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Strigol1/albumin/chitosan nanoparticles decrease cell viability, induce apoptosis and alter metabolomics profile in HepG2 cancer cell line

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ABSTRACT

Hepatocellular carcinoma is one [illegible] most com[illegible] causes of cancer-related deaths globally. Bioavailable, effective and safe therapeutic age[illegible] urger[illegible] needed for cancer treatment. This study evaluated the metabolomics profil[illegible]ti-proliferat[illegible]-apoptotic effects of strigol/albumin/chitosan nanoparticles (S/A/CNP) on HepG[illegible] diameter of S/A/CNP was (5 ± 0.01) nm. The IC_{50} was 180.4 nM and 47.6 nM for Strigol1 and S/A/[illegible], resp[illegible] after incubation for 24 h with HepG2 cells. By increasing the concentration of S/A/CNP, th[illegible]was[illegible]condensation, degranulation in the cytoplasm and shrinking in cell size indicating apoptotic[illegible]. Metabolomics profiling of the exposed cells by LC/MS/MS revealed that S/A/CNP [illegible] epigen[illegible] intermediates (spermine and spermidine) and down-regulated energy production pa[illegible]y and s[illegible]ficantly d[illegible]ased glutamine ($P < 0.001$). These findings demonstrated that S/A/CNP has anti-[illegible]erative[illegible]ptotic effects and modulate energetic, and epigenetic metabolites in the hepatocellular carcino[illegible]

1. Introduction

Liver cancer is a global [illegible]th problem and a [illegible]h leading cause of cancer-related deaths, [illegible] an est[illegible]ted incidence of more than one million cases by the y[illegible]. Hepatocellular carcinoma (HCC) represent the sixth most co[illegible] cancer [illegible] an estimated 841,080 new cases and 781[illegible]er re[illegible]dea[illegible] globally every year [2].

Cancer t[illegible]affec[illegible]e liver a[illegible]rahepatic bile duct represents the 13th mo[illegible]mmon [illegible]cer diagnos[illegible]d in the USA. It is estimated that in the year [illegible]a[illegible]2,810 new cases and 30,160 cancer-related death[illegible] occur in the USA [3]. The HCC is considered the second most le[illegible]type of cancer after pancreatic cancer, and the 5-years survival ra[illegible]only 18% [4].

The accumulation of intracellular reactive oxygen species (ROS), increases both the rate of DNA damage and peroxidation of membrane lipids resulted from side effects of treatment with chemotherapy. Chemotherapy is one of the most important treatment options for advanced HCC but its efficacy is still not satisfactory [6]. More safe molecules in conjunction with chemotherapeutic agents could reduce its doses and side effects. Recent alternative and complementary medicine are focusing on exploring chemo preventive natural products that inhibit carcinogenesis and exert pronounced anti-carcinogenic effects [7].

Strigolactones (SLs) are a class of phyto-hormones implicated in the regulation of shoots branching and contribute in signaling pathways involved in biotic and abiotic conditions [8]. One of these derivatives of SLs is (strigol 1) that consists of a fused-ring system connected *via* an enol ether bridge [9]. It has been reported that SLs induced cell cycle arrest and exhibited anticancer activity *in vitro* and *in vivo* studies [10–12]. Moreover, naphthalimide-polyamine conjugates reduced tumor volume and inhibited pulmonary metastasis in *Swiss* mice bearing hepatoma xeno-grafts [13].

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Many synthetic analogs of SLs induced pro-apoptotic effects in different cancer cell lines as prostate, colon, osteosarcoma and lung. These effects were mediated by activation of MAPKs, p38 and JNK and inhibition of ERK1/2 and PI3K/AKT, which resulted in the cell cycle arrest at the G2 phase [14].

We have previously showed that SLs analogs TIT3 and TIT7 significantly reduced HepG2 cell viability in a dose- and time-dependent manner and induced apoptosis likely through targeting microtubules [14]. Herein, we sought to enhance stability, hence efficacy of these compounds, by preparing strigol/albumin/chitosan nanoparticles.

