the samples were first gated for singlets stained with (Fluorescein isothiocyanate) dye, and then the expression of PCK-2 was determined from this gated population. Lower panel: Representative flow cytometric peak of HepG2 cells showing the percentage of PCK-2 grown in normal glucose (NG), high glucose (HG), HG+Asarone, and HG+Metformin. The middle table represents the details of singlet cells in each group.

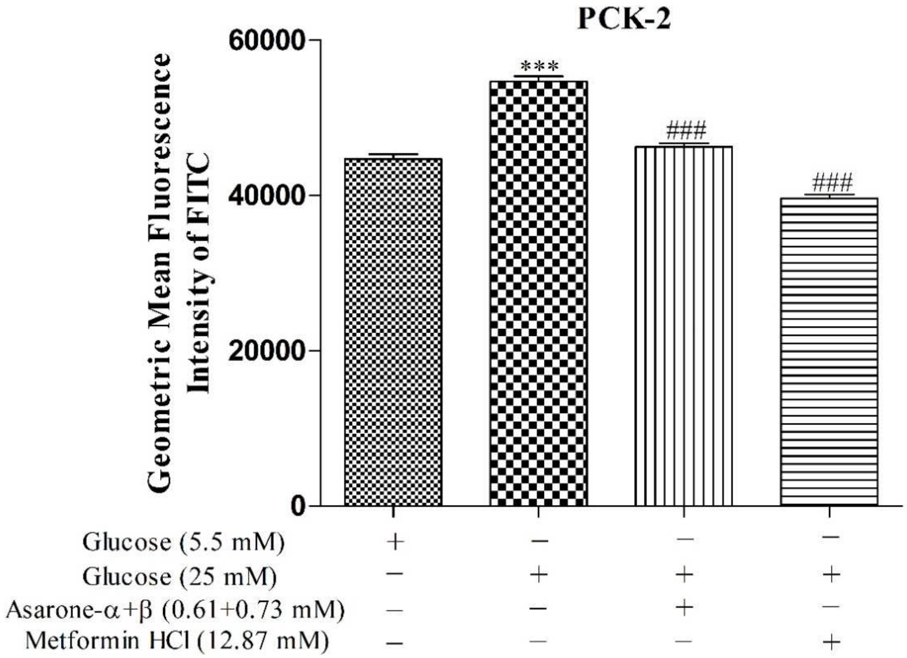
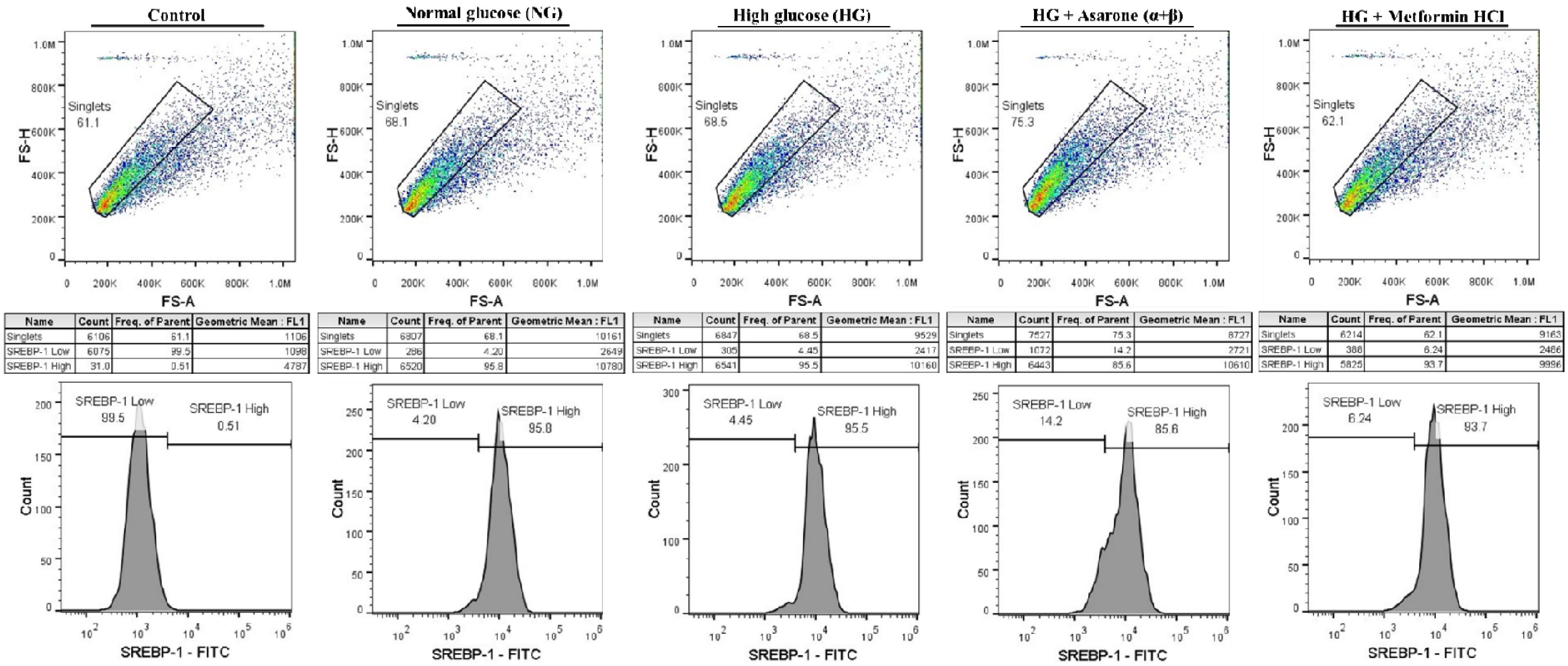


**Figure 50** (The treatment with asarone and metformin down-regulates the expression of PCK-2 during hyperglycemic condition)**:** Here, the bar figures represent the GMFI of PCK-2 in HepG2 cells grown in normal glucose (NG), high glucose (HG), HG+Asarone and HG+Metformin. The results were calculated and expressed as mean ± SEM for triplicate experiments, where $^{***}p<0.001$ in comparison to the normal glucose group and $^{\#\#\#}p<0.001$ in comparison to the high glucose group.



| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 6106 | 61.1 | 1106 |
| SREBP-1 Low | 6075 | 99.5 | 1098 |
| SREBP-1 High | 31.0 | 0.51 | 4787 |

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 6807 | 68.1 | 10161 |
| SREBP-1 Low | 286 | 4.20 | 2649 |
| SREBP-1 High | 6520 | 95.8 | 10780 |

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 6847 | 68.5 | 9529 |
| SREBP-1 Low | 305 | 4.45 | 2417 |
| SREBP-1 High | 6541 | 95.5 | 10160 |

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 7527 | 75.3 | 8727 |
| SREBP-1 Low | 1072 | 14.2 | 2721 |
| SREBP-1 High | 6443 | 85.6 | 10610 |

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 6214 | 62.1 | 9163 |
| SREBP-1 Low | 388 | 6.24 | 2486 |
| SREBP-1 High | 5825 | 93.7 | 9996 |

**Figure 51** (The expression of SREBP-1)**:** Upper panel: In this, the samples were first gated for singlets stained with FITC (Fluorescein isothiocyanate) dye, and then the expression of SREBP-1 was determined from this gated population. Lower panel: Representative flow cytometric peak of HepG2 cells showing the percentage of SREBP-1 grown in normal glucose (NG), high glucose (HG), HG+Asarone, and HG+Metformin. The middle table represents the details of singlet cells in each group.

