Ziziphus spina-christi leaves methanolic extract alleviates diethylnitrosamine-induced hepatocellular carcinoma in rats

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Ziziphus spina-christi leaves methanolic extract alleviates diethylnitrosamine-induced hepatocellular carcinoma in rats

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Cell: 00201030628070
Abstract

The present study aimed to evaluate the antitumor activity of Ziziphus spina-christi leaves extract (ZSCL) against diethylnitrosamine (DENA)-induced hepatocarcinogenesis in rats. The phytochemical constituents, in vitro antioxidant and cytotoxic activities of ZSCL extract were investigated. Male Wistar rats were divided into 6 groups: normal control, ZSCL1-administered rats (100 mg/ Kg body weight “b.w.”), ZSCL2-administered rats (300 mg/ Kg b.w.), DENA-induced hepatocarcinogenesis, hepatocarcinogenesis-bearing rats treated either with ZSCL1 or ZSCL2. Serum liver function & oxidative stress tests were assayed. The expression of hepatocyte growth factor, insulin-like growth factor-1 receptor, B cell lymphoma-2, and matrix metalloproteinase-9 oncogenes were quantified in liver. Histological examination of liver tissues was performed. ZSCL methanolic extract was rich in essential fatty acids, phytol, and polyphenolic flavones (luteolin and quercetin) with strong free radical & peroxide scavenging activities and cytotoxic activity. Administration of ZSCL1 and ZSCL2 to rats did not alter the studied parameters. DENA induced hepatocellular carcinoma and cholangioma by producing oxidative stress and upregulating liver oncogenes expression. Treatment of DENA-induced hepatocarcinogenesis with ZSCL2 ameliorated all the abnormalities induced by DENA except for cholangioma. In conclusion, ZSCL methanolic extract (300mg/ Kg b.w.) possessed a strong therapeutic activity against DENA-induced hepatocellular carcinoma via targeting oxidative stress and oncogenes.

Key Words

Ziziphus spina-christi; Leaves; Hepatocarcinogenesis; Oxidative Stress; Oncogenes
1-Introduction

Diethylnitrosamine (DENA) is a strong environmental carcinogen; present in the food chain (Mittal et al. 2006). The source of DENA could be endogenously or in occupational settings, tobacco smoke, processed meat, alcoholic beverages, agricultural chemicals, cosmetics, and pharmaceutical substances. Furthermore, metabolism of certain drugs can produce DENA (Sivaramakrishnan et al. 2008). In Egypt, hepatocellular carcinoma (HCC) contributes to 14.8% of all cancer mortality and is considered the second most common cancer type in Egyptian males after bladder cancer and the eighth most frequent in Egyptian females (Rashad et al. 2014).

A vital contributing mechanism to cancer development is altering the activation of proto-oncogenes which activate cells to enter the proliferation cycle, and inhibit both apoptosis and differentiation (Zhao et al. 2009). Several genetic and epigenetic alterations are involved in the HCC pathogenesis, including alterations in hepatocyte growth factor (HGF)/mesenchymal epithelial transition factor (c-MET) (Osada et al. 2008), insulin-like growth factor (IGF)/IGF receptor (IGF-R). Activation of these pathways produces resistance to apoptosis, increases the rate of cell proliferation, stimulates angiogenesis, invasiveness, and metastasis (Cervello et al. 2012).