Human serum albumin (HSA) was reported to be used in preparation of drug delivery system based on nanoparticles because it is non-toxic, cheap, easily available, non-antigenic, non-degradable and easy to prepare [15]. Chitosan is a hetero-polysaccharide that possesses a positive charge and is biodegradable easily. Due to its cationic property, chitosan can be attached to anionic nanoparticles such as albumin. Chitosan was used as a coating material for albumin nanoparticles. The chitosan-coated albumin-loaded drugs prolong the half-life of drugs in the blood [16].

Analysis of metabolomics profile has been recently used to understand the progression and chemotherapeutic effects of drugs [17]. Identification of these specific pathways was carried out to investigate the correlation between the metabolites and diseases. In this study, we investigated antiproliferative, proapoptotic, and metabolomics profile in HepG2 treated with strigol1/albumin/chitosan nanoparticles (S/A/CNP).

2. Material and methods

2.1. Chemicals and reagents

Strigol1 was purchased from Sigma (99.993% purity) and dissolved in DMSO at a concentration of 5 mM. The HAS, chitosan, solvents, culture media and NaOH used in this study were high-purity grade obtained from BIORAD, England.

2.2. Preparation of strigol/albumin/chitosan nanoparticles (S/A/CNP)

Briefly, 20 mg of HSA and 10 mg of strigol1 were dissolved in 2 ml of 10 mmol NaCl and stirred for 3 h for the synthesis of strigol1/albumin nanoparticles (S/A). Thereafter, 20 μl of glutaraldehyde was added as a cross-linking agent, and the solution was stirred overnight. The solution was evaporated under vacuum at 40 °C then centrifuged at 20,000 × *g* for 30 min to obtain strigol1/albumin (S/A) particles. Fifty mg of chitosan was dissolved in 5 ml of 1% glacial acetic acid, pH was adjusted to 4.5 with 0.1 M NaOH and this was added to the prepared S/A (20 mg/ml) in equal volume (1:1), with continuous stirring for 4 h. Finally, the prepared S/A/CNP was lyophilized for 24 h at −10 °C. The calculated strigol1 concentration was 8 μg in each 25 μg of S/A/CNP.

2.3. Characterization of S/A/CNP

The shape and size of S/A/CNP were determined by a Transmission Electron Microscope (TEM). The size, distribution, and zeta potential were determined by dynamic light scattering (DLS) using a Malvern Zeta size Nano-ZS-ZEN 1600. Fourier transform infrared (FT-IR) spectroscopy (Alpha-Centauri, Shimadzu IR Prestige-21) was used to obtain the infrared spectrum of transmission or absorption of S/A/CNP.

2.4. Cell culture

Hepatocellular carcinoma cell line (HepG2) and normal human liver fibroblast (NHLF) cells were obtained from KFMRC, Jeddah, Saudi Arabia. Cells were maintained in Dulbecco's Eagle's media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics in 5% CO_2 at 37 °C.

2.5. Determination of cell viability by WST-1

Cell viability was evaluated using tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate) reagent (Trevigen, USA). Briefly, the HepG2 cells were grown at 6×10^3 cells/well in 96-well micro titer plates, incubated for 24 h, then treated with different concentrations of S/A/CNP (9.24–92.4 nM). Thereafter, 10 μl of the WST-1 reagent was added and incubated for 2 h at 37 °C. The absorbance was measured at 450 nm by an ELISA reader. The untreated cells were considered as control (100% viable). The IC_{50} (50% inhibitory concentration) values were calculated from the dose-response curve.

2.6. Hoechst 33258 and acridine orange/ethidium bromide staining for cell morphology

We used ethidium bromide and acridine orange (EB/AO) morphological staining method as previously described [18]. HepG2 cells were left to grow for 24 h in each a confluent 2×10^4 cells/well. The cells were treated with different concentrations of S/A/CNP (3.6, 7.2, 14.4, and 57.7 nM). The cells was washed with PBS and fixed with ice-cold ethanol for 5 min. After 200 μl Hoechst 33258 stain was added and incubated at room temperature for 30 min. Cell death and the changes in nuclear morphology were analyzed, using a fluorescence microscope at 40×.