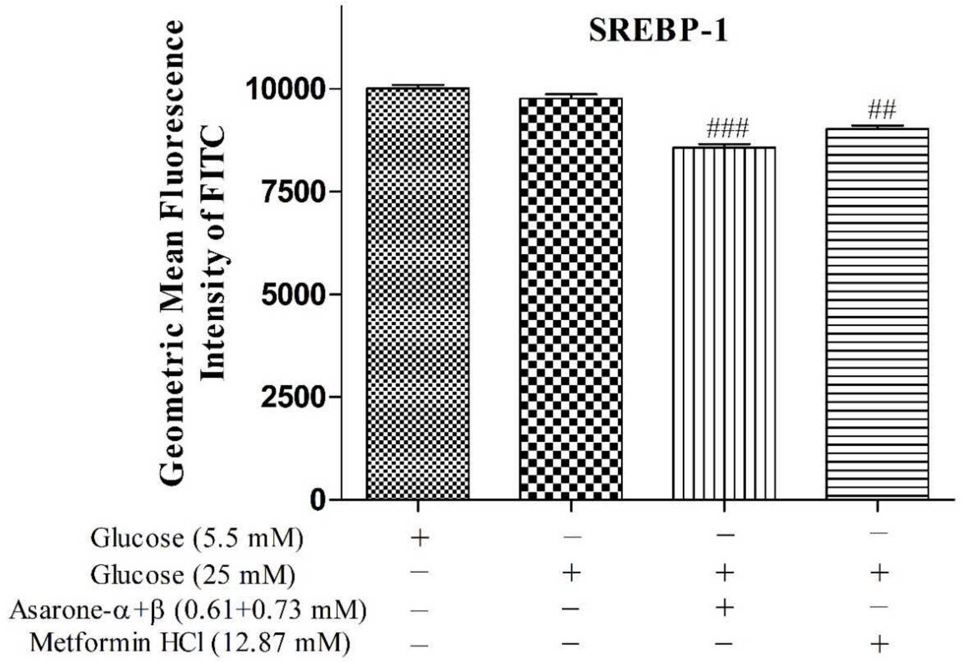
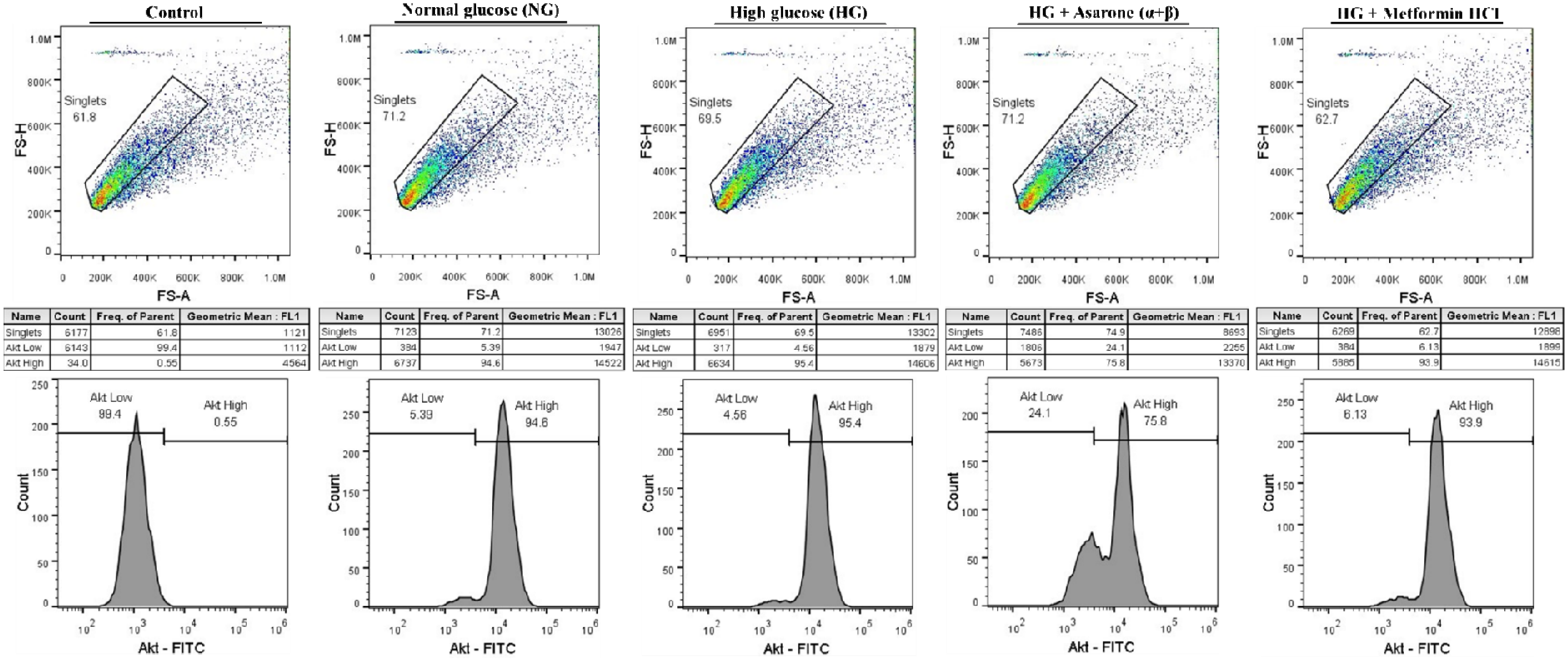


**Figure 52** (The treatment with asarone and metformin down-regulates the expression of SREBP-1 during hyperglycemic condition)**:** Here, the bar figures represent the GMFI of SREBP-1 in HepG2 cells grown in normal glucose (NG), high glucose (HG), HG+Asarone and HG+Metformin. The results were calculated and expressed as mean ± SEM for triplicate experiments, where $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ in comparison to the high glucose group.

### 3.2.6. Expression of Akt

The expression of PI3K/Akt, a prototypic signaling pathway, is associated with regulating growth and proliferation in hepatocarcinogenesis. Following treatment with asarone and metformin, the expression level of Akt was detected by flow cytometry. The results showed that the change in the glucose concentration did not show any significant difference in the expression of Akt in HepG2 cells. Nevertheless, as shown in figures 53 and 54, as indicated by GMFI, a significant decrease ($p<0.001$; $p<0.01$) in the expression levels of Akt was observed in asarone and metformin-treated groups compared with the high glucose group. This result suggested that asarone and metformin exert cytotoxic action on HepG2 cells, which may involve modulation of Akt beside the AMPK signaling pathway.



Control

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 6177 | 61.8 | 1121 |
| Akt Low | 6143 | 99.4 | 1112 |
| Akt High | 34.0 | 0.55 | 4564 |

Normal glucose (NG)

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 7123 | 71.2 | 13026 |
| Akt Low | 384 | 5.39 | 1947 |
| Akt High | 6737 | 94.6 | 14522 |

High glucose (HG)

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 6951 | 69.5 | 13302 |
| Akt Low | 317 | 4.56 | 1879 |
| Akt High | 6634 | 95.4 | 14606 |

HG + Asarone (α+β)

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 7486 | 74.9 | 8693 |
| Akt Low | 1806 | 24.1 | 2255 |
| Akt High | 5673 | 75.8 | 13370 |

HG + Metformin HCl

| Name | Count | Freq. of Parent | Geometric Mean : FL1 |
|---|---|---|---|
| Singlets | 6269 | 62.7 | 12898 |
| Akt Low | 384 | 6.13 | 1899 |
| Akt High | 5885 | 93.9 | 14615 |

**Figure 53** (The expression of Akt)**:** Upper panel: In this, the samples were first gated for singlets stained with FITC (Fluorescein isothiocyanate) dye, and then the expression of Akt was determined from this gated population. Lower panel: Representative flow cytometric peak of HepG2 cells showing the percentage of Aktgrown in normal glucose (NG), high glucose (HG), HG+Asarone, and HG+Metformin. The middle table represents the details of singlet cells in each group.

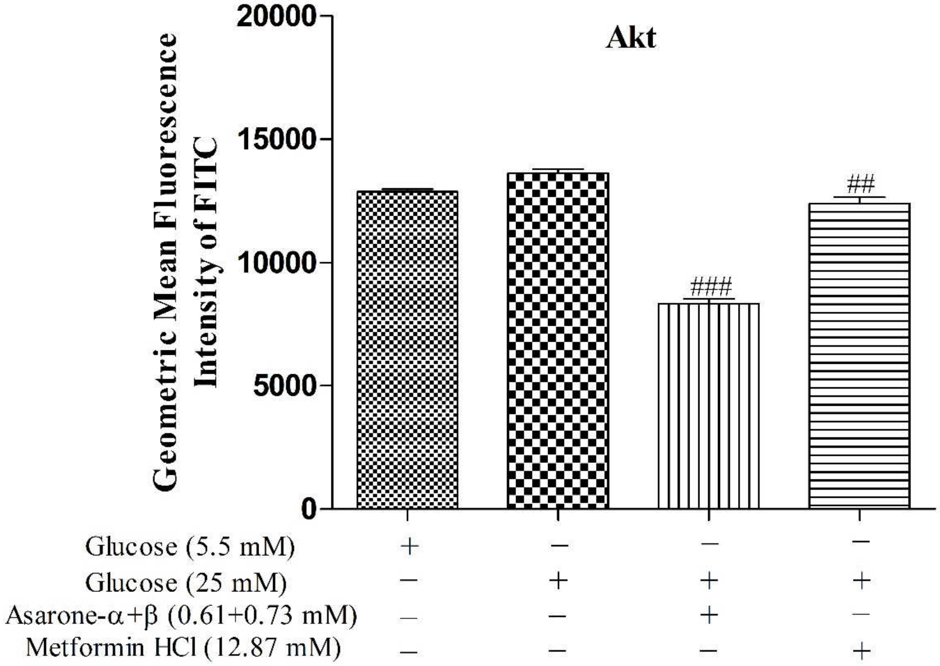


**Figure 54** (The treatment with asarone and metformin down-regulates the expression of Akt during hyperglycemic condition)**:** Here, the bar figures represent the GMFI of Akt in HepG2 cells grown in normal glucose (NG), high glucose (HG), HG+Asarone and HG+Metformin. The results were calculated and expressed as mean ± SEM for triplicate experiments, where $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ in comparison to the high glucose group.

# 4. DISCUSSION

A strong association co-exists between diabetes and hepatocellular carcinoma (HCC) to its relative risk.[200,201] Some of the risk factors related to diabetes (namely insulin, IGF-1, or inflammation) have been broadly studied with cancer progression and considered significant links. However, less attention has been paid to how hyperglycemia promotes tumorigenesis in cancer, specifically HCC.

There is limited evidence in the existing scientific literature concerning the impact of diabetes promoted hepatocarcinogenesis in an animal model. Henceforth this study focuses on the following objectives. One of the objectives of this work was to mimic an animal model to validate the scientific proof for the diabetic-HCC condition. The streptozotocin (STZ)-induced rats with chronic hyperglycemia did not develop HCC until the end of the 12-week study period. Conversely, hepatocarcinogenesis was observed in the diethylnitrosamine (DEN) alone and STZ+DEN-induced animals. These data of study design-I suggest that hyperglycemia alone has no impact on HCC development. Whereas, the pre-administration of the diabetogenic agent (STZ) before initiation of DEN promotes HCC by a few weeks earlier as supported by all biochemical and histo-morphological changes.

