RESEARCH PAPER

Evaluation of Anti-Cancer Activity of Sotagliflozin in HUH-7 Hepatoma Cell Lines and DEN–CCl_4 Model of Hepatocellular Carcinoma in Male Wistar Albino rats

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Abstract:
The objective of the study was to evaluate cytotoxic, antioxidant, and anti-cancer effects of Sotagliflozin in Hepatocellular carcinoma (HCC) using suitable in-vitro and in-vivo models. 3-(4,5-dimethylthiazol-2yl)-2,5diphenyltetrazolium bromide (MTT assay) and ferric reducing antioxidant power assay (FRAP) were done to assess the cytotoxic and the antioxidant property of Sotagliflozin on Human hepatoma cells. In MTT assay, a modest reduction in cell viability with Sotagliflozin was observed. In FRAP assay, a dose dependent antioxidant activity of Sotagliflozin was seen. For in vivo study, 27 Wistar rats were randomly assigned to five groups: Normal Control, Cancer Control, Cisplatin (6 mg/kg), Sotagliflozin (10 mg/kg) and Sotagliflozin (20 mg/kg). Body weight, Liver function tests and Alfa-fetoprotein were analysed at baseline, once in 2 weeks and at the end of the study (10^{th} week). At the end of 10 weeks, animals were sacrificed, liver tissues were collected for histopathological analysis and AMPK estimation. Sotagliflozin resulted in favourable changes in LFT & AFP and increased AMPK expression in HCC. Histopathological examination revealed attenuation of HCC features with Sotagliflozin. Hence, Sotagliflozin could be a promising therapeutic modality for HCC, which can be further explored in larger preclinical and clinical studies.
Keywords: Anti-cancer activity, DEN–CCl_4 rat model, Hepatocellular carcinoma, HUH-7 cell line, Sotagliflozin

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1. Introduction:

Hepatocellular carcinoma (HCC) accounts for the third most prevalent and eighth most common cause of cancer related deaths according to global cancer statistics- 2020 (1). In 2019, the Asia Pacific area recorded cases which exceeded more than half of the global HCC burden, with an approximate prevalence of 0.53 Million cases (2). The current modalities in HCC treatment include chemotherapy and surgical interventions such as hepatectomy, liver transplantation and laser ablation (3). However, complete cure in HCC is still questionable and also each treatment holds certain limitations. In surgical resection, limitations include elevated risk of post operative liver failure, high tumour recurrence rate (4, 5). Scarcity of donors, long waiting and strict selection criteria were some reasons limits liver transplantation (6, 7). Sorafenib, Lenvatinib, Regorafenib, Cabozantinib, and Samucirumab are some systemic drugs approved for the treatment of HCC but modest overall response, heterogenous patient response rates, high incidence of treatment related adverse events were some limitations seen with these drugs (8, 9). These limitations with current treatment modalities demands further research in looking for new treatment options to fight the morbidity and mortality due to HCC. When we look at the newer therapeutic targets for handling HCC, it is fascinating to note that despite adequate oxygen, cancer cells prefer glycolysis for ATP generation instead of oxidative phosphorylation in mitochondria. This shift in ATP generation in cancer cells from oxidative phosphorylation to glycolysis is known as Warburg effect (10). In HCC, this shift to glycolysis supplies rapid

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ATP, which was demonstrated by 18- FDG PET showing high glucose uptake (11). Thus, the "Warburg effect" insight finds to shape, metabolism-directed treatment strategies against HCC. In the molecular studies exploring the Warburg effect, several downstream regulatory pathways, viz. PI3K, HIF, P53, MYC, and AMPK have been identified and found to play a significant role in regulating the cell cycle. Drugs modulating these pathways have been shown to reduce cancer cell proliferation indirectly. One such downstream regulatory pathway is the Adenosine Monophosphate (AMP) – activated Protein Kinase (AMPK) pathway, activation of which causes cell cycle arrest at the G2/M phase in liver cells and imparts anti-cancer effect (12, 13). SGLT2 inhibitors reduce ATP production through inhibition of oxidative phosphorylation in cancer cells leading to increase in AMP/ATP ratio which further activates AMPK via phosphorylation by LKB1(14, 15). This activation then inhibits mTOR signalling, which is known to suppress tumour growth and proliferation (15).Sodium- glucose co transporter 2 inhibitors (SGLT2), like canagliflozin, empagliflozin, and dapagliflozin, not only block the glucose entry into the cancer cells but also indirectly activate the AMPK regulatory pathway providing anticancer effect (14). Hung MH et al,, in 2019 reported the anticancer potential of Canagliflozin (16). Vinay N et al., in 2019 also done a invitro study proved anticancer effects of Dapagliflozin and Canagliflozin (17). Compared to the conventional SGLT2 inhibitors, Sotagliflozin may show better anticancer effect due to its dual inhibition property of SGLT 1/2 (18). The molecular evaluation of certain tumour cells has shown both SGLT 1 and SGLT2 overexpression (19), which attributes to a better anticancer treatment outcome with a dual inhibitor like Sotagliflozin.

With this background, the study was conducted to assess the in vitro cytotoxicity and in vivo anti-cancer effect of Sotagliflozin using suitable in vitro assays and rat model of hepatocellular carcinoma respectively.

2. MATERIALS AND METHODS

2.1 Invitro Analysis:

2.1.1 Cell lines and reagent

HUH-7 human hepatoma cells were purchased from the National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)- low glucose supplemented with 10 % Fetal bovine serum (FBS) and 100 U/ml Penicillin and 100 µg/mL Streptomycin, to prevent contamination. The cells are incubated at 37˚C and in 5 % CO_2 humidified atmosphere. The culture medium was replaced every 2–3 days. After the cells attained 90% confluence, using standard trypsinization protocols, the cells were sub cultured.

