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The Relative Quantification of Hedgehog Signalling Components of Hepatocellular Carcinoma (Hepg-2) Cells Treated With *Sorghum bicolor* Leaf Stalk Ethanolic Extract

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ABSTRACT: Hepatocellular carcinoma (HCC) is the most frequent type of primary liver cancer. Patients with advanced HCC have a dismal prognosis due to the limited therapy choices available. Hence, this present study evaluates the cytotoxic potential of the ethanolic crude extract of *Sorghum bicolor* leaf stalk (SBELSE) against human hepatocellular carcinoma (HEPG2) cell line, and its effect in the expression of its Hh signaling component-*GLI1* and *PTCH*. The leaf stalk of *Sorghum bicolor* (SB) was pulverized and extracted with 70% ethanol. HEPG2 cells were treated with different concentration of ethanol extract of SBELSE which was subjected to 2-fold serial dilution to achieve a graded concentration (9.77-5000 µg/ml). Cell viability was performed using CCK-8 assay. The cells were treated with 8 µg/mL and 23 µg/mL concentration of SBELSE for 48 hour, after which RNA was isolated for downstream gene expression studies using RT-qPCR. The expression level of *GLI1* and *PTCH* genes in cell line was examined relative to the untreated group. SBELSE exerted a cytotoxic effect on HEPG-2 cells with an inhibitory concentration (IC_{50}) of 150 µg/mL. After 48 hours of treatment and evaluation, Expression of *GLI1* and *PTCH* genes was seen in all cell group but was increased in groups treated with 8µg/mL concentration of *S. bicolor* extract than in the control group. The findings of this study suggest the possibility of the extract to contain agonistic component that might support cancer cell proliferation.

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Keyword: Hedgehog; *Sorghum bicolor*; Hepatocellular carcinoma; HEPG-2; Gene Expression.

Liver cancer is ranked the world second leading cause of cancer-related mortality. It is also the sixth most common cancer type (White *et al.,* 2017; Wu *et al.,*2019), with Hepatocellular carcinoma (HCC) as the most frequent type of primary liver cancer and one of the deadliest cancers in the world (Rawla *et al.,* 2018). The prevalence of Hepatocellular carcinoma (HCC) have increased in recent years, with viral hepatitis B (HBV) or viral hepatitis C (HCV) infection being a common risk factor for HCC (El-Serag, 2012). Despite the immunization programme, the incidence of HCC still remains high due to additional risk factors such as alcoholic and non-alcoholic fatty liver disease, as well as obesity, which is getting increasingly out of control (Maria *et al.,* 2017). The therapeutic options available for HCC are limited. Surgery, transcatheter arterial chemoembolization, and chemotherapy are currently the available treatment options for HCC. This has made HCC patients have poor prognosis. The focus of recent studies has been to identify novel molecular entities which can be targeted to block oncogenic signals associated with the initiation, proliferation, and progression of HCC (Maria *et al.,* 2017). The Wnt and Hedgehog (Hh) pathways are two key signalling pathways shown to be disrupted in HCC (Ingham and McMahon, 2001). The Hedgehog (Hh) pathway is a

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signaling cascade that is involved in a variety of basic functions, which includes embryogenesis, stem-cell maintenance, and cancer (Amakye *et al.,* 2013). Research evidence have shown a link between abnormal Hh activation and neoplastic changes, malignancy, and resistance to medicine in several cancer type. it has also been found to aid cancer growth by influencing the destiny of cancer stem cells (CSCs) at the molecular level (Sari *et al.,* 2018). In a differential gene expression assay conducted in 2001, Hh pathway transcripts were discovered in the liver microarray from patients with cholangiopathies. It was noted as the first indicatio that Hh might have a role to play in liver disease (Shackle *et al*., 2001). Efficacy of agents that selectively target critical signalling pathways has been evaluated in several clinical trials, but no significant improvement has yet been achieved. As a result, it is critical to identify therapeutic strategies for Hh signalling dysregulation caused diseases of which hepatocellular carcinoma, (Li *et al*., 2019) , Chronic myeloid lymphoma are implicated amongst others in order to provide a more effective cancer treatment. The importance of isolating active compounds with therapeutic potential from plants has been emphasized due to its potency and lack of side effects (Akolade *et al*, 2019; Chinsembu and Hedimbi, 2010; Iroanya *et al*., 2008; Willcox *et al.,* 2011). Sorghum (*Sorghum bicolor*) belongs to family *Poaceae,* commonly known as the grass family subfamily *Panicoideae,* tribe *Andropogoneae*, subtribe *Sorghinae,* and genus *Sorghum Moench* (Clayton and Renvoize, 1986). Sorghum is rich in phytochemicals, this which includes tannins, phenolic acids, anthocyanins, fosters, and policosanols, which are integral cellular components or secondary plant metabolites. These phytochemicals are known to significantly affect human health. Epidemiological evidences have linked high concentration of proanthocyanidins in sorghum to its prophylaxis effect in cancer risk, and have suggested that consumption of sorghum-based food could reduce the risk of certain types of cancer (Awika and Rooney, 2004). In this research, we investigated cytotoxic potential of ethanolic crude extract of Sorghum bicolor leaf stalk against human hepatocellular carcinoma cell.