Further, this study aimed to demonstrate the role of test compound asarone and anti-diabetic drug metformin against experimentally induced diabetic-HCC and attempted to elucidate the cancer-associated pathways both *in-vivo* and *in-vitro*. The results demonstrated that asarone and metformin treatment either reduced or reversed the severity of hepatocarcinogenesis during the diabetic condition, indicating their chemo-preventive effect. This was supported by all biochemical, $^{1}$H-NMR based metabolomics and histo-morphological evidence. The *in-vitro* study results suggest that asarone and metformin treatment reduces the HepG2 cells proliferation in hyperglycemic state by arresting the $G_0/G_1$ stage of the cell cycle. This was mediated by activating AMPK (5´adenosine monophosphate-activated protein kinase) and inhibiting protein kinase B/Akt signaling pathways. This study also proved that the inhibition of PCK-2 (phosphoenolpyruvate carboxykinase-2) and SREBP-1 (sterol regulatory element-binding protein-1) indicate an association between the glucose metabolic pathway and HepG2 cell proliferation.

## 4.1. *In-vivo study*

### 4.1.1. General observations

It is well-established that the STZ exerts its diabetogenic effect by acting on the insulin-secreting pancreatic beta-cells through the glucose transporter-2 (GLUT-2) protein, which causes cell swelling and impairment of mitochondrial function and production of nitric oxide and free radicals. Further, induction of DNA methylation leads to degressive glucose uptake and utilization and increases blood glucose and decreased insulin levels.[202-204] In general, hyperglycemia, hypo-insulinemia, severe loss in body weight, polyuria, polydipsia, and polyphagia characterize STZ-induced diabetes similar to the clinical symptoms of diabetic patients. The present study results confirmed a continuous state of increased blood glucose levels after a single injection of STZ. They remained in the hyperglycemic state until the end of the experiment. The experiential reduction in average body weight along with the increased consumption of relative food and water in the study are consistent with these observations in diabetic rats. The decline in insulin due to the administration of STZ causes impairment in the regulation of muscle protein metabolism and muscle wasting.[205] The characteristic weight loss observed in STZ-induced animals is most likely due to increased muscle wasting caused by accelerated catabolism of structural proteins and reduced protein synthesis due to defective insulin secretion.[206,207] Further, the elevated glucose levels result in glucosuria along with loss of water and electrolytes as the capacity of the kidneys to re-absorb glucose is surpassed. This condition is termed polyuria. The overall decline in the volume of water from the body leads to the activation of the thirst mechanism, known as polydipsia. As a result of glucosuria and defective regulation of proteins and fats, it ensues in negative energy balance, leading to an increase in appetite or food intake as observed in the case of the STZ-induced rats, termed as polyphagia.[208,209] The present study also showed increased relative liver weight in the STZ-treated rats even though the average body weight of the animals was reduced. This could be due to low insulin levels, resulting in the increased influx of fatty acids and triglyceride accumulation into the hepatocytes.[210-212] Furthermore, in the present study, the elevated glycosylated hemoglobin ($HbA_{1c}$) levels in the STZ-treated rats indicate a continuous state of long-standing hyperglycemia as observed in study design II. This is responsible for oxidative degradation and non-enzymatic glycation of proteins and is measured as a tool for diagnosing diabetes and its related complications.[213]

The liver is a fundamental metabolic organ mainly involved in maintaining the regulation of glucose metabolism through glycogenic and gluconeogenic pathways, and high glucose is considered one of the main characteristics of diabetes.[214] The long-standing hyperglycemia during diabetes leads to severe classical health complications and negatively impacts various tissues and organs, including the liver.[215,216] Many epidemiological reports strongly associate diabetes-mediated hyperglycemia with the increased progression of malignant hepatocarcinogenesis.[7,86] Experimental studies to mimic the HCC involve using different chemicals, including a representative compound of the nitrosamine family, the diethylnitrosamine (DEN). The liver is the leading site of metabolism of the most potent carcinogen, DEN. It's been linked to reactive oxygen species (ROS) production, causing oxidative stress and cellular damage.[217,218] It takes a longer duration when used alone; however, when combined with just a promoting agent, it can significantly shorten the time required for the incidence of hepatocarcinogenesis.[219] In the present study, tumor-associated proteins produce subcapsular nodules in DEN alone or STZ+DEN-induced rats. Subsequently, uncontrolled proliferation of cells increases the liver and relative liver weight. In both rats and humans, an increase in the number, growth, and size of hepatocytic nodules on the surface could increase tumour volume, confirm the presence, and act as a probable precursor to HCC. Previous studies in experimental and human disease correlate with the number and size of hepatocyte nodules and increased HCC.[220-223] However, oral treatment of asarone and metformin alleviated the STZ+DEN induced increased liver weight, suggesting that it has suppressed the tumor growth (Table 30, 31). Furthermore, the significant decrease in the average body weight of DEN alone or STZ+DEN administered rats doesn’t correlate with the increased food consumption compared to normal rats. Hence, the weight loss observed in these groups could be independent of food consumption. One of the reasons for polyphagia and a decrease in average body weight might be the pre-administration of STZ in the diabetic-HCC group, which results in negative energy balance and increased muscle wasting.[205,207]

It has been established from the data presented in the results section of the *in-vivo* study that oral administration of asarone and metformin has caused a substantial decrease in the blood glucose concentration and an upsurge in the insulin levels in the diabetic-HCC rats (Figure 32; Table 30). The asarone-mediated hypoglycemic effect might be due to the stimulus of plasma insulin release from the existing β-cells in STZ+DEN induced rats, resulting in increased glucose utilization by the extrahepatic tissues. Moreover, reducing $HbA_{1c}$ in the STZ+DEN-treated rats indicates the potential of asarone and metformin to avert the diabetic-related complications mediated by the glucose-lowering effect, thus contributing towards the neutralization of free radicals (Table 30).

### 4.1.2. Liver function markers

The result of reactive oxygen species (ROS)-mediated oxidative damage caused by an injury in the liver releases specific enzymes/markers into the bloodstream from the damaged hepatocytes. The levels of serum liver markers were higher in different diseased conditions and are considered the pathophysiological indicator of the liver, including diabetes mellitus.[224-227] The STZ+DEN-induced oxidative stress is responsible for its carcinogenic effects due to ROS production in the liver. In this study, the STZ and/or DEN treated rats showed significantly elevated levels of aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT), and bilirubin in the serum, which is indicative of hepatotoxic damage due to both the inducing agents (STZ and DEN). The aminotransferases viz., the ALT and AST are involved in amino acid metabolism's transamination and oxidation process. The ALT catalyzes the transfer of amino group from alanine to α-ketoglutarate (α-KG) and engages in the formation of glutamate and pyruvate. While, the AST, a tissue enzyme located in the cytosol of the liver, catalyzes the exchange of $\alpha$-ketoglutaric acids and aspartic to glutamic acids and oxaloacetate. Besides amino acid metabolism, it also correlates with carbohydrate and protein metabolism by exchanging active substances.[228,229] The ALP, a membrane-bound enzyme of the liver, increases with the noxious effect of the inducing agents. It is the resultant of the variation in the membrane permeability, thereby leading to disarrangement in the transport of essential metabolites.[217,230] Further, a significant increase of bilirubin levels in the STZ+DEN administered group may be attributed to the failure of normal uptake, conjugation, excretion, or leakage of bilirubin into the circulatory system resulting from severe hepatic parenchymal damage. This leads to variation in the membrane permeability, altering the build-up of unconjugated bilirubin in the blood, as in concurrence with previous studies.[230,231] Further, the generation of ROS due to STZ and DEN decreases the total serum protein content. This variation is due to the intoxication of a carcinogenic agent, which causes disturbance and dissociation of polyribosomes on the endoplasmic reticulum, thereby decreasing protein biosynthesis.[232,233] Albumin and globulin, the two-serum protein, are routinely measured and known as a prognostic indicator in several types of cancer, including liver cancer. As observed, the decreased levels of this serum protein in the STZ+DEN-induced rats suggest impairment in the liver protein biosynthesis.[234,235] In contrast to the STZ+DEN group, both treatment groups demonstrated a significant reduction in ALT, AST, ALP, and bilirubin levels, as well as an overall increase in protein albumin and globulin levels (Table 32). This finding supports the stabilizing activity of the plasma membrane, or the ability to repair the liver injury by inhibiting the leakage of enzymes through membranes and protecting the polyribosomes through the restoration of the protein synthesis, thus providing hepatoprotective action and inhibiting the hepatocarcinogenesis.