2.1.2 Assessment of cytotoxicity- MTT assay

To assess the cytotoxicity of Sotagliflozin, MTT assay was performed. Initially, HUH-7 cells were inoculated into a 96-well plate at a density of 5×10^3 cells/well and kept for 24 hours for the cells to attach to the plate. The cells were then rinsed with Phosphate buffered saline (PBS). Following that, the cells were treated with various concentrations of Sotagliflozin (1µM, 5µM, 10µM, 15µM, 20µM, 30µM, and 50µM) and Cisplatin (2.5 µg/mL) for 24 hours and 48 hours. The assay was conducted in five replicates for each dose and each duration (24 hours and 48 hours) of Sotagliflozin and Cisplatin (70 wells for 7 doses of Sotagliflozin and 10 wells for Cisplatin), 10 wells (wells containing HCC cells without treatment) served as control and 6 wells containing Dimethyl sulfoxide (DMSO) served as blank. After incubating the wells for respective time period of 24 and 48 hours, MTT solution (5mg/mL) was added to each well and then plates were incubated at 37^0 C for another 4 hours. After 4 hours, the media was carefully removed and 100 µL of DMSO was added to dissolve the MTT-formazan crystallites. Later, the absorbance of each well, was measured using ELISA reader at 570 nm. The percentage of the cell viability was assessed using the following formula:

"Cell viability (%) = (Absorbance 570 nm of Sample − blank) x 100"
(Absorbance 570 nm of Control − blank)

2.1.3 Assessment of Antioxidant activity- FRAP Assay

The Ferric Reducing Antioxidant Power (FRAP) assay is used to assess the antioxidant ability of the test substance. It is the measure of ability of the test substance to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). A visible colour change is seen during reduction, which is recorded using a microplate reader. The FRAP assay in this study was conducted using the **EZAssay™ Antioxidant Activity Estimation Kit** (**Product Code: CCK072**) according to the manufacturer's protocol. The HUH-7 cells treated with the two doses of Sotagliflozin (20 µM, 30 µM) and Cisplatin 2.5 µM/ml were incubated. To 100 µL of each sample, standard ($FeCl_2$), control, and blank was added in triplicate into a 96-well microplate. To each well, 100 µL of the chromogenic substrate was added. The plate was incubated at room temperature in the dark for 10 minutes to allow colour development. After incubation, the absorbance was measured at 560 nm using a microplate reader. The average absorbance values were calculated for each set of triplicates, and the mean blank value was subtracted to obtain the corrected absorbance: "Corrected Absorbance =Abs560nm (sample/standard) −Abs560nm (blank)" The FRAP value of each test sample was calculated as Fe^{2+} equivalents for which a standard curve was plotted using Corrected absorbance of $FeCl_2$ in Y – axis and known $FeCl_2$ concentrations in X- axis. The linear regression equation is (y= mx + c), derived from the graph (y= Corrected absorbance, m=slope, c= intercept).Using the derived data, $Fe2^+$ equivalents and FRAP value is derived using the following formula:

"**Fe(II) equivalents (µM)** = (Corrected Absorbance – I ntercept) / Slope"

Since each of the test sample will have varying protein concentration it is essential to normalise the protein

content. The following formula is used to normalise the protein content:

"FRAP Value = Fe(II) equivalents (μM) / Protein concentration (μg)"

Protein concentration of each sample was determined separately using a standard protein quantification method (e.g., BCA). Final results were reported as FRAP value Fe(II) μM/μg of protein.

All samples were analysed in triplicate, and the results were expressed as mean ± standard deviation.

2.2 **In Vivo Study:**

The in vivo experiment was initiated after obtaining approval from the institutional animal ethics committee (IAEC1/ proposal: 155/A.Lr: 117/dt: 20.02.2024). A total of 27 Male Wistar Albino rats were used in this experiment. Animals were randomly allocated to five groups (Groups 1- 5). HCC was induced using DEN and CCl_4 (20). The total duration of the study was 11 weeks including 1 week of acclimatization period (Week 0 to week 1) and experiment period (Week 1 to week 10).2 – 3 animals per cage was housed in a wire mesh cages and maintained between 23 - 25 degrees Celsius with 50 to 60 percent humidity. The rats were subjected to a 12-hour cycle of light and dark. Throughout the study, rats had free access to food and water. The experiments was carried out as per the Committee for Control and Supervision of Experiments on Animals (CCSEA) guidelines. The induction of HCC and interventions during the experiment period given to the rats were as follows **(Table 1)** Rats in all the five groups were sacrificed at the end of 10 weeks using overdose of halothane and Liver and Kidney were dissected and preserved for histopathological analysis.

Table 1 Group wise interventions

Groups	Treatment		
	Week 1 (Baseline)	Week 2- week 8	Week 9- week 10
1- Normal Control (n=3)	Single IP injection of Normal Saline (0.5 ml)	Normal Saline (0.5 ml) orally daily once, 0.5 ml S/C weekly once and 0.5 ml I/P weekly once	No treatment
2- Cancer control (n=6)	Single IP injection of DEN (200 mg / kg) (20)	CCl_4 (2 mg / kg) S/C weekly once (20) + Normal Saline (0.5 ml) orally daily once	No treatment
3- Standard treatment (Cisplatin) (n=6)	Single IP injection of DEN (200 mg / kg)	CCl_4 (2 mg / kg) S/C weekly once + Cisplatin (6 mg / kg) I/P weekly once	No treatment
4- Sotagliflozin (10 mg / Kg) (n=6)	Single IP injection of DEN (200 mg / kg)	CCl_4 (2 mg / kg) S/C weekly once + Sotagliflozin (10 mg / kg) orally daily once	No treatment
5- Sotagliflozin (20 mg / Kg) (n=6)	Single IP injection of DEN (200 mg / kg)	CCl_4 (2 mg / kg) S/C weekly once + Sotagliflozin (20 mg / kg) orally daily once	No treatment

2.2.1 Dose Selection of Sotagliflozin

The low dose Sotagliflozin (10mg/kg/day) was selected based on the other previous studies done with Sotagliflozin (21, 22). The high dose Sotagliflozin (20 mg/kg/day) was calculated using the Food and Drug Administration (FDA) human to animal dose conversion formula, considering the recommended clinical dose of Sotagliflozin in humans (200 mg/day), which corresponds to approximately 3.33 mg/kg/day based on an average human body weight of 60 kg, the equivalent dose of Sotagliflozin in rats is 20 mg/kg/day (multiplying the Km value of 6 for rats to the Human equivalent dose of 3.33 mg/kg/day)(23).