MATERIALS AND METHODS

Plant Collection: Sorghum bicolor (guinea corn) leaf stalk was obtained from a farm about 200 m from Shere Hills, Jos, Plateau State (9°57’N, 9°3’N). The voucher specimen were authenticated by Dr. Nodza George at the Herbarium of Botany Department, University of Lagos with voucher number 7417. The leaf stalk of *Sorghum bicolor* was dried at room temperature and pulverized using a mechanical grinder. The powdered plant was weighed 1000 g of the weighed plant extracted with 70% ethanol for 72 hours with intermittent shaking. Thereafter, Muslin cloth and filter paper were used to filter the extracts. The filtrates was concentrated using a rotary evaporator under reduced pressure to a viscous liquid and was left to dry completely in a waterbath at 40 °C. Two hundred milligrams of the crude extract of *sorghum bicolor* was weighed and dissolved in 150 µL of DMSO and made up to 20 mL of PBS to prepare a stock of 10mg/mL. It was filter sterilized through 0.22 µM PDVF membrane syringe filter in a Class II biosafety cabinet.

Cell Culture And Conditions: The Human Hepatocellular Carcinoma (HEPG-2) cells were purchased from Cell Line Services (CLS), Germany. The cells were cultured in EMEM or DMEM supplemented with 10% FBS, 1% Antibiotics/ Antimycotics in a tissue culture flask and incubated in a humidified 5% CO_2 incubator at 37 °C till confluent was reached.

Cell Viability Assay: HEPG-2 cells were brought to suspension at a seeding concentration of 5000 cells per well in a 96 well plate. The plate was sealed and incubated for about 24 hour to allow for the cells to attach. After 24 hour, the cells were treated with *SBELSE* in a 2-fold serial dilution at a start concentration of 5000µg/mL and incubated for another 24 hour. Afterwards, 10 µl of Cell Counting Kit-8 (CCK-8) reagent was added to each well and incubated for 3 hour. Post incubation, the optical density (OD) was determined by measuring the absorbance at test wavelength of 450 nm and reference wavelength of 630nm on a microplate reader. The percentage half-maximal inhibitory concentration (IC_{50}) values was calculated as the concentration of extract resulting in 50% reduction of cell population relative to untreated cells as deduced from the equation below:

$$\%\ \text{Cell Viability} = \frac{ODTest - ODBlank}{ODControl} \times 100$$

$$\%\ Cytotoxicity = 100 - \%\ Viability$$

Gene Expression Profile Studies: The mRNA expression levels of *PTCH* and *GLI1* genes were determined via quantitative real time -polymerase chain reaction (RT-qPCR) assay.

Table 1: cDNA Synthesis Conditions

Cycle Step	Temperature (°C)	Time (Min)	Cycles
Primer Annealing	25	2	1
cDNA Synthesis	55	10	1
Heat Inactivation	95	1	1

The HEPG-2 cells were cultured in a 6 well plate at a concentration of 5 x 10^5 cells per well and maintained in DMEM medium for 48 hour after which it was treated with 30 ug/mL and 100ug/mL of the SBELSE, representing the low and high concentration of IC_{50}. Total RNA was isolated from the treated and untreated cells after 48 h using RNeasy mini kit, following the manufacturer's protocol. cDNA was synthesized from total isolated RNA according to the protocol of the Luna Script RT Super Mix Kit (E3010). Then, 800ng of cDNA was subjected to qPCR assay for amplification of *PTCH* and *GLI1* target genes using specifically designed primer sequences.