### 4.1.3. Lipid profile

The body's homeostasis gets disturbed during metabolic syndrome (MetS), which is linked to the pathophysiology of various disorders, including diabetes mellitus.[236] The complex pathophysiology of MetS involves multiple organs, including the liver, as it is one of the most important organs playing a pivotal role in energy metabolism. Along with carbohydrates, proteins, and nucleic acid, it plays a vital role in maintaining the homeostasis of endogenous lipids, lipoproteins, and plasma apolipoproteins metabolism.[237,238] Further, the progression of hepato-carcinoma has also been linked with the alteration in plasma lipid and lipoprotein metabolism. As a source of energy and support of cell division and fatty acid derivatives, the tumor cells are highly dependent on the metabolism of the lipids, thereby directly linking to cell survival and growth.[239,240] In this study, the STZ and/or DEN treated rats showed significantly altered lipid profile levels as TC, TG, LDL-c and HDL-c. During diabetes, STZ-induced animals rely on other fuels for energy, such as lipids or free fatty acids, due to impaired glucose metabolism. This suggests the breakdown of lipids and further mobilization of free fatty acid from the peripheral fat repository. Henceforth, this results in triglycerides, cholesterol, and other lipids, as indicated in the study. This increase can also be explained due to the lack of insulin (resultant of STZ administration), which fails to activate the lipoprotein lipase enzyme, thereby causing elevated triglycerides and cholesterol levels.[241-243] Furthermore, during the diabetic-HCC conditions, a significant increase in the lipid levels directs the supplement of energy source for the proliferation of tumor cells besides glucose metabolism. Together, the survival and growth process with the alteration in lipid levels directly affects the tumour cells' fluidity, cellular process, and membrane integrity.[244,245] However, following treatment with asarone and metformin to STZ+DEN-induced rats, it showed a significant reduction in TC, TG, and LDL-c levels along with an increase in HDL-c levels (Table 33). These alterations might be due to the stabilizing function of the enzyme lipoprotein lipase as a result of the increased levels of insulin.

### 4.1.4. Liver tumor bio-markers

The γ-glutamyl transferase (GGT), a well-known membrane-bound molecule and diagnostic marker which transports gamma-glutamyl functional groups, is found in numerous tissues, with the liver being the most prominent.[246] The rise in tumor mass linearly increases the levels of GGT in the serum. The increased GGT levels are linked to an increase in nodule incidence and a greater distribution of foci and nodules in liver tissue.[247,248] An increase of this enzyme activity observed in DEN alone and STZ+DEN administered groups indicates a response due to toxic cellular injury in the hepatocytes. This is why the higher nodule incidence, multiplicity and an increased percentage of the relative nodular size were observed in this study.

It is a well-known fact that serum alpha-fetoprotein (AFP) is a sensitive detecting bio-marker and is found to be elevated in germ cell cancers and HCC.[249] Some reports confirm that it can stimulate the proliferation of human hepatocytes and play an important role in regulating cell differentiation and tumor growth.[250,251] The vigorous production of AFP during fetal life by the hepatocytes of the liver and the visceral endoderm of the yolk sac decreases intensely after birth to reach only trace quantities in adulthood.[252] A significant rise in the AFP levels detected in DEN alone and STZ+DEN administered rats indicates the presence of HCC when compared to the normal group. This increase in AFP levels is in concurrence and is observed in adult animals when exposed to hepatocarcinogens. The elevation of AFP levels during HCC can be explained due to post-translational modification affecting AFP production or increased transcription of the AFP gene.[253] However, asarone and metformin significantly decreased the rise in serum alpha-fetoprotein level compared to the STZ+DEN-induced group (Table 33). This reduction of AFP in the treatment group might be responsible for the inhibitory effect, thereby regulating the cell differentiation and tumor growth, thus confirming its chemo-preventive activity.

### 4.1.5. $^1$H-NMR based metabolomics

Nuclear Magnetic Resonance (NMR)-based metabolomics, a novel approach in the biomedical area, identifies the alterations in the metabolic profiles of various biological samples and provides crucial insights into the pathophysiological condition of various tumorigenesis and diabetes mellitus. It can detect subtle metabolic perturbations under different pathophysiological conditions and be a highly effective approach for identifying the biomarkers.[254-256] The impairment of insulin deficiency or function during the STZ-induced hyperglycemia exacerbates the regulation of glucose, lipids, and amino acids metabolism. This persistent metabolic alteration during long-standing hyperglycemia is linked with various vascular complications and affects multiple organs and tissues throughout the body, including the liver. Under normal functional conditions, the energy derived through glucose oxidation, glycolysis, and lipid peroxidation in ATPs primarily utilizes substrates, namely glucose, ketone bodies, amino acids, or fatty acids.[257,258] However, following STZ injection, the diabetic animals lead to impaired glucose uptake and utilization. The metabolites associated with the glucose metabolism pathway, namely pyruvate and lactate, are involved in diabetes mellitus. A decrease in pyruvate and lactate levels observed in the $^1$H-NMR spectra of STZ-induced diabetic rats is in concurrence with previous studies.[259,260] The glycolysis can explain this, and aerobic metabolism pathways involved in building energy through ATP production are impaired and result in hyperglycemia. Furthermore, due to decreased glucose metabolism and increased glucose production, diabetic animals depend on other fuels for energy, namely lipids and free fatty acids. This, in turn, leads to the accumulation of triglycerides and cholesterol as specified in the biochemical assays. In this study, the enhanced levels of acetate in the STZ-treated rats are due to an increase in the beta-oxidation pathway of fatty acids, which agrees with a previous study.[261] This increase in the intracellular concentrations of lipid metabolites activates a serine/threonine kinase downstream signaling pathway and results in decreased insulin sensitivity to tissues along with mitochondrial dysfunction.[262,263] Likewise, the impairment in the amino acids metabolism pathway after STZ injection reduces amino acids, such as valine, alanine, and glutamine, which enhances the gluconeogenesis process during the diabetic state. The altered levels of alanine and glutamine are suggestive for maintaining fasting hyperglycemic conditions in STZ-induced diabetic rats, which is in accord with previous studies.[261,264,265] This can also be correlated between the decreased body weight in STZ-induced rats and the attenuation of protein synthesis accompanied by the changes in the metabolism of amino acid pathways. Henceforth, the alterations of the substrates for energy synthesis in the body might be a significant event in studying the pathological condition of diabetes.

The results of $^1$H-NMR-based tumor metabolomics related to the progression of HCC during diabetic condition confirms that the supply of energy is vital for the growth or proliferation of tumor cells as evident through the aberrant metabolism in glucose, lipid and amino acids pathways.[266] The process of glycolysis or tricarboxylic acid (TCA) cycle provides the necessary support for the cells in the form of glucose. However, augmented glycolysis is considered one of the hallmarks of the progression of cancer.[239] This study confirms the maintenance of a continuous state of hyperglycemia due to the administration of a single injection of STZ till the end of the study, which indicates that the ample availability of glucose in the diabetic-HCC group supports the tumor cells for proliferation as a result of augmented glycolysis.

This results in a substantial amount of pyruvate and lactate as the energy demand for the cells increases during hepatocarcinogenesis, which is achieved through glycolysis. Further, for maintenance of the fluctuating energy demands, the tumor cells rely on another substrate of glucose for energy, namely creatine. In this study, the significantly elevated levels of creatine in the STZ+DEN-induced group correlate with the tumorigenic transformation of the hepatocytes in the liver tissue compared to normal rats. This increase in creatine levels can also be attributed to the parenchymal damage of the liver and is indicative of necrosis of the hepatocytes.[267] The need for further energy for the growth or proliferation of the tumor cells directs the supplemental source through the metabolism of lipids and amino acids. Henceforth, a significant increase in the acetate levels in the STZ+DEN treated rats reflects the hepatic lipid metabolism supported by the rise in serum lipids levels and could be used as a potential biomarker for diabetic-HCC condition. It is known that glutamine, the highly heterogeneous and non-essential amino acid depends on several factors and plays a role in cancer metabolism.[268] In this study, the abnormal alterations in glutamine levels may be attributed to meeting the needs of energy to the fast-proliferating hepatocytes in STZ+DEN-induced rats. However, both the treatment groups showed a substantial alteration in metabolites level compared to STZ+DEN-induced rats (Table 34, Figure 33 and 34). This highlights the importance of serum metabolites for the altered energy metabolism during the complex pathophysiological process of diabetic-HCC conditions.

### 4.1.6. Oxidant-antioxidant imbalance

Oxidative stress plays a vital role and acts as a co-pathological factor contributing to and accelerating hepatocarcinogenesis progress during the diabetic condition.[269,270] The oxygen and nitrogen-based free radicals, which are typically unstable and highly reactive, have unpaired electrons. The addition of non-radicals such as hypochlorous acid, hydrogen peroxide, or ozone to oxygen-based free radicals, namely hydroxyl radicals, superoxide, or peroxyl radicals produced during oxygen metabolism, is known as reactive oxygen species (ROS). Furthermore, the nitrogen-based free radicals include non-radicals and nitrogen-based radicals. These include nitric oxide radicals and nitrogen dioxide produced by NADPH oxidase and synthase processes from superoxide and nitric oxide and are known as RNS.[269] Under normal functional conditions, these reactive species (ROS/RNS) are required for performing certain physiological functions and are not indeed a risk for human-being since the body can eradicate these reactive species to a certain extent. This includes defense mechanisms against different microorganisms, the involvement of various signal transduction pathways, or the expression of the other genes to promote growth or death.[269,271] Henceforth, the balance between the free radical generation and subsequent elimination is significant for maintaining oxidative cellular stress. This may lead to cell toxicity, degradation of proteins, DNA damage, and carcinogenesis. This imbalance occurs when the concentration of ROS exceeds the anti-oxidant potential of the cells.[269-271] In this study, the STZ alone and STZ+DEN-treated animals exhibited a significant decrease in the levels of scavenging enzymes such as catalase (CAT) and superoxide dismutase (SOD). The SOD scavenges superoxide radicals ($O_2^-$) and converts them to hydrogen peroxide ($H_2O_2$) and oxygen ($O_2$), and the CAT, haem-containing ubiquities enzyme detoxifies hydrogen peroxide ($H_2O_2$) into water ($H_2O$) and oxygen ($O_2$). The involvement of glutathione peroxidase (GPx) in detoxifying $H_2O_2$ similar to CAT is also an important defense mechanism against ROS-induced oxidative stress.[272,273] The present study reveals decreased GPx levels in the STZ alone and STZ+DEN-induced rats, as reported to be relatively low in hepatoma conditions.[274] The deleterious impact of inducing agents is responsible for the decrease in these scavenging enzymes, which inactivates free radicals and prevents the human body from oxidative damage. The increased levels of lipid peroxidation (LPO) of unsaturated fatty acids confirm the oxidative stress-mediated damage. It directly interacts with oxygen and lipids to produce radical intermediates and semi-stable peroxides such as malondialdehyde (MDA). The increase in MDA levels in the STZ alone and STZ+DEN-treated rats indicate enhanced lipid peroxidation leading to injury of the hepatocytes and failure of the defense mechanism. The MDA also acts as a mutagen in mammals by interacting with the deoxynucleotides through the formation of adducts. This is in concurrence with earlier reports where elevated levels of LPO in the hepatic tissues of both the diabetic and cancer models are observed.[275,276]