2.2.2 Study assessment

Body weight, Liver Function tests- Aspartate transaminase (AST), Alanine transaminase (ALT) and Alkaline Phosphatase (ALP) and HCC biomarker-Alpha Feto Protein (AFP) were assessed at baseline (Week 1), once in 2 weeks (Week 2, 4, 6 and 8) and at the end of the study (Week 10). At the end of 10^{th} week all animals were sacrificed using high dose halothane and Liver & Kidney were dissected out and used for histopathological analysis and to assess AMPK gene expression.

2.3 AMPKα [Pt172] ELISA Estimation:

The expression levels of phosphorylated AMPK were analysed using a commercially available ELISA kit **(AMPKα [pT172] ELISA Kit; Catalogue Number:**

KHO0651). The assay was performed according to the manufacturer's instructions and protocols.In this study the rats liver specimen were used in quantification of AMPK. The frozen tissue samples (Liver) were homogenised using Radioimmunoprecipitation assay (RIPA) lysis buffer which lyse the cells and extract proteins. Further the homogenates were incubated on ice for 30 min and centrifuged at 13,000 rpm. The supernatant of the centrifuged lysates containing the soluble proteins was then collected and used for further analysis. The 100 µL of each supernatant samples and standards provided with the kit were added to 96 well plate precoated with antibodies specific for phosphorylated AMPK α, incubated for 2 hours under standard conditions to ensure proper binding of the target protein to the plate-bound antibodies. Following that, a series of washing steps were carried out to remove the unbound components. Sequentially Antibody detector, Anti rabbit IgG horseradish peroxidase (HRP) conjugate were added according to the kit's protocol. Subsequent addition of the substrate chromogen solution led to the development of calorimetric reaction. Once sufficient colour developed, a stop solution was added to terminate the reaction. The intensity of the colour was directly proportional to the concentration of phosphorylated AMPKα present in the samples. The optical density (OD) of each well was then measured at 450 nm using a microplate reader. The concentrations of phosphorylated AMPK in the samples (expressed in Units/mL) were determined by plotting a standard curve, generated by plotting the OD values (Y axis) against the known concentrations of the standard protein (X axis). The concentrations in the test samples were calculated by interpolating their OD values on this standard curve and used for comparative analysis between experimental groups.

3. STATISTICAL ANALYSIS:

Statistical analysis was done using GraphPad Prism software version 8.0.2. The quantitative variables were summarized as Mean ± standard deviation (SD) and compared across the groups using one- way analysis of variance (ANOVA) followed by Tukey's Post hoc multiple comparison test and within the groups using repeated measures ANOVA. $P < 0.05$ was considered significant. The total sample size is 27 (n=3 in normal control group and n= 6 in all other groups).

4. RESULTS

4.1 Invitro cellular toxicity (MTT assay):

After 24 hours of treatment (**Figure 1**Error! Reference source not found.), Sotagliflozin in all tested concentrations resulted in reduced cell viability compared to untreated control. The higher concentrations (10, 15, 20, 30, 50 µM) showed lesser cell viability compared to 1 and 2 µM of Sotagliflozin. The cell viability was 83.95 ± 1.35%, 83.22 ± 1.80% 77.30 ± 1.15%, 79.12 ± 2.13%, and 82.17 ± 1.565% with 10, 15, 20, 30 and 50 µM respectively, whereas it was 89.34 ± 1.49% and 93.92 ± 1.70% with 1 and 2 µM respectively. Upon extending the treatment duration to 48hrs (**Figure 2**), same trend was noted with all the tested doses of Sotagliflozin. The cell viability did not drop below 50% in any of the doses of Sotagliflozin but with Cisplatin, the cell viability dropped to 27.88 ± 1.880% ($p < 0.001$ vs. control and vs all doses of Sotagliflozin).IC50 value could not be derived for Sotagliflozin, as the cell viability did not drop below 50%. Based on the results of MTT assay, the higher doses of Sotagliflozin (10, 15, 20 µM, 30 µM and 50 µM) almost had similar effect on the cell viability. Hence, we had taken 2 doses, 20 µM and 30 µM for further assays and evaluations.

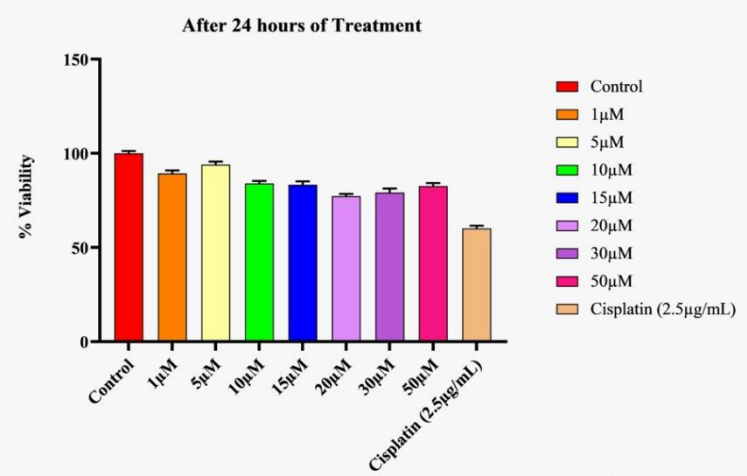

Figure 1 MTT Assay after 24 hours

Intergroup comparison - One way ANOVA ($p< 0.0001$),
Post hoc: Control vs all test concentrations ($p < 0.0001$), lower concentrations (1 µM, 5 µM, 10 µM) vs higher concentrations (20 µM, 30 µM, 50 µM) ($p < 0.0001$), 20 µM vs 50 µM ($p = 0.0002$), 30 µM vs 50 µM ($p= 0.0291$), Cisplatin 2.5 µg/mL vs all doses of Sotagliflozin ($p < 0.0001$)

