ABSTRACT
Hepatocellular carcinoma (HCC) has a range of risk factors and Hepatitis B Virus infection considers as one of these factors which lead to develop HCC. This type of carcinoma is still classified as a complex disease due to many risk factors and the patients with HCC diagnosed always at advance stage. The surgery and transplantation are still the most effective treatment while the response to chemotherapy and radiotherapy has poor effect. Our aim is to study the effect of β-Sitosterol, which is found in many plants on HBsAg and on hepatocellular carcinoma. To address this issue, Huh7, HepG2.2.15( harbouring four copies of HBV DNA ) and HepG2 cell lines were used. The Cell viability assay, Enzyme – Linked Immunosorbent Assay, and Gene Expression were used to evaluate the effect of β-Sitosterol. The result of Gene Ontology shows it is effect on HepG2-HBsAg by stimulating the phagocytosis we conclude β-Sitosterol has a positive regulation of phagocytosis to HepG2 Transfected with HBsAg this process could be help full to eliminate the dead cells caused by the apoptotic effect of β-Sitosterol on hepatocellular carcinoma cell infected with hepatitis B virus.

KEYWORDS: Beta-sitosterol, phagocytosis, HCC, Hepatitis B virus.
INTRODUCTION

The poor prognosis of HCC due to late and advance stage and a wide range factors classified it as complex disease. Moreover cannot responsive to standard chemotherapy and resist to radiotherapy while in the most cases the surgical and liver transplantation remains only the choice for treatment. There for the development of novel effective treatment is prime important.\textsuperscript{[1]} The recent studies using a combination of different technologies, like genomics, proteomics, metabolomics, MicroRNA studies, and Bioinformatics, are providing new understanding of the gene expression and protein profiles to develop novel cancer therapeutics.\textsuperscript{[2]} A 30\% of the global populations are infected with HBV and they are at risk of developing liver fibrosis, HCC, and other illness.\textsuperscript{[3, 4, and 5]} In this study, we used DNA microarray to evaluate the effect of Beta sitosterol on Hepatoma cell HepG2 transfected with HBsAg. The HBsurface (HBs) proteins, consist of large, middle and small (SHBs) (LHBs, MHBs, SHBs) proteins and it are possibly distinguished by their different domains and glycosylation status.\textsuperscript{[6]} The carboxyl terminal domain containing SHBs is present in all surface-proteins, preS1 N-terminal extension only in LHBs, preS2 in LHBs and MHBs.\textsuperscript{[7]} These three forms of HBV surface proteins represent HBsAg.\textsuperscript{[8]} and synthesized in the endoplasmic reticulum and could lead to the situation that amounts of protein produced exceed those required for virion assembly. Excess surface proteins undergo multimerization resulting in their budding from the ER/Golgi compartment as both non-infectious spherical and filamentous sub viral particles (SVP) or as versions.\textsuperscript{[9]} The SVPs typically outnumber the virions, could be components of circulating immune complexes,\textsuperscript{[10]}, and may induce immune tolerance by a mechanism of “viral apoptotic -like mimicry”\textsuperscript{[11]}. The domains of HBV surface proteins are S, preS2 domain is 55 aa long, and preS1. PreS1 contains 108, 118, or 119 amino acids (aa), depending on the genotype, and S domain (S) PreS extensions of LHBs and MHBs possess certain features that are very important for HBV infectivity such as binding the nucleocapsid during virus envelopment\textsuperscript{[9]} HBsAg could be involved in the arrangement, of the immune responses, especially in disturbances of the appropriate immune responses.\textsuperscript{[12, 13, 14 and 15]} The HCC carcinogenesis caused by HBV has been studied and the relations with HBV were proof as a multiple factors process that includes a direct mechanism involving viral protein, indirect mechanisms through the chronic inflammation, and the integration of HBV DNA.\textsuperscript{[16]} The direct mechanism of the viral protein reported in the HBx gene\textsuperscript{[17, 18]} and PreS2 gene\textsuperscript{[19]} based on a transgenic mouse model and force expression model of the cell lines act as promoters of carcinogenesis, the Pre S2 protein is encoded by HBsAg. It activates mitogen-
activated protein kinase (MAPK), which is involved in cell proliferation\textsuperscript{[20]} Moreover, PreS2 protein accumulates in the endoplasmic reticulum (ER) of hepatocytes, and DNA injury is caused in the cell by ER stress \textsuperscript{[21, 22]} we subjected the Hepatoma cell lines Huh7, HepG2 and HepG2.2.215 to β-Sitosterol to evaluate it is an effect of this compound which can be found in many plants it will be important to evaluate its function on HBV and HCC.