The oxidative-stress mediated liver damage due to STZ and DEN administration depleted the reduced glutathione (GSH) and Vitamin-C levels. These non-enzymatic anti-oxidants act together to neutralize free radicals and defend the human body from injury associated with oxidative stress and contrast to the LPO.[277] Furthermore, vitamin C, a known hydrophilic anti-oxidant, also detoxifies and scavenges the free radicals from the cytosol in combination with glutathione and vitaminE.[278] The availability of vitamin-C can also protect the lipoprotein particles and cellular membrane from oxidative damage and is responsible for malignant invasiveness.[279] Thus, in the present study, the decreased levels of these non-enzymic anti-oxidants in the STZ alone and STZ+DEN-treated groups may be due to the utilization of anti-oxidant to scavenge the oxidative stress caused by both the inducing agents. The asarone and metformin-treated group significantly higher levels of SOD, CAT, GPx, GSH, and Vitamin-C as well as a lower level of LPO compared to the

STZ+DEN-induced group (Table 35). This helps inhibit all the biochemical actions, which can cause ROS-mediated oxidative stress and a favorable environment for the proliferation of the tumor cells.

### 4.1.7. Histopathological evidence

The changes observed in this study for all the biochemical parameters as well as the $^1$H-NMR-based metabonomics were also confirmed by the detailed histopathological sequence of events in the hepatic tissue. There are studies in diabetic patients which revealed prominent alterations in liver histopathology.[280] Reports suggest that the STZ, initially developed as an anti-biotic and anti-cancer agent, also acts as a carcinogenic agent.[281] The reason for the inclusion of the STZ alone group in study design II was to observe the pathological changes of the liver for the development of HCC. In this study, the STZ alone group progressed to hepatic fibrosis but did not develop hepatocarcinogenesis at the end of 18 weeks. The chemical injection of a diabetogenic agent induces mild inflammation as the key pathogenesis of any metabolic disorder. Further, congestion in sinusoids (S), portal triad (PT) and central vein (CV), as well as infiltration of lymphocyte, necrotic cells and fibrosis (stage 1) observed in the liver tissue indicates the ROS-mediated oxidative stress. These changes may be attributed to oxidative-stress mediated inflammatory damage by the STZ with the generation of pro-inflammatory mediators such as IL-1β, TNF-α, and IL-6. The activation of pro-fibrogenic factors may be responsible for developing stage 1 fibrosis, as verified by Sirius red stain and the loss of cellular function.[10,12,280] The single injection of STZ and DEN in the diabetic-HCC group stimulates oxidative stress and inflammation, contributing to the complete loss of hepatic architecture with deformed cellular margins. The infiltration of inflammatory mediators promotes the stress-mediated hepatic cells into necrotic cells, followed by pre-HCC stages, and the appearance of many neoplastic cells confirms the hepatocarcinogenesis. The importance of intra- and inter-animal heterogeneity in histo-morphological features was established in this STZ+DEN model system. The occurrence of the development of various pre-HCC steps, such as fibrosis and cirrhosis, in STZ+DEN-induced rats, was confirmed using non-tumoral hepatic tissues. The STZ+DEN-induced model showed the status of fibrotic cells that progress into cirrhosis and finally develop HCC. The growth, division, and activation of hepatic stellate cells (HSC) are linked to the aggregation of extracellular matrix components, such as collagen and laminin, resulting in fibrosis. Furthermore, the degree of collagen deposition in nearly all central and portal regions, including the portal to portal (P-P) and portal to central (P-C) rises and accentuates fibrosis.[282] The increased fibrosis leads to progressive cirrhosis and, eventually, HCC. This is due to immune cell infiltration and neovascularization, which is followed by encapsulation. The hypercellularity (nuclear crowding), microacinar formation, hyper-basophilia, or hyper-eosinophilia of the cytoplasm and trabecular/nested/solid growth pattern of the hepatocytes are also taken into consideration for the confirmation of HCC. Furthermore, cases of STZ+DEN-treated rats developed lung metastases, indicating that the diseased status is irreversible. Finally, the STZ+DEN-induced diabetic-HCC model sequentially depicts central vein and portal triad congestion, inflammation, necrosis, ballooning hepatocytes, pre-HCC stages (fibrosis and cirrhosis) and eventually HCC. Treatment with asarone and metformin has shown recovery of the hepatocytes along with the other histopathological features compared to STZ+DEN-induced rats (Figure 36 to 38). This restoration ability of the hepatocytes may be due to the protection of the test compounds from oxidative mediated stress, signs of reduced inflammation, and other characteristic pathological evidence (Table 36).

The schematic illustration of the mechanism of action of streptozotocin and diethylnitrosamine-induced hepatocellular carcinoma (HCC) and chemo-preventive effects of asarone and metformin on the liver in experimental rats is depicted in figure 55.

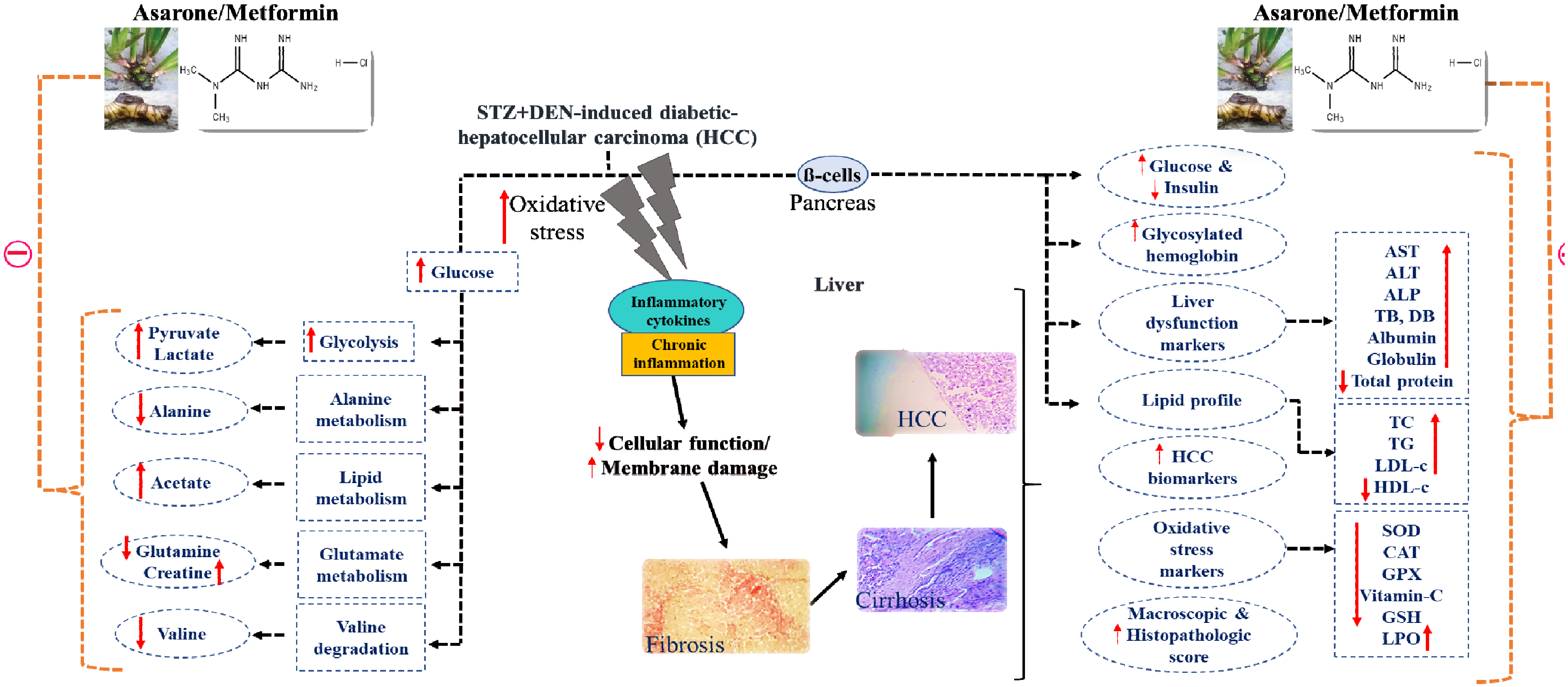


**Figure 55:** Schematic representation of the mechanism of action of streptozotocin (STZ) and diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC) and chemo-preventive effects of asarone and metformin on the liver in experimental rats.