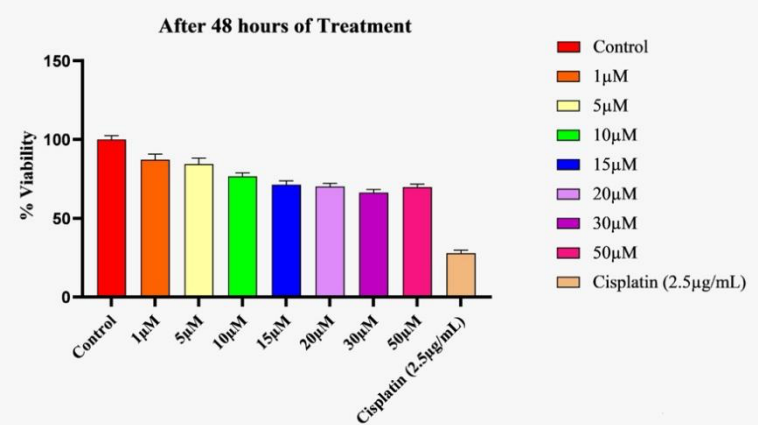

Figure 2 MTT Assay after 48 hours

Intergroup Comparison One way ANOVA ($p < 0.0001$), Post hoc: Control vs all test concentrations: $p < 0.0001$, lower concentration (1 µM, 5 µM, 10 µM) vs higher concentration (20 µM, 30 µM, 50 µM) ($p < 0.0001$), 20 µM vs 30 µM ($p = 0.99$), 30 µM vs 50 µM ($p=0.50$), Cisplatin 2.5 µg/mL vs all doses of Sotagliflozin ($p < 0.0001$).

4.2 In vitro antioxidant assay (FRAP assay)

The Ferric reducing antioxidant power (FRAP) assay was used to assess the antioxidant ability of Sotagliflozin. Treatment with 20 µM concentration of Sotagliflozin resulted in FRAP value of 0.29 ± 0.006 µM Fe(II)/µg of protein, which was significantly higher than the control, 0.26 ± 0.007 µM Fe(II)/µg of protein ($p < 0.001$). 30 µM Sotagliflozin raised the FRAP value to 0.366 ± 0.004 µM Fe(II)/µg of protein which was significantly high, compared to both the control ($p < 0.001$) and 20 µM Sotagliflozin ($p < 0.001$). The highest antioxidant activity is seen with Cisplatin (2.5 µg/mL) concentration with a FRAP value of 0.42 ± 0.009 µM Fe(II)/µg of protein. The results of FRAP assay showed that Sotagliflozin exhibited antioxidant activity in a dose dependent manner **(Figure 3)**

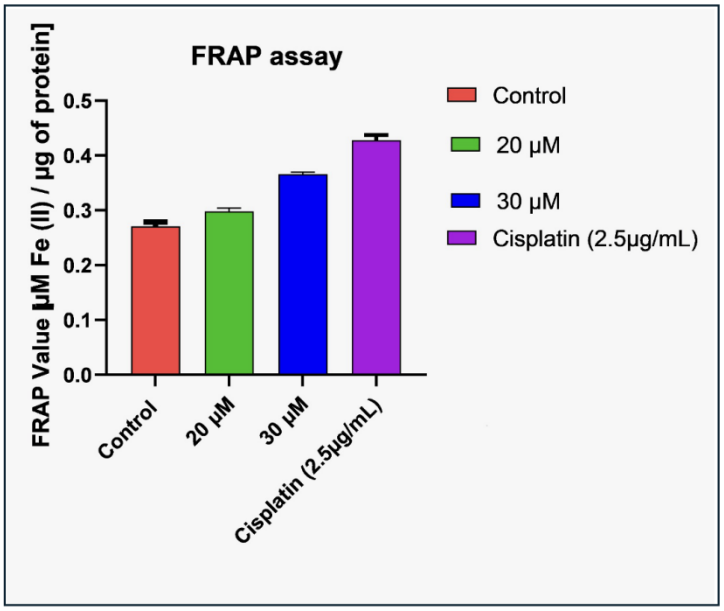

Figure 3 FRAP Assay

Intergroup Comparison One way ANOVA: $p < 0.0001$, Post hoc: Control vs all test concentrations ($p < 0.001$), Cisplatin 2.5 µg/mL vs 20 µM & 30 µM ($p < 0.001$), 20 µM vs 30 µM ($p < 0.001$).

4.3 In vivo Experiment:

4.3.1 Body weight

Body weight was recorded at baseline and once in 2 weeks till the end of the study (10 weeks) and values were recorded as Mean ± SD (**Table 2**). There was a significant difference observed in body weight across the groups ($p < 0.001$). The Normal control rats gained weight progressively, reaching 283.00 ±12.52 g at the end of 10 weeks, whereas the rats in cancer control group lost weight steadily reaching 160.17± 2.13 g at week 8,

with almost similar weight (164.17 ± 1.72 g) at the end of 10th week. The same trend was observed in Cisplatin treated rats till 8 weeks (157.33±1.63 g), but there was a marked increase in weight to 183.83±2.92g at the end of 10 weeks. In Sotagliflozin 10mg/kg group, the weight gain from week 8 to week 10 is partial (170.67±3.14 g), as comparable to Cisplatin group. Sotagliflozin high dose group showed slightly more pronounced weight gain (174.00 ± 5.36 g) comparable to low dose Sotagliflozin group. With Cisplatin and both the doses of Sotagliflozin, the body weight was significantly high compared to cancer control group ($p < 0.001$). The effect on body weight was similar with both the doses of Sotagliflozin ($p = 0.99$).