2.7. Liquid chromatography-mass spectrometry (LC-MS/MS) metabolomics analysis

The HepG2 cells treated with S/A/CNP for 48 h were collected for metabolomics analysis by liquid chromatography-mass spectrometry (LC-MS/MS). LC-MS/MS allowed the determination of specific metabolic pathways' intermediates such as glycolysis, Krebs cycle and some amino acids. The LC-MS/MS data were calibrated using internal standards (IS) and quality control (QC) samples. Briefly, 200 μl of cell lysate was extracted with methanol: acetonitrile: water at 2/1/1 (v/v/v), vortexed and incubated for an hour, then centrifuged for 5 min at 10,000 rpm. The clear supernatants were analyzed by LC-MS/MS. 20 μl of supernatant from each sample was injected into the Hypersil gold column (150 mm × 4.6 mm, 5 μm), and the flow rate was 0.30 ml/min. The mobile phase (A) was: 99.9% acetonitrile/0.1% formic acid and the mobile phase (B) was 2% formic acid ultrapure water. The metabolites were identified using the database from the metabolomics line [23].

2.8. Statistical analysis

Data are presented as means ± SE. Statistical significance was determined with an unpaired, two-tailed *t*-test using the GraphPad software Prism version 7.0. (GraphPad Software, USA, www.graphpad.com). P value of ≤0.05 was considered significant.

3. Results

3.1. The characterization of the prepared S/A/CNP

We first characterized the shape and size of S/A/CNP, which was 5–10 nm, and the FTIR spectrum showed transmittance maxima at 1592 as shown in Fig. 1.

3.2. The cytotoxicity of S/A/CNP on HepG2 cells

To examine the efficacy of S/A/CNP, we compared the cytotoxic effect of strigol1 with S/A/CNP in HepG2 cells using a WST-1 assay. IC50 for S/A/CNP was 47.6 nM while it was 180.4 nm for strigol1, indicating the superiority of the S/A/CNP over strigol1 (Fig. 2a). We further tested the cytotoxicity of S/A/CNP on NHLF as a control to confirm its safety on normal liver cells. S/A/CNP showed no cytotoxic

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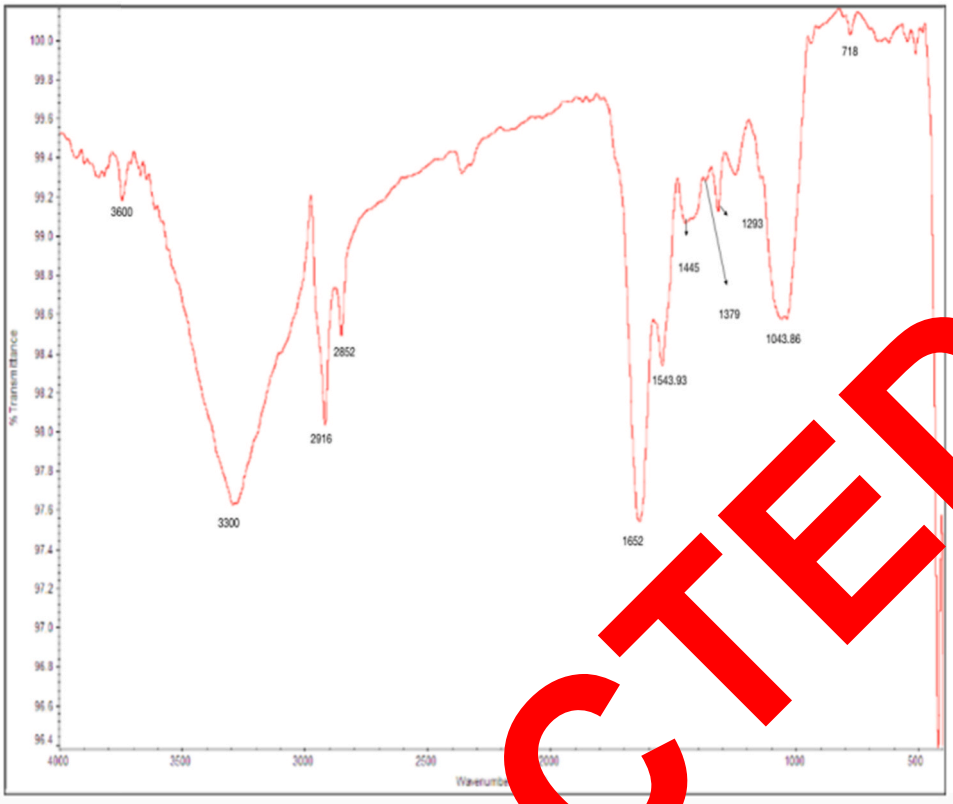

Fig. 1. The FTIR spectrum of S/A/CNP with transmittance maxima at 1592.