STZ, Streptozotocin; DEN, Diethylnitrosamine; TG, Triglycerides; AST, Aspartate aminotransferase; GSH, Reduced glutathione; DB, Direct bilirubin; ALT, Alanine aminotransferase; TB, Total bilirubin; TC, Total cholesterol; SOD, Superoxide dismutase; LDL-c, Low-density lipoprotein cholesterol; ALP, Alkaline phosphatase; CAT, Catalase; GPx, Glutathione peroxidase; HDL-c, High-density lipoprotein cholesterol; LPO, Lipid peroxidation; ↑ Increase; ↓ Decrease.

## 4.2. *In-vitro study*

Asarone and metformin successfully suppressed the growth of human HepG2 cells during a glucose-rich environment. It has been established from the data presented in the results section of the *in-vitro* study that asarone (α and β) and metformin inhibit the growth (expressed as percentage cell viability) and modify the morphology of HepG2 cells (Figure 40 and 41). The cause for the concentration-dependent decrease in cell viability is due to the toxicity of the test compounds, which helps suppress the growth of HepG2 cells. Further, the results indicated that the test compounds arrested the cell cycle specifically at the $G_0/G_1$ phase due to activating AMPK and suppressing protein kinase B/Akt transducer pathways.

The ability of the cancer cells for rapid uptake and utilization of glucose favors the anabolic process and provides a proliferative advantage for the tumor cells. Further, this condition might be amplified under the glucose-rich condition, as observed in diabetes mellitus. Studies indicate that elevated serum glucose condition is strongly linked to a higher incidence of cancer.[94,104,200] In this study, the glucose-rich state increases the proliferation of HepG2 cells, as evident with a time-dependent increase in proliferation or cell viability along with the changes in the morphology (Figure 42). High glucose (HG) triggers ROS generation, which causes glucose toxicity and alteration in the expression of genes.[20] The HG causes ROS production and promotes and favors the oncogenic signaling pathways for cell proliferation, adhesion, mutations, migration, and survival in several tumor cell lines.[20,98] In this direction, the AMPK and Akt transcriptional factors, as well as their cascade regulators serve a significant part in the metabolism of glucose and cancer.[124]

The AMPK is an important regulatory indicator that helps maintain cellular energy balance and is a known pharmacologic target for treating a metabolic condition like diabetes. Nevertheless, emerging evidence documents AMPK as a probable metabolic tumor inhibitor and contributes to the prevention and treatment of tumor growth.[120,144] The dysregulation of AMPK has been observed in different conditions, including diabetes, hypertension, obesity, heart failure, aging, liver diseases, and certain cancers.[126] It has been demonstrated that the activation of AMPK can regulate numerous cell and tissue-specific downstream markers responsible for proliferation, apoptosis, and autophagy.[119,121,126] Asarone and metformin have been proven to produce anti-proliferative activity by significant upregulation of AMPKα1 activity (Figures 47 and 48). This is in agreement with other research that links the AMPK activation to tumor growth inhibition.[119,121,123] These findings imply that the activity of AMPK in high glucose conditions is inversely correlated with the progression of the HepG2 cell line and may serve as a regulator contributing to the proliferation and differentiation of the hepatocytes.

The phosphoenolpyruvate carboxykinase (PCK or PEPCK) genes, a gluconeogenic factor and a downstream regulator in the AMPK pathway of the liver are associated with the growth and differentiation of HCC. The PCK-2, a mitochondrial isoenzyme, catalyzes the reaction of altering oxaloacetate (OAA) to phosphoenolpyruvate (PEP) and is considered a possible target hepatocarcinogenesis.[140] The results (Figures 49 and 50) suggest that HepG2 treated cells in hyperglycemic conditions upregulates the expression of PCK-2 compared to normoglycemic conditions. The observed increased expression of PCK-2 causes an upsurge in glucose uptake and utilization,which supports the anabolic metabolism and promotes HepG2 cell proliferation. As per numerous studies, the elevated expression of PCK-2 is observed in various types of cancer and is correlated to increased anabolic metabolism and proliferation of the cancer cells.[131-133] However, asarone and metformin treatment downregulate the expression of PCK-2 in high glucose conditions (Figures 49 and 50). This result indicates the functional significance of PCK-2 in contribution to the glucose metabolism pathway by reducing the proliferation of human HepG2 cells. Thus, this directs an insight underlying the anti-proliferative role of asarone and metformin in HepG2 cells with the function of PCK-gene during hyperglycemic conditions.

Furthermore, increasing evidence suggests that SREBP-1 (sterol regulatory element-binding protein-1), the crucial regulatory component controlling glucose uptake to glycolysis and *de novo* lipogenesis, upregulates in various types of cancers been associated with playing an integral role in the oncogenic signaling pathways.[125,135,136] The results suggest that asarone and metformin-treated high glucose cells decreased the expression of SREBP-1 (Figure 51 and 52) compared to the hyperglycemic condition, which could be possibly responsible for abating the cancer cell growth. Furthermore, AMPK phosphorylates SREBP-1 as an upstream regulator, thereby signifying the connection between AMPK-SREBP-1 signaling pathways through cell proliferation and differentiation.

The deregulation of the prototypic survival Akt/PI3K signaling pathway has been linked with various functions of the cells, notably cell growth and proliferation. Reports suggest that anomalous Akt signaling pathway activation is common in HCC carcinogenesis.[127-129] Henceforth, targeting Akt for hepatocarcinogenesis offers the clinical relevance of this particular pathway, as evidenced by both clinical and pre-clinical studies. The HepG2 cells in high glucose conditions treated with asarone and metformin significantly suppressed the proliferation of the cells, as evident from the decreased expression of Akt (Figures 53 and 54). Their findings suggest that metformin acts through Akt's inhibition on various cancer cell lines in support of other workers.[26,140,283]

The progression of the cell cycle during the proliferation of hepatocarcinogenesis is controlled by the balance between the different growth suppressor proteins and cyclin-dependent kinases.[126] Myoshi *et al.* have reported arrest of the cell cycle in HCC cells regulated by a dynamic balance between the up-regulation of p27 and p21 and inhibition of G1 cyclins.[151] Besides these, the cell cycle is controlled by modifying both the AMPK and Akt-mediated pathways. Following this, the flow cytometry analysis suggests that asarone and metformin inhibit the proliferation of HepG2 cells, specifically at the $G_0/G_1$stage of the cell cycle in glucose-rich conditions (Figure 45 and 46). This is due to the modulation of the cell cycle-specific proteins, which allows the cancer cells for proliferation.[284]

The schematic illustration by which asarone and metformin decrease the proliferation of HepG2 cells is depicted in figure 56.

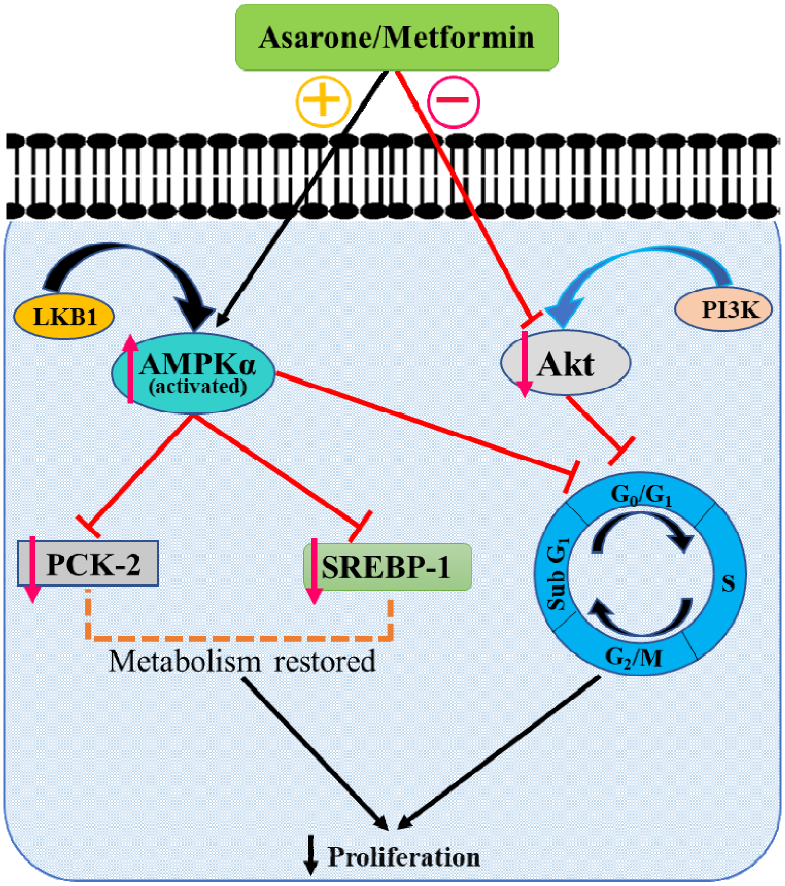


**Figure 56:** Schematic illustration of asarone and metformin, which decreases the proliferation of HepG2 cells.

LKB1, Liver kinase B1; AMPK, 5´adenosine monophosphate-activated protein kinase; PCK-2, Phosphoenolpyruvate carboxykinase-2; SREBP-1, Sterol regulatory element binding protein-1; PI3K, Phosphoinositide 3-kinase; Akt, Protein kinase B (PKB).