Table 2 Primer Sequence for *PTCH and GLI1* gene

Target Genes	Nucleotide Sequence	Length
GLI-1	F: 5'-TTC CTA CCA GAG TCC CAA GT-3'	20
	R: 5'-CCC TAT GTG AAG CCC TAT TT-3'	20
PTCH	F: 5'-GGT GGC ACA GTC AAG AAC A-3'	19
	R: 5'-ACC AAG AGC GAG AAA TGG-3'	18

Table 3: Amplification conditions for *PTCH and GLI1* target genes

Cycle Step	Temperature (°C)	Time (sec)	Cycles
Initial Denaturation	95	60	1
Denaturation	95	15	40-45
Extension	60	30 (+ plate read)	
Melt curve	65	Varied	1

Statistical Analysis: All data were expressed in mean ±SD and processed using Microsoft Excel Spreadsheet (2016) and Graph pad Prism (9.0). Relative quantification of the Ct value was determined using established method described by Pfaffil (2007). The Ct Value of the gene of interest in the treated group was expressed as a fold difference relative to the untreated sample, which served as the calibrator. Tukey's multiple comparisons test was used to test the mean difference of the fold difference. Analysis of variance was used to test the levels of confidence and statistical significance was considered at $p < 0.05$.

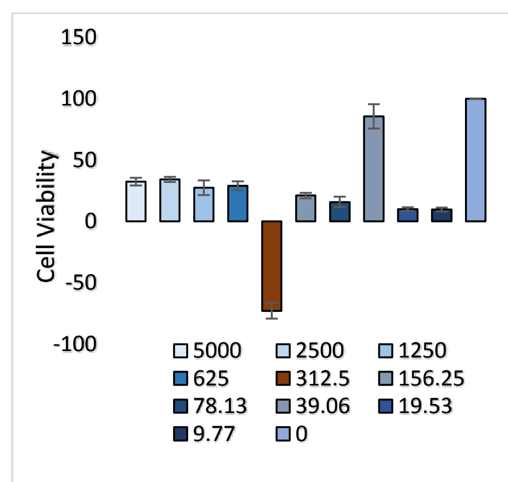

Fig 1: Cell viability dose response graph of SBELSE on HepG2 cells. Following 24 h treatment of HEPG-2 with SBELSE, cell viability assay was conducted and cytotoxicity was determined. The IC_{50} was determined at 150 µg/mL.

RESULTS AND DISCUSSION

Activity of SBELSE on HepG-2: Relative Quantification Gene Expression Analyses: The gene expression analysis was carried out on a relative normalized expression of the target genes (*PTCH* and *GLI-1*) in different doses of the treated sample to the calibrator negative control. The expression of the target gene is expressed as an increase or decrease relative to the level of expression of the control. Using the formular below, we derive the fold change of 9.15 in the *PTCH* gene of samples treated with low dose of extract while the samples treated with high dose had 2.50-fold higher than the negative control.

For the *GLI1,* the low dose had 1.33-fold increase, while the high dose treatment had 1.20-fold increase when compared with the negative control.

$$Ratio_{test\ calibrator} = 2^{[ct(calibrator)-ct(test)]}$$

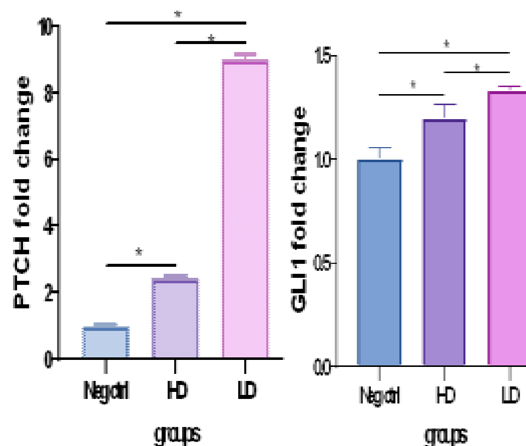

Fig 2: qPCR analysis of HEPG2 mRNA expression of *GLI1* and PTCH target genes 48 h after treatment with *SBELSE.*

Gene Expression Quantification: The quantity of the RT-qPCR product was measured using SYBR Green fluorescence chemistry which detect accurate quantification of the genes expressed. The Gene quantification results of the treated samples when compared to the Control sample showed that the expression level of both *GLI1* and *PTCH* was higher in Low dose treatment compared to the negative control.