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effect at most of the doses tested (9.24–92.4 nM). However, it induced slight inhibition on NHLF at 100 μmol suggesting it has slight cytotoxicity against normal cells at high concentrations (Fig. 2). To demonstrate the extent of cytotoxicity of S/A/CNP, the effect of different doses of S/A/CNP on HepG2 cell viability was examined. Our results showed that growth inhibition was dose-dependent for 24 h (Fig. 2). There was a significant decrease in the cell viability at concentration 9.24 nM (81%) and at a concentration of 18.4 nM (62%, $p < 0.001$), 46.2 nM (45%, $p < 0.001$), 64.7 nM (28%, $p < 0.001$) and 92.4 nM (15%, $p < 0.001$).

3.3. The effect of S/A/CNP on cell morphology of HepG2 cells

The morphological alterations of HepG2 cells in response to different concentrations of S/A/CNP (100 μg) were evaluated using Hoechst 33258 and Acridine orange/Ethidium Bromide. The untreated cells exhibited diffused blue stained nuclei. However, treated cells appeared blue after Hoechst 33342 staining and stained more intensely (Fig. 3). Moreover, AO showed green nuclei, and the treated cells appeared orange stained nuclei, indicating the disruption of the cell membrane. Furthermore, there was a remarkable shrinking of the cells and pores in the cell membrane, associated with the initiation phase of apoptosis (Fig. 3).

3.4. Metabolomics profiling

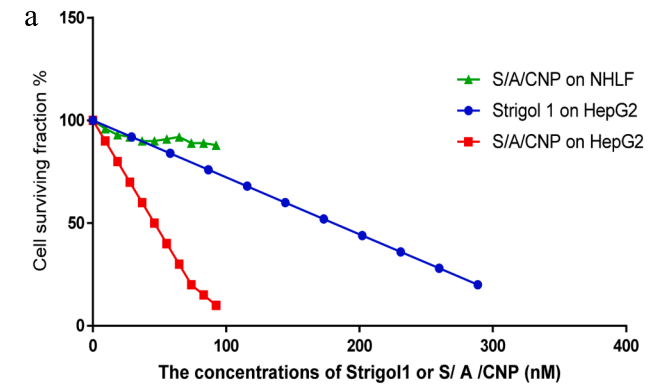
The metabolomic profiling at specific pathways, including the Krebs cycle, amino acids, fatty acids and biogenic amines, was done to compare treated and untreated cells (Table 1). These metabolites play significant/key/major roles in cancer cell proliferation, energy production, cell signaling, DNA modification, and neurotransmitters in the cell. The differences were observed in metabolites involved in epigenetic changes such as glutamines, methionine polyamines, and biogenic amines that are also important for the immune system. Notably, many metabolites such as fumarate, isocitrate, malate and succinyl CoA were significantly decreased ($p < 0.001$). Also, there was about a two-fold decrease in glutamine ($p < 0.001$). Moreover, there was a significant decrease in the levels of spermine and spermidine compared to treated cells ($p < 0.001$).

4. Discussion

Strigolactones are growth factors that play an important role in cell division, proliferation, DNA replication and growth. We previously reported that strigolactone analogs showed antitumor activity against HepG2 cell lines [14]. In this study, we investigated the antitumor activity of nanoparticles prepared from strigol1 with albumin and chitosan (S/A/CNP) as they are biocompatible to enhance efficacy and reduce the toxicity of strigolactones. Our results showed that S/A/CNP exhibited cytotoxic effect against HepG2 greater than strigol1 alone, indicating that the nanoparticles enhanced their antitumor activity. Moreover, we previously demonstrated pro-apoptotic effect of strigolactone analogs on HepG2 [18,19]. In this study, as the concentration of S/A/CNP increased, morphological changes were observed in HepG2, the nuclear condensation increased, cytoplasmic degranulation was accompanied by the formation of apoptotic bodies.