Table 2 Body weight in grams expressed as Mean ± SD

Groups	Base line	Week 2	Week 4	Week 6	Week 8	Week 10	P-value (Within group) Repeated measures ANOVA	P value (Intergroup) One-way ANOVA
Normal control n=3	184.33±6.02	204.67±2.51	225.00±2.64	244.33±3.21	262.67±3.05	283.00± 12.52	P<0.0001	P<0.0001
Cancer Control n=6	182.17±2.63	178.00±2.09	172.83±2.31	164.67±2.94	160.17±2.13	164.17± 1.72	P=0.0002	
Cisplatin group n=6	184.00±3.34	178.00±2.82	168.50±2.25	160.50±1.51	157.33±1.63	183.83± 2.92	P<0.0001	
Sotagliflozin 10mg/kg group n=6	182.50±2.88	176.83±2.56	171.17±2.63	166.33±2.33	163.67±2.73	170.67± 3.14	P<0.0001	
Sotagliflozin 20mg/kg group n=6	183.00±2.82	178.17±2.78	173.67±2.73	169.50±2.42	167.17±2.04	174.00±5.36	P<0.0001	

4.3.2 Liver Enzymes (AST, ALT and ALP)

AST is a key prognosis enzyme in hepatocellular injury, elevation of which indicates ongoing necrosis and inflammation. **Figure 4** shows AST levels of all groups at different time points. Normal control rats did not show any significant variation in the AST levels from baseline till the end of 10 weeks, while the other 3 groups had a significant elevation of AST, as a result of hepatic damage induced by DEN+CCl_4. Cancer control rats had exceedingly high AST values at each time point reaching up to 335.00±9.07 IU/L the end of 10 weeks. Cisplatin treatment resulted in the restoration of elevated AST levels from 162.67±7.06 IU/L at 8 weeks to 93.17±4.02 IU/L at 10 weeks. Sotagliflozin exhibited dose dependent effect on reduction in AST, where 10 mg/kg treated rats had AST level of 284.67±8.91IU/L at week 8, which then declined to 218.17 ± 6.08 IU/L at week 10 and 20 mg/kg group resulted in a more effective reduction of AST from 213.83 ± 5.94 IU/L at week 8 to 145.33 ± 3.98 IU/L at week 10. Though Sotagliflozin (both doses) did not reduce AST as effective as Cisplatin ($P<0.05$), the reduction of AST with both 10 and 20 mg/kg of Sotagliflozin was significantly high compared to cancer control (**Figure 4**).Similar to AST, ALT levels increased significantly after DEN + CCl_4 administration in cancer control and intervention groups. Unlike cancer control rats, where the ALT values elevated to 312.33±9.83 IU/L, Cisplatin and Sotagliflozin (10 mg/kg and 20 mg/kg) treated animals had significantly lower levels of ALT at 10 weeks. The reduction was more with Cisplatin, followed by Sotagliflozin 20 mg/kg and 10 mg/kg, reflecting the dose dependent effect of Sotagliflozin on ALT (**Figure 4**)The trend of changes in the ALP levels from baseline to 10 weeks is similar to AST and ALT across the groups (**Figure 4**)

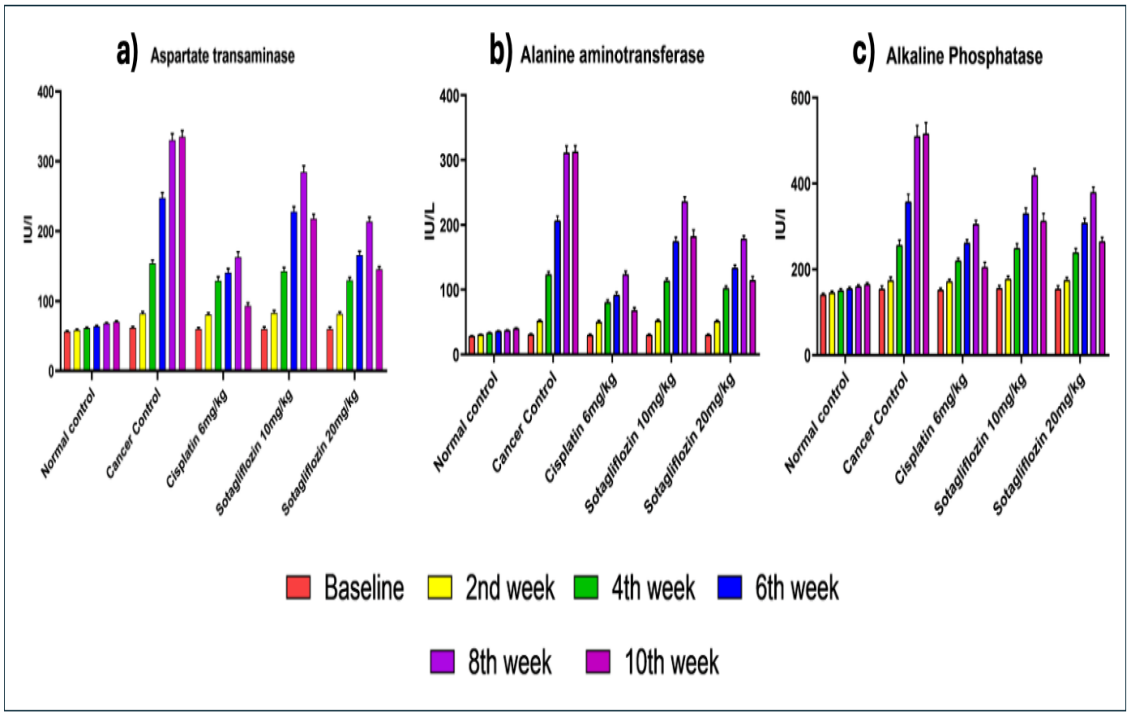

Figure 4 Effect on Liver function test

Intergroup comparison (2^{nd}, 4^{th}, 6^{th}, 8^{th} and 10^{th} Week) for AST, ALP, ALT– One way ANOVA (p value < 0.001),

Post hoc: Normal control vs Cancer control ($p<0.001$), Cancer Control vs all treatment groups ($p < 0.0001$) at week 10, High dose vs Low dose Sotagliflozin group ($p< 0.0001$).