Materials

β-Sitosterol (C\textsubscript{29}H\textsubscript{50}O) (Sigma Aldrich analytical standard) prepared by dissolved 0.995mg β-Sitosterol in 10 ml Ethanol HepG2.2.15 cells (CRL-11997; ATCC) harboring four copies of HBV-DNA. HepG2 from American Type Culture Collection (ATCC). Cell counting kit 8 CCK8, DOjinDO laboratories, Japan. G418 Geneticin anti-biotic (Invitrogen)

Method

Cell culture HepG2.2.15, Huh 7and HepG2 Cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) with 10\%FBS (Fetal Bovine Serum) from Gibco® and G418 Geneticin (Invitrogen) at 380 μg/ml added to HepG2.2.15

Cell viability assay: Cell Counting Kit 8 (CCK8, DOjinDO laboratories, Japan) was used for the Cell viability determination. 8x10\textsuperscript{3} cells from Huh7 HepG2 and HepG2.2.15were counted (Count star Biotech Automated Cell Counter Shanghai) and cultured in 100 μl DMEM with 10\% FBS in 96 wells plate for 24 hours, β-Sitosterol added in different concentration from 0.0, 60, 120, 240 μM . Incubated for 24 hours to determine the cell viability in selected time. One hour before measuring the viability of the cells, The DMEM were changed with 100 μl of 1:10 CCK-8 solution and DMEM and incubated for one hour at 37°C in 5\% CO2 incubator. Then we measured the absorbance at 450 nm using a micro plate reader (ELx800 Absorbance Reader). Care was taken not to introduce bubbles to the wells during changing the medium, since they interfere with the O.D. reading.

Enzyme – Linked Immunosorbent Assay (ELISA).

Detection of Hepatitis B Surface Ag: We incubate the HepG2.2.15 cells in plate till a 90\% confluence was reached .Then β-Sitosterol in ethanol at 60 μM concentration were added and incubated for 24 hours at 37°C in 5\% CO\textsubscript{2} incubator. the reagents kit used from Wantai Beijing China and we followed the company protocol. One hundred μl taken from the cell culture medium in one well of 96 wells plate ( in duplicate ) In next well 50μl added from positive control and next well named negative control a 50μl from negative
control were added. Then 50μl from diluents were added to all the wells, the plate were shook slowly, cover and incubated for one hour at 37°C in 5% CO2 incubator. 50μl of conjugate added and incubated for 30 minutes at 37°C in 5% CO2 incubator. The reagent was decanted and the plate was tapped to remove any reagent from the wells. Cells were washed by adding wash solution four times; incubated after addition of wash solution for two minutes each time. Then 50μl of Chromogen A and 50μl Chromogen B solution were added incubated for 30 minutes at 37°C in 5% CO2 incubator. We stopped the reaction by adding 50μl from the stop solution and read at 450nM. Then we measured the absorbance at 450 nm using a microplate reader (ELx800 AbsorbanceReader). Care was taken not to introduce bubbles to the wells during changing the medium.