# 5. SUMMARY

One of the objectives of this research was to mimic an animal model to validate the scientific proof for the diabetic-HCC condition. Further, this study assesses the role of test compound asarone and anti-diabetic drug metformin against experimentally induced diabetic-HCC and attempts to elucidate the cancer-associated pathways both *in-vivo* and *in-vitro*.

The *in-vivo* study was carried out through two study designs in male Wistar rats. The study design I was to investigate the impact of STZ-induced hyperglycemia on the development of DEN-induced HCC for 12 weeks. In study design II, the combined role of asarone and metformin HCl was examined separately on STZ+DEN rats for 18 weeks. In study design I, the STZ induced animals administered at a single dose of 55 mg/kg exhibited severe hyperglycemia and did not show any sign for the development of HCC until the end of 12-weeks. Conversely, hepatocarcinogenesis was observed in the DEN (200 mg/kg; *i.p.*) alone and STZ+DEN-induced animals. These data of study design-I suggest that hyperglycemia alone has no impact on HCC development. In contrast, the pre-administration of the diabetogenic agent (STZ) before initiation of DEN promotes HCC by a few weeks earlier as supported by all biochemical and histo-morphological changes.

However, in study design II, oral treatment of asarone (50 μg/kg; prepared in 1:1 ratio) and metformin (250 mg/kg) either reduced or reversed the severity of hepatocarcinogenesis during the diabetic condition, confirming a chemotherapeutic efficacy. This was supported by all biochemical, $^1$H-NMR based metabolomics and histo-morphological evidence. This could be attributed to the ability of the test compounds to repair the hepatic injury by maintaining the structural integrity of the plasma membrane by reducing the toxic effects of free radicals and confirming its chemo-preventive activity.

Furthermore, the *in-vitro* study was carried out on human HCC cell line HepG2. This study design also attempted to investigate whether a change in the concentration of glucose aids in the proliferation of HepG2 cells. We also explored whether the asarone and metformin-mediated cytotoxic effect on HepG2 cells were regulated through AMPK and Akt regulatory pathways during high glucose conditions. A time-dependent growth of HepG2 cells was observed as the glucose concentration increases. The asarone and metformin treatment treated groups reduce the proliferation of HepG2 cells during high glucose conditions due to arrest at the $G_0/G_1$ phase of the cell cycle. This reduction is mediated due to an increase in the AMPK and suppressing protein kinase B/Akt cell-signaling pathways. This study also proved that the inhibition of PCK-2 and SREBP-1 indicated a connection involving HepG2 cell proliferation and glucose metabolic process. Further, studies investigating the involvement of other cancer-signaling pathways may strengthen these findings.

# 6. CONCLUSION

The present study concluded that the streptozotocin (STZ)-induced diabetic rats developed upto hepatic fibrosis in study design II but failed to progress to hepatocarcinogenesis (HCC) till the end of the study period. In contrast, the pre-administration of the diabetogenic agent (STZ) before initiation of DEN resulted in the development of HCC at least a few weeks earlier. This suggests that hyperglycemia due to STZ alone has no impact on HCC development but can precipitate the development of HCC when combined with DEN. Furthermore, asarone and metformin treated groups exhibited chemo-preventive activity in diabetic conditions as confirmed through an *in-vivo* study. The *in-vitro* study results showed that the treated groups reduce the proliferation of HepG2 cells during hyperglycemia due to arrest at the $G_0/G_1$ phase of the cell cycle. This reduction is mediated due to an increase in the AMPK and suppressing protein kinase B/Akt cell-signaling pathways. In addition, the inclusion of PCK-2 and SREBP-1 indicated a connection involving HepG2 cell proliferation and the glucose metabolic process. Nevertheless, further studies involving the other cancer-associated pathways for its anti-proliferative effect may strengthen these findings.

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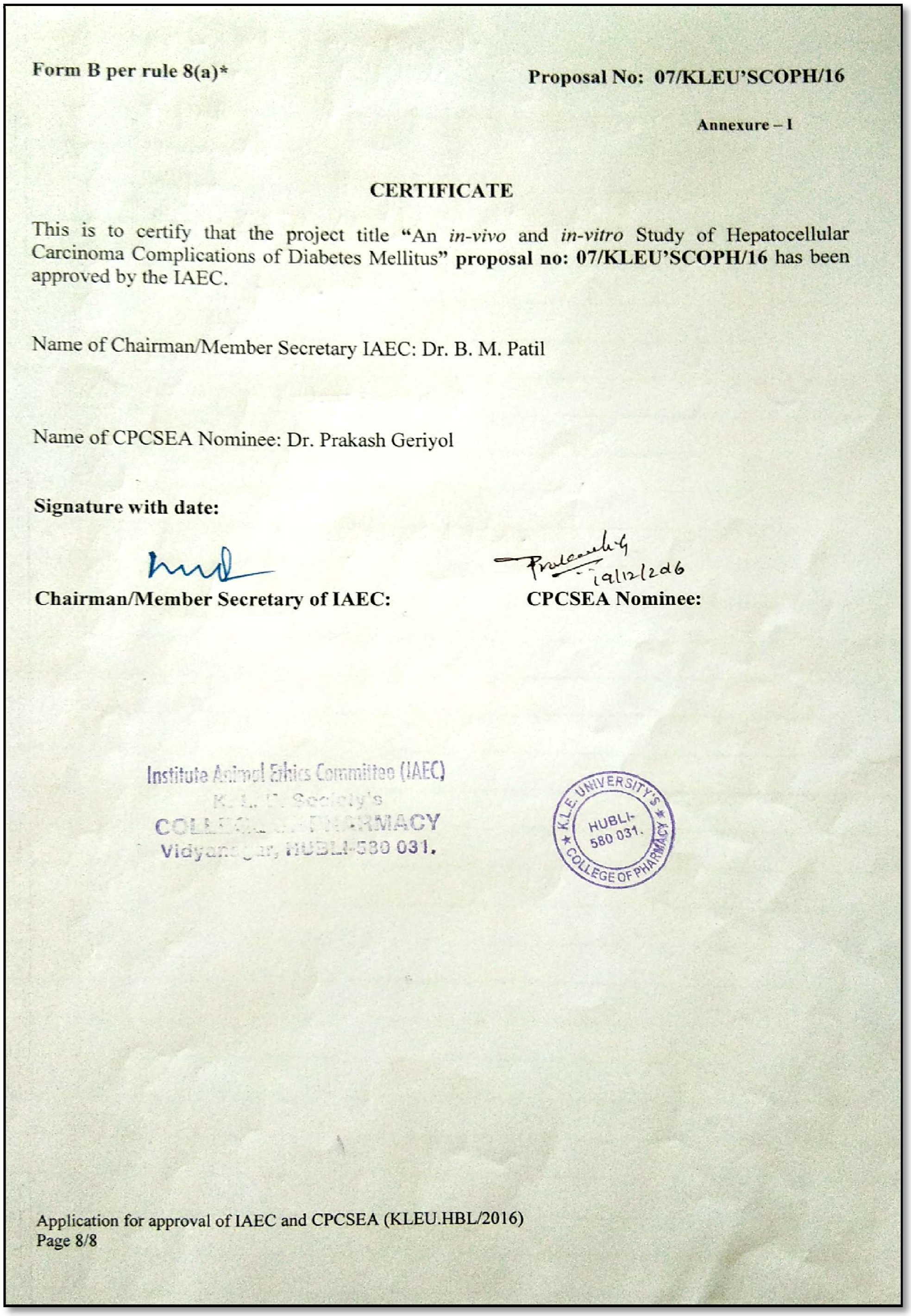
# 8. ANNEXURES

## 8.1. Animal ethical clearance certificate



**Form B per rule 8(a)*** **Proposal No: 07/KLEU'SCOPH/16**

**Annexure – I**

**CERTIFICATE**

This is to certify that the project title **"**An *in-vivo* and *in-vitro* Study of Hepatocellular Carcinoma Complications of Diabetes Mellitus**" proposal no: 07/KLEU'SCOPH/16** has been approved by the IAEC.