Within the group comparison (baseline to end of study) Repeated measures ANOVA: AST ($p <0.0001$) for all groups, ALP ($p < 0.0001$) for all groups, ALT ($p < 0.0001$) for all groups.

4.3.3 Alfa Feto Protein (AFP)

A well-established tumour marker for hepatocellular carcinoma is Alpha Feto protein (AFP). AFP measurements were done at 3 time points: Baseline, at the end of week 4 and week 10. Normal control rats had low levels of AFP throughout the study period. Cancer control rats exhibited steep and significant rise in AFP from 3.33 ± 0.51 ng/ml at baseline to 137.83 ± 6.30 ng/ml at week 4 and 606.00 ± 36.79 ng/ml at the end of the study (week 10), indicating tumour progression. In Cisplatin and Sotagliflozin groups, AFP increased significantly at week 4 compared to baseline. Cisplatin effectively reduced the AFP levels from 100.50±5.75 ng/ml at week 4 to 69.17±5.63 ng/ml at week 10. In Sotagliflozin (10 and 20 mg /kg) groups, the AFP values were high at week 10 compared to week 4, though the increase was negligible with high dose of Sotagliflozin. However, the AFP levels at the end of 10 weeks were significantly less with both the doses of Sotagliflozin as compared to cancer control ($P<0.001$) (**Figure 5**)

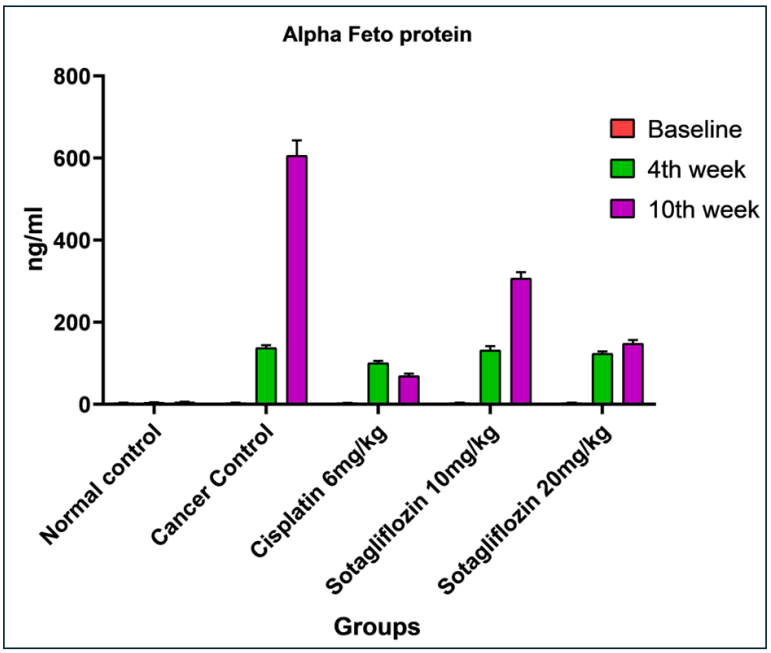

Figure 5 Effect on Alfa Feto protein

Intergroup comparison (Baseline, 4th and 10th Week) – One way ANOVA shows significant difference among the groups, (p value < 0.0001),Post hoc: Normal control vs Cancer control (p<0.0001), Cancer Control vs all treatment groups (p < 0.0001) at week 10, High dose vs Low dose Sotagliflozin group (p< 0.0001). Within the group comparison (baseline to end of study) Repeated measures ANOVA: Normal Control (p = 0.02), Cancer Control (p=0.004), Cisplatin 6mg/kg (p < 0.0001), Sotagliflozin 10mg/kg (p < 0.0001), Sotagliflozin 20mg/kg (p < 0.0001).

4.3.4 AMPK a [pT172]

The levels of phosphorylated AMPK (AMPKa [pT172]) in the liver tissue homogenates were quantitatively assessed at the end of the study in all the groups. AMPK activity was significantly reduced in Cancer control group compared to normal control rats. The liver tissues of Sotagliflozin treated rats showed significantly higher AMPK levels for both the doses when compared to cancer control. Cisplatin treatment did not improve the AMPK activity comparable to Sotagliflozin (2.06 ± 0.02 U/ml) **(Figure 6)**

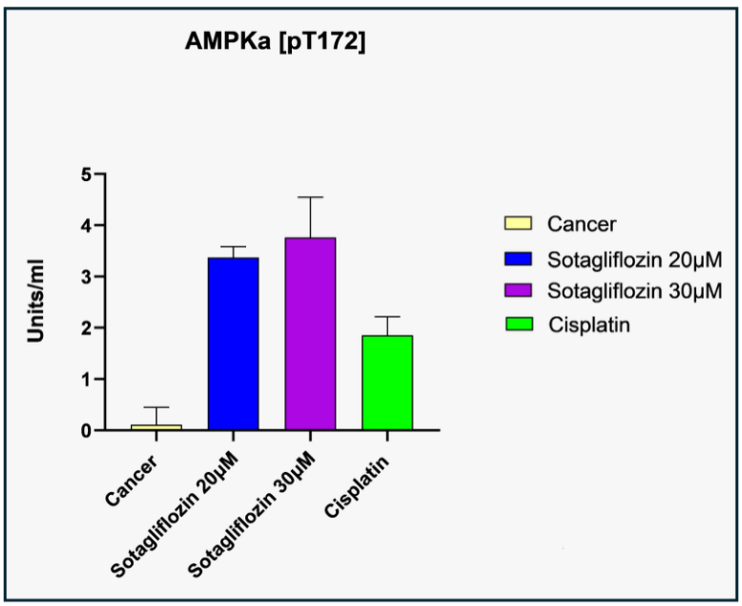

Figure 6 Quantitative Assessment of AMPK

Intergroup Comparison One way ANOVA: $p < 0.0001$
Post hoc: Control vs all test concentrations ($p < 0.001$), Cisplatin 2.5 µg/mL vs 20 µM & 30 µM ($p < 0.001$), 20 µM vs 30 µM ($p = 0.66$)