The metabolomics profiling of specific pathways showed changes in phenotype and alterations in gene expression, protein synthesis, and cellular function [17]. The observed metabolites give clues about the pathogenesis of diseases and can be used as biomarkers for disease progression and management. Some metabolites were detected in HepG2 treated with S/A/CNP compared to untreated cells. The altered levels of metabolites involved in epigenetic regulation, energy generation, neurotransmitters, and signal transduction were found in treated cells.

The biomarkers related to epigenetic changes were increased in treated cells while those involved in energy production was significantly decreased. Polyamines as spermine and spermidine involved in DNA



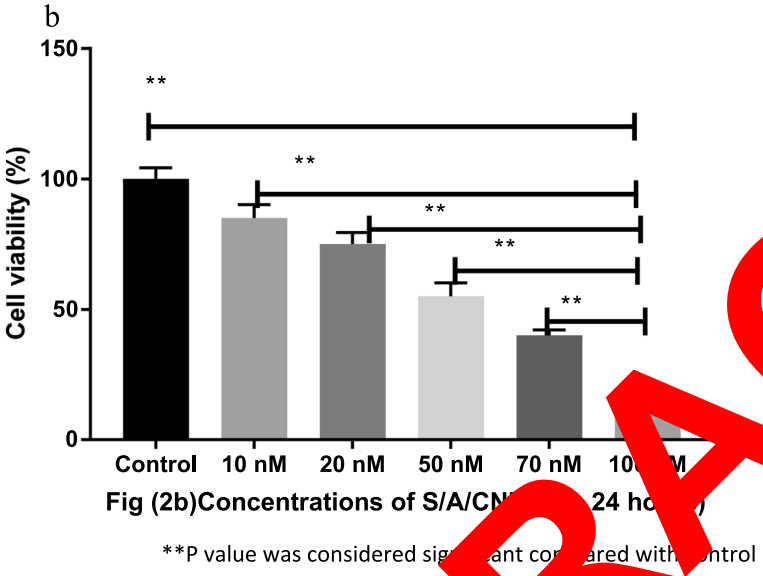


Fig. 2. (a) Cytotoxic effect of strigol1 or S/A/CNP treatment on HepG2 and normal human liver fibroblast (NHLF) cells. Cell survival was determined after 24 h of exposure to strigol1 or S/A/CNP. Each point is mean ± SE of two independent experiments conducted in triplicate. IC50 for Strigol 1 was 180.4 nM and that was 47.6 nM for S/A/CNP (b): A dose-dependent effect of S/A/CNP on cell viability of HepG2 after 24 h of incubation. *Significant different from control # Significant different from the corresponding S/A/CNP.

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replication were down regulated in ce eated with NP.

Methionine is mainly metaboliz y liver where converted to S-adenosylmethionine (SAM) [20]. It been shown that SAM decreased in human HCC and levels of SAM associated with HCC progression [21]. We spe te that the increas ethionine in this study would lead to in sed SA ynthesis and hence reduced cell proliferation and induc f a ptosis of HepG2 cells.

However, the concomi crease ginine in response to S/A/CNP is still pa It has sh that L-arginine increased the synthesis a ecret of nitri e in IGF-1 *in vitro*, and induces MAPK s ing pa way in cultured hepatocytes [22]. Moreover, arginine i mitochondrial damage and led to apoptosis in 2 cells [23]. Increased arginine led to increased cell proliferation, bu er biological effects of S/A/CNP might counteract this with an ove decrease in cell proliferation and increased apoptosis. Further studies are still needed to delineate the role of increased arginine in response to S/A/CNP.