**Plasmid construction:** Plasmids included HBs cDNA, were constructed and used as templates for polymerase chain reaction (PCR) amplification. PCR-generated HBx genes were inserted into the KpnI and XhoI sites of the pcDNA3.1/Hygro (+) plasmid (V870–20, Invitrogen), the resultant vectors were called pcDNAhygro+- HBs the primers used for PCR amplification is shown as followed:

S-hygro (+) cloneF: 5’- CGGGGTACC GCC ACC ATG GAGAACATCGC ATCAGG-3’
S-hygro (+) cloneR: 5’ - CCGCTCGAG CTA CTTGTCGTCA TCGTCTTTGT AGTCAATGTA TACCCAAAGA-3’

**Transfection:** HepG2 cell line were cultured in DMEM supplemented with 10% FBS in 10 cm plate till a 90% confluence reached then the 2ml trypsin were added for 3mints and removed 3ml of DMEM were added, HepG2 cells were counted and seeded in 6-wells plate at the density of 5×10^5 per well 24 hours before transfection. The cells would reach 90% confluent and the medium changed to DMEM without FBS and incubated for one hour before transfection. Plasmids were transected into HepG2 cells using lipofectamine 3000 (Invitrogen, Carlsbad, CA) prepared in two test tubes one as reference containing 2.87 μl pcDNAhygro+- plasmid and Salmon sperm DNA 12.4 μl, P3000™ 4 μl, Opti-MEM 80.73 μl the total amount 100.00 μl and the second tube PcDNAhygro+- HBs 2.56 μl, Salmon sperm DNA 0.0 μl, P3000™ 4μl, Opti-MEM 93.44 μl the total amount 100.00 μl. In another two tubes we prepared lipofectamine as in table 1 five minutes before transfection we added the tubes of lipofectamine with the tubes containing PcDNAhygro+- HBs and pcDNAhygro+- plasmid respectively. And transfer it to HepG2 cells in 10 cm plate. 24 hours after transfection the Hygromycin were added. The cells were monitored by changing
the medium with the addition of Hygromycin 800μg/ml till reached the clear clone, and then we picked the clones into 24 wells plate after the cells propagated, the cells were passage into 12, 6 wells, 6 cm, and 10cm plate and propagated.

Table – 1 two tubes prepare with lipofectamine 3000

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>Lipo3000</td>
<td>3μl</td>
</tr>
<tr>
<td>Opti-MEM</td>
<td>97μl</td>
</tr>
<tr>
<td>Total amount</td>
<td>100μl</td>
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Gene chip
After transfection the HepG2-HBsAg, were cultured in DMEM with 10% FBS in 10 cm plates till reached a 90% confluenence .Then the 60 μM of β-sitosterol were added and incubated at 37 °C, 5% CO₂ for 24 hours then the DMEM removed and 1.5 ml of Trizol (Invitrogen) added to each plate We used Affymetrix gene chip and Agilent gene chip. Total RNA quality control, mark, chip hybridization, washing, staining and scanning, and data extraction and analysis procedure as company protocol. Affymetrix micro array for detection of mRNA expression profiles of total RNA samples as the starting, and biotin-labeled in vitro amplification, markAmbion #1792 cRNA expansion tags Kit. Data extraction and analysis software using the AGCC (Affymetrix® Gene Chip® Command Console® Software) chip . Fluorescent Scanned image is saved as DAT files and analyzed with KEGG PATHWAY, P ID, PANTHER, REACTOME, BIOCART, BIO CYCL software.

RESULT
Cell viability: Cell viability: Cell viability by CCK8 result shows decreases in viable cells related to concentration of β-Sitosterol in three cell lines In previous study we proofed that β-sitosterol has effect on HepG 2 and HepG2.2.15 cell lines by reducing viable cells .It is effective on Huh7 shows in figure 1.
Figure 1 shows the viable cells in Huh7 Cell line y axis represented the number of viable cell, x axis represented the concentration of β-Sitosterol column 1- No β-Sitosterol were added, column 2- 60 μM β-Sitosterol , column 3-120 μM β-Sitosterol, column 4- 240 μM β-sitosterol

Effect of β-Sitosterol on HBsAg
The optical reading (OD) was indicating that 60 μM of β-sitosterol has an effect on HepG2.2.15by reducing the total amount of HBsAg. as shown in figure 2.