4.3.5 Histopathological analysis of liver

Histopathological examination of liver in all the groups showed the normal hepatic architecture in normal control group. In cancer control, complete effacement of architecture with nodules of atypical multinucleated cells were seen along with prominent nucleoli and abundant eosinophilic cytoplasm. Nuclear cytoplasmic ratio was increased with prominent nucleoli with areas of fibrosis and necrosis suggestive of hepatocellular carcinoma. The liver of Cisplatin treated rats showed minimal congestion with sinusoidal dilatation with mild periportal inflammation and signs suggestive of healing. In Sotagliflozin (both doses) treated rats, the liver showed hydropic degeneration with minimal inflammation, suggesting healing (**Figure 7**)

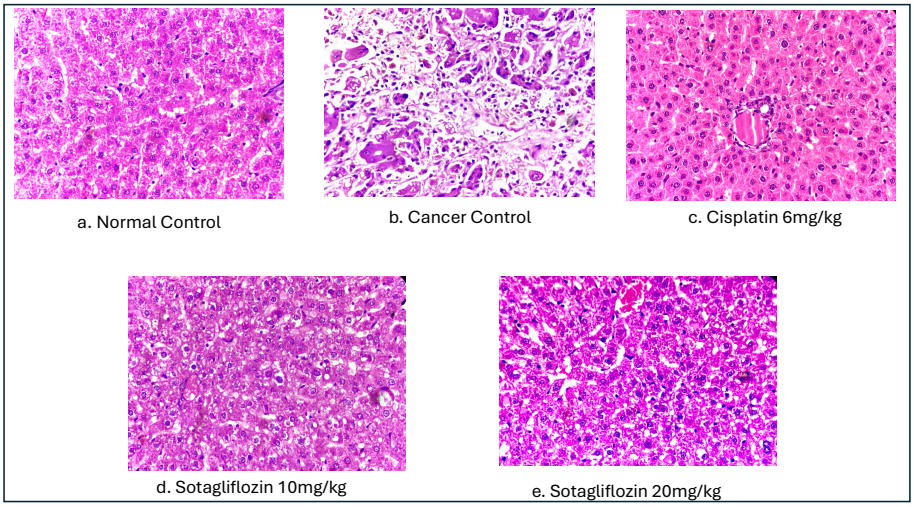

Figure 7 Histopathology of Liver

5. DISCUSSION

This study evaluated the anticancer and antioxidant effects of Sotagliflozin, a dual inhibitor of SGLT1 & 2 in in vitro HUH -7 Hepatoma cell line model and in in vivo DEN-CCl_4 rat model of HCC. We found that Sotagliflozin produced a modest reduction in cell viability compared to pronounced cytotoxic effect of Cisplatin, dose dependent antioxidant property and was found to be hepatoprotective in rat model of HCC, restoring the AMPK signalling and favourable histopathological changes in the liver. Sotagliflozin demonstrated only a modest reduction in cell viability in vitro when compared with the robust cytotoxic response elicited by Cisplatin. This shows that the therapeutic benefits of Sotagliflozin may extend beyond direct cytotoxicity, potentially involving metabolic modulation and preservation of hepatic architecture. Similar invitro study done by Karim S et.al., to study the cytotoxicity of Canagliflozin, Empagliflozin and Dapagliflozin on MCF-7 breast cancer cells reported moderate cytotoxicity with Canagliflozin, whereas Empagliflozin and Dapagliflozin did not induce any cytotoxicity, similar to what is observed in our present study (24). Dutka et al., reviewed and reported the varied mechanisms of SGLT2 inhibitors in cancer treatment apart from direct cytotoxicity aligning with the results of the present study (14).In FRAP assay, a notable antioxidant potential of Sotagliflozin was seen with both tested concentrations with higher activity observed at 30µm. This property makes relevance in HCC, in which oxidative stress plays an important role in disease progression (25). Chronic oxidative stress is directly linked to the development of HCC through genomic instability, mitochondrial DNA damage, chronic inflammation, which is mediated by reactive oxygen species (ROS) stimulation of oncogenic signalling pathway, including Wnt/ β- catenin and notch (26). Antioxidant potential of a drug attenuating the oxidative stress not only reduces hepatotoxicity but also creates a less favourable microenvironment for the progression of HCC (27). Thus, antioxidant property of Sotagliflozin supports its therapeutic benefit in HCC. The antioxidant effect of Sotagliflozin was 1-1.4 times higher when compared to Canagliflozin, Dapagliflozin, and Empagliflozin, as reported De A et al., (28). Anoush M et al., studied the neuroprotective effects of Empagliflozin against memory impairment and oxidative stress in rats found significantly increased FRAP levels with Empagliflozin indicating its antioxidant property enhancing memory performance in animal models (29). Unlike other SGLT2 inhibitors, the dual inhibition (SGLT1/2) property of Sotagliflozin may contribute to enhanced antioxidant effects, as SGLT1 inhibition decreases the intestinal glucose absorption and subsequent oxidative burden (19).Sotagliflozin resulted in a favourable effect on HCC in the rat model, as evidenced by a significant decline in serum AFP levels, enhanced AMPK activity, and clear hepatoprotective features on histopathological examination. Multiple pathways are involved in the development of HCC and anticancer drugs are being used targeting one or more than one pathways. One of