Name of Chairman/Member Secretary IAEC: Dr. B. M. Patil

Name of CPCSEA Nominee: Dr. Prakash Geriyol

**Signature with date:**

19/12/2016

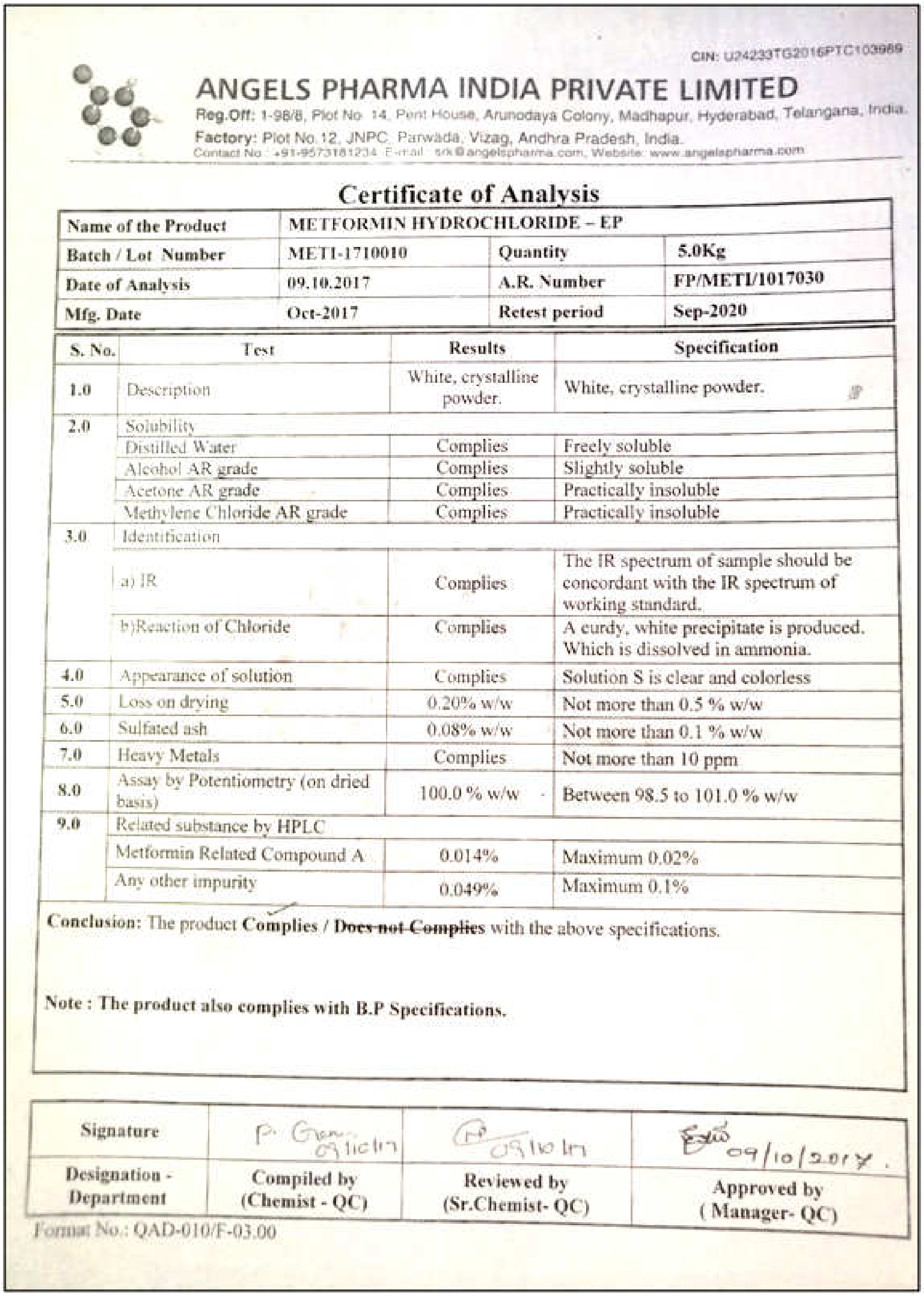
**Chairman/Member Secretary of IAEC:** **CPCSEA Nominee:**

Institute Animal Ethics Committee (IAEC)
K. L. E. Society's
COLLEGE OF PHARMACY
Vidyanagar, HUBLI-580 031.

HUBLI-580 031.

Application for approval of IAEC and CPCSEA (KLEU.HBL/2016)
Page 8/8

## 8.2. Certificate of analysis of Metformin HCl



CIN: U24233TG2016PTC103989

**ANGELS PHARMA INDIA PRIVATE LIMITED**

**Reg.Off:** 1-98/8, Plot No. 14, Pent House, Arunodaya Colony, Madhapur, Hyderabad, Telangana, India.
**Factory:** Plot No.12, JNPC, Parwada, Vizag, Andhra Pradesh, India.
Contact No.: +91-9573181234 E-mail: srk@angelspharma.com, Website: www.angelspharma.com

### Certificate of Analysis

| | | | |
|---|---|---|---|
| **Name of the Product** | METFORMIN HYDROCHLORIDE – EP | | |
| **Batch / Lot Number** | METI-1710010 | **Quantity** | **5.0Kg** |
| **Date of Analysis** | 09.10.2017 | **A.R. Number** | **FP/METI/1017030** |
| **Mfg. Date** | Oct-2017 | **Retest period** | **Sep-2020** |

| S. No. | Test | Results | Specification |
|---|---|---|---|
| 1.0 | Description | White, crystalline powder. | White, crystalline powder. |
| 2.0 | Solubility | | |
| | Distilled Water | Complies | Freely soluble |
| | Alcohol AR grade | Complies | Slightly soluble |
| | Acetone AR grade | Complies | Practically insoluble |
| | Methylene Chloride AR grade | Complies | Practically insoluble |
| 3.0 | Identification | | |
| | a) IR | Complies | The IR spectrum of sample should be concordant with the IR spectrum of working standard. |
| | b)Reaction of Chloride | Complies | A curdy, white precipitate is produced. Which is dissolved in ammonia. |
| 4.0 | Appearance of solution | Complies | Solution S is clear and colorless |
| 5.0 | Loss on drying | 0.20% w/w | Not more than 0.5 % w/w |
| 6.0 | Sulfated ash | 0.08% w/w | Not more than 0.1 % w/w |
| 7.0 | Heavy Metals | Complies | Not more than 10 ppm |
| 8.0 | Assay by Potentiometry (on dried basis) | 100.0 % w/w | Between 98.5 to 101.0 % w/w |
| 9.0 | Related substance by HPLC | | |
| | Metformin Related Compound A | 0.014% | Maximum 0.02% |
| | Any other impurity | 0.049% | Maximum 0.1% |

**Conclusion:** The product **Complies** / ~~**Does not Complies**~~ with the above specifications.

**Note : The product also complies with B.P Specifications.**

| | | | |
|---|---|---|---|
| **Signature** | 09/10/17 | 09/10/17 | 09/10/2017. |
| **Designation - Department** | **Compiled by (Chemist - QC)** | **Reviewed by (Sr.Chemist- QC)** | **Approved by ( Manager- QC)** |

Format No.: QAD-010/F-03.00

## 8.3. Publications

**Research Article:**

1. Das, B.K., Choukimath S.M., Gadad, P.C., 2019. Asarone and metformin delays experimentally induced hepatocellular carcinoma in diabetic milieu. Life Sci., 230:10-8. **(Impact Factor: 5.03)**
2. Das, B.K., Gadad, P.C., 2020. Asarone and metformin modulates the oxidant-antioxidant imbalance on experimentally induced hepatocellular carcinoma during diabetic condition. Indian J. Pharm. Educ. Res., 54:1039-1045. **(Impact Factor: 0.68)**
3. Das, B.K., Knott, R.M., Gadad, P.C., 2021. Metformin and asarone inhibit HepG2 cell proliferation in a high glucose environment by regulating AMPK and Akt signaling pathway. Futur. J. Pharm. Sci., 7:43.
4. Das, B.K., Jayalakshmi. K., Gadad, P.C. $^1$H-NMR based serum metabolomic study to evaluate the effect of asarone and metformin on experimentally induced diabetic-hepatocellular carcinoma. (Under review).

**Review Article:**

1. Das, B.K., Swamy, A.H.M.V., Koti, B.C., Gadad, P.C., 2019. Experimental evidence for use of *Acorus calamus* (asarone) for cancer chemoprevention. Heliyon. 5:e01585.
2. Das, B.K., Gadad, P.C. 2021. Impact of diabetes on the increased risk of hepatic cancer: An updated review of biological aspects. Diabetes Epidemiol. Manag., 4:100025.

**8.4. Details of Seminars/Conferences/Workshops attended**

1. Participated as delegate in 5 days workshop on "Sample Size and Multivariate Analysis by SPSS/Excel" from 22nd to 26th August 2016, organized by KLE University, Department of Epidemiology & Biostatistics, Belagavi, Karnataka, India.
2. Participated as a delegate in a workshop on "Pharmacognostic Approach in Drug Discovery" on 22nd November 2016, organized by KLE University, UDEHP, Belagavi, Karnataka, India.
3. Participated as delegate in CME on "Research in Cancer Biology-Breast Cancer Biomarkers" on 17th February 2017, organized by SDM College of Medical Sciences & Hospital, Manjushree Nagar, Sattur, Dharwad, Karnataka, India.
4. Participated as delegate in a workshop on "Hands-on Training in Basic Molecular Biology Techniques" on 18th February 2017, organized by SDM College of Medical Sciences & Hospital, Manjushree Nagar, Sattur, Dharwad, Karnataka, India.
5. Participated as delegate in a workshop on "Good Laboratory Practices and Laboratory Safety" on 18th April 2017, organized by KLE-BSRC, UDEHP, Belagavi, Karnataka, India.
6. Participated in International CME on "Scientific Manuscript Writing" on 26th & 27th April 2017, organized by USM-KLE, IMP, Belagavi, Karnataka, India.
7. Participated as delegate in a workshop on "Ethical Guidelines in Biomedical Research" on 24th & 25th November 2017, organized by KLE-COP, UDEHP, Hubballi, Karnataka, India.

8. Participated as delegate in a webinar on “Lt Platform: Virtual Experimental Physiology and Pharmacology Lab” on 13th June 2020, jointly organized by AD Instruments and KLE-COP, Hubballi, Karnataka, India.