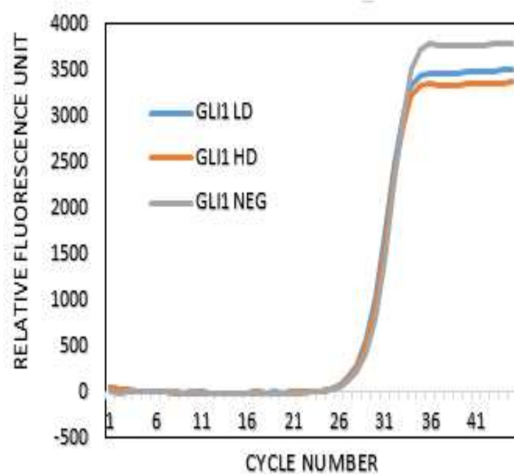

Fig 3: Amplification plot of PTCH target genes in HEPG-2 cells

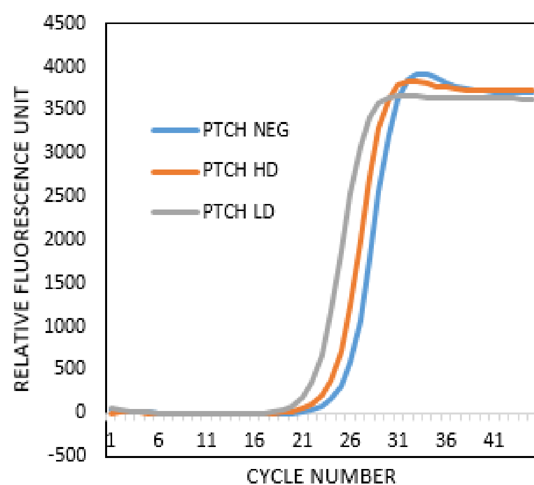

Fig 4: Amplification plot of PTCH target genes in HEPG-2 cells

Dysregulation of the Hh signalling system has been identified as one potential cause for hepatocarcinogenesis (Sicklick *et al.*,2006)closely linked to the prevalence of chronic liver diseases. Despite the fact that the global incidence of liver cancer is predicted to increase over the next decade, HCC already has one of the highest cancer-related mortality and prevalence rates (GLOBOCAN, 2020). Research is still on going to identify the mechanisms of tumor initiation and progression.Hedgehog (Hh) is a signalling cascade that is involved in a number of essential processes, which includes embryonic development and tissue homeostasis. Although it is involved in the hepatic determination of endodermal progenitors during embryonic development (Deutsch *et al.*, 2001), Hh pathway is not activated mature hepatocytes , hence it has not been considered a growth regulator in adult livers. Hh signalling has been demonstrated to promote the progression of cancer at the molecular level, by controlling cancer cell proliferation, malignancy, metastasis, and the expansion of cancer stem cells (CSCs) (Sari *et al.*, 2018). As a result, targeting the Hh pathway could provide therapeutic alternatives for a variety of human malignancies. Anti-Hh antibodies to inhibit receptor activation or small-molecule antagonists that inhibit downstream Hh signalling, such as *PTCH* and *GLI1*, can be used to effectively shutdown the Hh signalling pathway. In this study, we evaluated the activity of SBELSE on HEPG-2 cell lines, focusing on its effect on the component of the Hh pathway. The HEPG-2 cells which was treated with different concentration of SBELSE, was assayed to determine the cytotoxic effect of the extract on HCC. Total RNA was also extracted and reverse transcribed to cDNA which was then used for relative gene expression studies comparing the effect of the plant extract on Hh pathway component of treated and untreated HEPG-2 cell lines. Cytotoxicity is the level of damage caused due to the action of a chemotherapeutic agent on cells. The cytotoxic effect of any chemical agent towards the cell lines is presented as the average of half the maximal inhibitory concentration (IC_{50}) of the agent used. In this investigation, SBELSE, had moderate activity against HEPG-2 cells with an IC_{50} value of 150 µg/mL (Fig. 1). Correlating this finding with previous studies, *in-vitro* potential of eight sorghum varieties were assayed by Awika *et al.* (2009), their study declared the tannin containing sorghum extract had the strongest anti proliferative activity against both OE33 and HT-29 cells with an IC_{50} of 38-105 µg/ml which is slightly close to SBELSE used in this study. According to Suganyadevi *et al.* (2013) whose cytotoxic study of 3-deoxyanthocyanins extracted from sorghum on MCF7 cells via MTT assay gave an IC_{50} value of 300 µl/mL had an inhibitory effect on human breast cancer cell proliferation. This findings goes to show that different cell types may differ in their physiological responses to treatment from the same natural products owing to the agonistic, synergistic and/or antagonistic bioactive components in the extracts being studied. Hh signaling has a role to play in the development of different malignanciesand abnormal activation of Hh signaling in liver tumours which includes HCC has been well characterized