A significant finding of this study is the pronounced decrease in glutamine in HepG2 cells after treatment with S/A/CNP. Indeed, glutamine is also a major energy substrate for many cancers [24]. Glutamine degradation can lead to mitochondrial phosphate synthesis through the stepwise conversion of glutamine into succinyl CoA and succinate, which will eventually generate mitochondrial substrate-level phosphorylation that is activated by succinate ligase. Moreover, glutamine inhibition through 6-diazo-5-oxo-L-norleucine (DON) combined with inhibition of glycolysis generated promising results in different animal models and human glioblastoma [25]. This not only explains at least in part the decrease of some of Krebs cycle metabolites in this study as well as the anti-proliferative properties of S/A/CNP. Also, we speculate that the decrease of both succinyl CoA and glutamine is crucial for decreasing substrate-level phosphorylation in the Krebs cycle, which provides cancer cells with ATP. Furthermore, the pronounced decrease of Krebs cycle metabolites was in line with previous results showing that this is important for the deprivation of intermediates needed for essential biosynthesis pathways [26].

Since glutamine is involved in protein synthesis and acts as a carbon and nitrogen donor, the decrease in Krebs cycle metabolites could be due to its pronounced reduction in our study. Recently, a novel combination targeting glutamine reduction showed promising results inhibiting liver cancer both *in vitro* and *in vivo* [27].

The arachidonic acid was downregulated in HepG2 cells treated with S/A/CNP compared with untreated cells. Arachidonic acid plays an important role in the production of diacylglycerol and phosphatidylinositol phosphate which is involved in signal transduction and stimulates proliferation [28]. In line with our results, it has been shown that Berberine suppressed the arachidonic acid (AA) metabolic pathway led to the reduction of the viabilities of H22, HepG2 and Bel 7404 hepatoma cell lines, and increased the number of apoptotic cells [29]. Therefore, this could account for the anti-proliferative effects of S/A/CNP.

Finally, the decrease in the levels of spermidine and spermine observed in this study is consistent with previous results that showed that spermidine/spermine N1-acetyltransferase which decreases their cellular content leads to significant inhibition of cell proliferation, migration, and invasion in HCC cell lines including HepG2 [30]. In