Figure- 2 ELISA test for HBsAg quantification 1 is a reference shows the amount of HBsAg in HepG2.2.15 Column 2shows the amount of HBsAg in HepG2215with 60 μM beta-sitosterol this figure shows the decreased in HBsAg amount in cells treated with beta-sitosterol

Gene Expression
PCR quantification of RNA
Figure 3 Electrophoresis for quantification of the protein in the samples for microarray test. Lane 6 HepG2 HBsAg, Lane 10 HepG2-PcDNA-hygro treated with β-sitosterol, M Lane Hela Cell Control RNA.

Figure 4 - Log-log scatter gives a visual comparison of gene level variation between two cell lines. S6 refer to HepG2-HBsAg, the S10 is HepG2 cell line as reference both the cell lines were treated with β-sitosterol red color represented up regulation of genes, while green color down regulation and black equal expression in both reference and sample.

Gene Ontology
The result of Gene Ontology (GO) biological process increased the phagocytosis and activation c3 and c5 pathways and increasing cell adhesion the –log (corrected p-value) shows there are increased expression on several genes as shown in figure 7 and enrichment GO hierarchy biological process shown in DAG Graph file.
DISCUSSION

Phagocytosis is the way toward taking in microorganisms, parasites, dead cells, and foreign debris by a cell [23]. When β-sitosterol add to HepG2-HBsAg stimulate the phagocytosis by positive regulation of phagocytosis/GO: 0050766 in HepG2 cell line our previous study showed β-sitosterol has an effect on HepG2-HBx by inducing the extrinsic apoptotic signaling pathway more over ; it has senescence inducing effect to HepG2-HBSSP, This herbal compound (β-sitosterol) has anti-proliferative effect on Huh7 cell line and its effect on HepG2, HepG.2.2.15 shown in previous study. The extensive study by DNA microarray and Gene Ontology studies under the biological process revealed that β-sitosterol can stimulate the phagocytosis in HepG2-HBsAg. The important of the Phagocytosis of dying cells is a major process that be the final stage of cell death in a tissue under basal conditions, in a diseased tissue like cancer or after anticancer therapies. [24] The cluster of differentiation (CD47) is a commonly expressed molecule on all cancers. Its function to block phagocytosis is known, and blockade of its function leads to tumor cell phagocytosis and elimination. CD47 is therefore a validated target for cancer therapies. [25] Studies from the I.L.W. laboratory have shown that malignant cancers up-regulate the “don’t eat me” signal CD47, presumably in their progression to allow escape from endogenous “eat me” signals that were induced as part of apoptosis and programmed cell removal. [25, 26, 27 and 28]. Treatment of Caski and HeLa cells with β-sitosterol significantly suppressed the expression of genes involved in cell proliferation and oncogenic transformation, while augmenting the expression of genes involved in apoptosis and tumor suppression. Ultrastuctural characterization of Caski and HeLa cells treated with β-sitosterol further confirmed the anti-
proliferative and anti-cancer activity of this natural product isolated from traditional Chinese herbs.\(^{[29]}\) We believe that \(\beta\)-sitosterol can stimulate the phagocytosis in hepatocellular carcinoma infected with Hepatitis B Virus and by this process can eliminate the defragmented and dead cells which occurs due to apoptotic effect of \(\beta\)-sitosterol on hepatocellular carcinoma and HBV.

CONCLUSION
\(\beta\)-sitosterol has anti-proliferation effect on HCC cell lines Huh7, HepG2 and HepG2.2.15 and stimulate the positive regulation of phagocytosis process in HepG2.2.15 by direct effect on HBsAg. Therefore \(\beta\)-sitosterol has great effect on hepatitis B Virus replication and this is the first study to evaluate the effect of \(\beta\)-sitosterol on HepG2-HBsAg as for our knowledge and could open new areas of research on hepatitis B virus pathogenesis.

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