which is Warburg effect, characterised by preferential metabolism through glycolysis even in the presence of oxygen rather through oxidative phosphorylation, in the cancer cells. This switch in metabolism is hallmark of cancer cells (30). Modulating this effect by forcing cancer cells to rely on less efficient energy production pathways could be used for arresting the cancer cell proliferation. It is found that there is higher expression of SGLT 1 protein in HCC tissues than normal liver tissues (31). Jojima et al., demonstrated stronger expression of SGLT2 in liver tumour cells compared to normal hepatic lobules. Additionally, the authors also reported the diffuse expression of SGLT1 in hepatic lobules in HCC cells (32). Hence, SGLT1 and SGLT2 can be used as targets in the management of HCC.A few studies reported the favourable effect of SGLT 2 inhibitors in decreasing the tumour burden in HCC. Hung MH et al., studied the in vivo anticancer effect of Canagliflozin, an SGLT2 inhibitor in HCC and observed that Canagliflozin, by preventing the glucose influx, attenuated the tumour growth by blocking glucose mediated β – catenin activation (16). Unlike Canagliflozin which inhibits only SGLT 2, Sotagliflozin blocks both SGLT1 and SGLT2 (33), and it would have an additional benefit in HCC, as the HCC cells have increased expression of both SGLT1 and SGLT2.The present study observed the hepatoprotective effects of Sotagliflozin in HCC, as evidenced by the reduction in the levels of hepatic enzymes and AFP. These findings are consistent with other observational studies conducted in humans, which reported the hepatoprotective effects of SGLT2 inhibitors in patients with diabetes. Hendryx et al. conducted a large scale epidemiological study in patients with HCC and diabetes. They compared the overall survival rate in HCC patients who were taking SGLT2 inhibitors for diabetes with patients who were on other oral hypoglycaemic drugs for diabetes. The authors reported that SGLT2 inhibition was associated with significant improvement on overall survival in HCC patients with pre – existing diabetes (34). Another study conducted by Kim et al. found that the incidence of HCC is less in patients with fatty liver disease and diabetes who received SGLT2 inhibitors (35).Amjad W et al., did a meta-analysis and evaluated the effect of SGLT2 inhibitors on liver enzymes in patients with non-alcoholic fatty liver disease (NAFLD) and found that there were reduction in ALT and AST levels with the use of SGLT2 inhibitors which indicates the hepatoprotective effect of Sotagliflozin, which is observed in the present study also (36).AFP plays an important role in development of HCC and a higher expression of AFP is directly linked to HCC progression (37). In the present study, we noticed a significant reduction in AFP levels in HCC induced rats, from week 8 to week 10 with Sotagliflozin treatment, establishing the anticancer potential of Sotagliflozin. Jojima et al., observed a similar effect on AFP, where Canagliflozin significantly decreased AFP mRNA expression in HCC liver tissue (32).Histopathological findings is one of the necessary evidence in assessing HCC. Unlike the cancer control group, where the liver showed distorted architecture, increased nuclear cytoplasmic ratio, fibrosis and necrosis, Sotagliflozin treatment showed prominent signs of healing in the liver tissues. Akuta N et al., evaluated the effect of SGLT2 inhibitor, Canagliflozin on the histopathological features of liver biopsy samples in patients with NAFLD. Six out of seven patients had an evidence of reduction in inflammation and attenuation of fibrosis (38).AMPK acts as a metabolic sensor that promotes autophagy, through mTOR inhibition, activating tumour suppressor pathways including p53 (39). Zhou et al. demonstrated that SGLT2 inhibitors activate AMPK that promotes cell cycle arrest and apoptosis in breast cancer cells through mTOR blockade (40). The present study shows enhanced AMPK activity with Sotagliflozin which was significantly higher than the standard drug Cisplatin. This may be attributed to varying mechanism of action of Cisplatin and Sotagliflozin. The SGLT 1/2 inhibitor Sotagliflozin limits the glucose uptake, thereby reducing the intracellular ATP levels (41). This activates LKB1 – AMPK -mTOR signalling cascade, which triggers AMPK mediated inhibition of anabolic pathways and promotes apoptosis of cancer cells (42, 43). On contrary, Cisplatin exerts its anticancer effects via direct DNA damage, through generation of ROS and through activation of P53 apoptotic cascade (44). In this scenario, AMPK activation is transient or secondary and it may be suppressed at the later stages due to excessive ROS generation (45) or caspase mediated cleavage of AMPK -related proteins (46). This may be the reason that despite having strong cytotoxic effect, Cisplatin is less effective in modulating AMPK signalling, which explains the lower AMPK activity seen with Cisplatin compared to Sotagliflozin. The limitations of the current study were shorter duration of 10 weeks, which be insufficient to assess long term cancer effects and non-exploration of potential signalling pathways like PI3K/AKT, Wnt/β-catenin, or p53 cascades other than AMPK, that may also contribute to the anticancer effects of Sotagliflozin. However, with the findings of this study, future research can be done in other animal models and for a longer duration to explore the full therapeutic potential of Sotagliflozin as monotherapy or adjuvant to other anti-cancer drugs, in HCC, before proceeding to clinical studies.

6. CONCLUSION

This study provides strong evidence for the anticancer potential of Sotagliflozin in HCC through in vitro and in vivo investigations. This study also demonstrates antioxidant property, hepatoprotective effect and restoration of AMPK signalling which make Sotagliflozin a promising candidate for HCC treatment. However, further research may be required in larger animal models and human clinical trials to elucidate its efficacy and optimal application.

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8. CONFLICT OF INTEREST:

The authors declare that there are no conflicts of interest that could have appeared to influence the work reported in this manuscript.

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