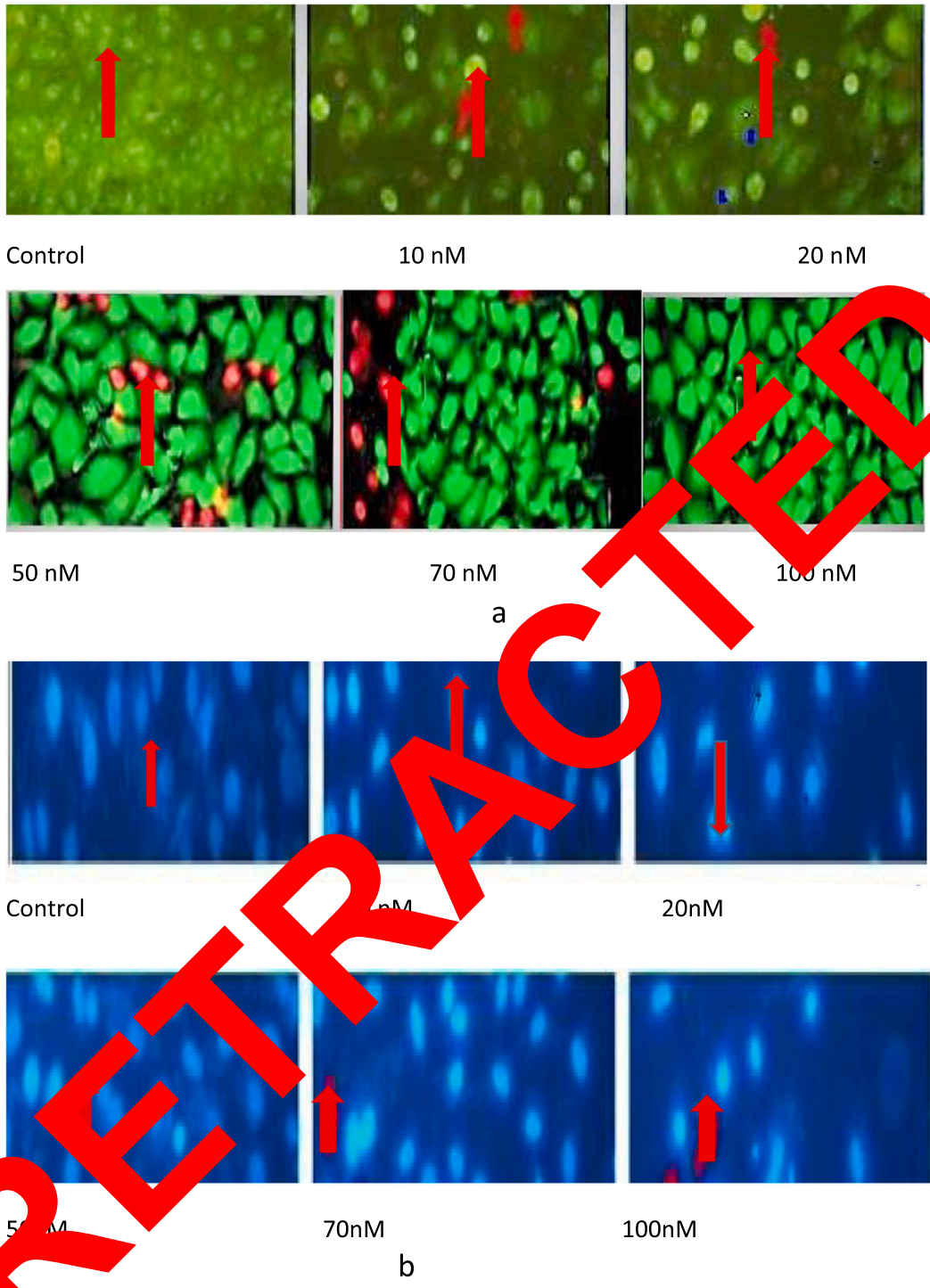

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Fig. 3. (A) Micros changes in nucleus of HepG2 cells exposed to S/A/CNP evaluated using Hoechst 33258 and Acridine Orange/Ethidium Bromide. (a) The untreated cells showe ep blue-stained nuclei. Cells treated with S/A/CNP showed blue color after Hoechst 33342 staining but stained more intensely. (b) The untreated cells showed green nuclei, and the treated cells with S/A/CNP appeared orange/red nuclei, indicating the disruption of the cell membrane.

Table 1
Metabolomics profiling in HepG2 in response to S/A/CNP.

Category	Compound	Log2FC	*p*-value
Epigenetic metabolites	Methionine	2.10	<0.001
	Arginine	1.91	<0.001
	Glutamine	−2.11	<0.001
Krebs cycle metabolites	Fumarate	−1.53	<0.001
	Isocitrate	−1.98	<0.001
	Malate	−0.42	0.01
	Succinyl CoA	−1.75	0.001
Amino acids and derivatives	Histidine	1.82	<0.001
	Nitric oxide	1.61	<0.001
	L-Citrulline	0.81	0.001
	L-Lysine	0.55	0.01
	Cystathionine	0.51	0.01
	L-Homoserine	0.63	0.01
	L-Serine	1.14	<0.001
	3-Hydroxyphenylacetic acid	−2.37	<0.001
Fatty acid metabolites	Gamma-Linolenic acid	−2.31	<0.001
	Arachidonic acid	−3.45	<0.001
	Alpha-Linolenic acid	−0.55	<0.001
	Hexanoyl-CoA	−1.31	0.0004
Biogenic amines	Spermidine	−0.33	<0.001
	Spermine	−0.53	<0.001
	Dopamine	0.86	<0.001
	Acetylcholine	0.95	<0.01

conclusion, S/A/CNP showed pronounced cytotoxicity and induced apoptotic activity in HepG2. This inhibition could be mediated by glutamine which is thought to constitute an important energy source for many cancers. However, further studies are still needed to get better understand the molecular mechanisms, metabolic pathways involved in S/A/CNP anti-carcinogenic effect.

CRediT authorship contribution statement

This is certify that, all authors included in this manuscript "Strigo albumin/chitosan nanoparticles decrease cell viabili apoptos and alter the metabolomics profile in HepG2 ca cell l share qually in this work.

Conflict of interest

The authors declare that they h flict of inter

Data Availability

All data sets genera or anal during this study are included in the manuscript.

Acknowledge

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