(Zheng *et al., 2013*). The findings of Patil *et al.*(2006) detected transcripts of Hh pathway signaling molecules in HCC cell lines and tumor and found that Hip1, an inhibitor of Hh signaling is downregulated in HCC samples relative to normal cell sample. In normal adult livers, Hh signalling is not expressed (Gao *et al., 2018*). However, injured hepatocytes, activated hepatic stellate cells (HSCs), Kupffer cells, endothelial cells, progenitor cells, and natural killer cells all express Hh ligands, and activated Hh signalling is required for tissue repair (Machado and Diehl, 2018). During organogenesis, Hh signalling is transiently expressed in hepatoblasts and suppressed when they mature to hepatocytes (Hirose *et al.,* 2009), indicating that it is essential for liver progenitor cell differentiation and maturation. Hh signalling is maintained at a low level in adult liver and may play a role in the maintenance of progenitor cell pool, hepatocellular zone difference, and lipid hemostasis (Ding *et al*.,2021). In a study conducted by Huang *et al.* (2006) on the analysis of Hh markers in HCC tumour tissues and adjacent normal liver tissues via *in situ* hybridization and polymerase chain reaction to measure the expression of SHH and its target genes. They found that Shh, PTCH1, and Gli1 expression were active in 115 cases of HCC and 44 liver tissues close to the tumour. Shh expression was found in about 60% of the HCCs studied while Hedgehog target genes *PTCH-1* and *GLI-1* are expressed in over 50% of HCCs, indicating that the hedgehog pathway is frequently active in these cancers. The Glioma-associated oncogene (*GLI-1)* is a transcription factor at the terminal end of the Hh pathway. In healthy tissue, it has a relatively low expression, however, its aberrant activation have been linked with the promotion of hallmarks of cancer. Increased expression in terminally differentiated cells is a known biomarker for a number of cancer subtype making it an ideal drug discovery target (Gupta, 2010).

In this study, the activity of the extract on the component of hedgehog pathway in HEPG-2 cells were investigated and a relative quantification was conducted by comparing the extent of gene fold in the treated cells with respect to the untreated cells (Pfaffl, 2007). The expression of Hh components -Receptor *PTCH-1* and the Transcription factor *GLI-1* in the qPCR assay of the cell line further ascertain the report by Patil *et al.* (2006) who detected transcript of Hh pathway signaling molecules in HCC cell lines. The genes had a varying expression level. The expression of *PTCH* gene is 9.15 -fold higher in samples treated with 30 μg/mL concentration of SBELSE, while the samples treated with 100 μg/mL concentration is 2.50 fold higher compared to the untreated group ($p<0.0001$). For the *GLI-1* gene the low concentration of SBELSE exerted a 1.33 fold increase in its expression, while the high dose had a 1.20 fold increase ($P<0.005$). While the activity of SBELSE on HEPG-2 cells is unknown, this research finding suggest that the crude extract may have promoted HEPG-2 progression. This is as a result of the higher level of fold change in the treated cells when compared to the untreated group. The ethanolic extract component of the *S. bicolor* might be supporting the proliferation of the cancer cells through an agonistic activity.

Conclusion: The SBELSE exhibited pro-oncogenic feature by supporting the proliferation of the cells. The extract is suggested to exert the agonistic effect due to varying components it is composed of creating a need for more detailed studies of the plant. Further bioassay guided screening which includes identifying the most active fraction(s) with most potent agonistic effect, as well as identifying the fraction(s) that may be able to deactivate the Hh pathway, in order to provide patients with a treatment option for HCC.

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