*Ziziphus spina-christi* (L.) Desf. (christ’s thorn Jujube, Rhamnaceae) is a tropical evergreen tree producing small, orange-yellow fruits. It is commonly called Nabk and Sidr and widespread in the Middle Eastern countries (Michel et al. 2011). Phytochemical investigations reported that *Ziziphus spina-christi* contains tannins, essential oils, phytosterols, flavonoids, saponins, triterpenoid sapogenins, and alkaloids (Kadioglu et al. 2016). Previous studies have reported the cytotoxic effects of *Z. spina-Christi* crude leaves (ZSCL) extract against numerous cancer cell lines (Jafarian et al. 2014 and Farmani et al. 2016). ZSCL is known with its antiinflammatory, antimicrobial, hypoglycemic, immunostimulatory, and hepatoprotective activities (Michel et al. 2011, Kadioglu et al. 2016, and Dkhil et al. 2018a, b).
Up till now, there are no reports about the therapeutic activity of *Ziziphus spina-christi* leaves extract *in vivo* against hepatocarcinogenesis. So, the present study was undertaken based on the ancient medicinal value information about *Ziziphus spina-christi* to prepare leaves crude methanolic extract to fulfill the following points; characterization of *Ziziphus spina-christi* leaves (ZSCL) methanolic extract, evaluating the *in vitro* antioxidant as well as cytotoxic activities of ZSCL, studying the possible hepatotherapeutic targets of ZSCL against hepatocarcinogenesis induced in rats by diethylnitrosamine through studying the oxidative stress and the expression of hepatocyte growth factor (Hgf), insulin-like growth factor-1 receptor (Igf-1r), B cell lymphoma-2 (Bcl-2), and matrix metalloproteinase-9 (Mmp-9) oncogenes in liver tissues.

2- Materials and Methods

2.1. Preparation of *Ziziphus spina-christi* Leaves (ZSCL) Extract

Fresh Leaves of *Ziziphus spina-christi* were collected from Al Orman garden, Giza, Egypt. ZSCL extract was prepared according to the method of Hafiz and Mubaraki (2016). Air-dried leaves (500 g) were ground and the active ingredients were extracted from the dried leaves using 70% methanol at 27°C. Then the extract was dried in a vacuum evaporator at 40°C giving 50g yield. For experimental use, sterile saline was used to dissolve the residue.

2.2. Determination of Total Phenolics and Flavonoids Contents of ZSCL

The contents of phenolics and flavonoids in ZSCL methanolic extract were determined according to the methods of Singleton et al. (1999) and Chang et al. (2002), respectively. Triplicate assays were performed.

2.3. Gas Chromatography- Mass Spectrometry (GC-MS) Analysis of ZSCL

The phytochemical constituents present in the methanolic extract of ZSCL were identified using GC-MS in the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt, using Thermo Scientific TRACE 1310 Gas Chromatography attached to ISQ LT single
quadrupole mass spectrometry, equipped with DB5-MS, 30m x 0.25 mm ID column (J&W Scientific). ZSCL sample was dissolved in absolute methanol (Sigma, USA) to obtain a concentration of 100μg/ml, filtered using a membrane discfilter (0.2 μm), and then subjected to GC-MS analysis. Oven temperature was programmed from 50 ºC (1 min) to 150 ºC (2 min) at 10ºC/min. -250 ºC (1 min) at 5ºC/min.-270 ºC (2 min) at 3.5ºC/min. Ionization source EI with 70ev voltage. The carrier gas was helium at a flow of 1.5 ml/min. The temperatures of the detector and injector were adjusted at 300 and 250ºC, respectively. The individual GC peaks were identified by mass spectra using a computer search of the commercial libraries (WILEY & NIST mass spectral data).

2.4. In vitro Antioxidant Activity of ZSCL

The peroxide and free radical scavenging activities of ZSCL crude methanolic extract were assayed according to Smirnoff and Cumbes (1989) and Yen and Pin-Der (1994), respectively. The assays were performed in triplicates.

2.5. In vitro Cytotoxic Activity of ZSCL

The cytotoxic effect of ZSCL was performed according to the methods of Mosmann (1983) Elaasser and Abdel-aziz (2011). The assay was carried out on normal melanocyte cell line (HBF4) and well differentiated hepatocellular cancer cell line (HepG2) that were kindly provided by Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. The cell lines were suspended in Roswell Park Memorial Institute-1640 (RPMI-1640) medium at concentration of 5x10^4 cell/well in Corning® 96-well tissue culture plates; where RPMI-1640 were supplemented with 10% inactivated fetal calf serum and 50µg/ml gentamycin. The cells were then incubated for 24 h and ZSCL extract was added at concentration range of 15.6-500 µg/ml. Six vehicle controls with media or 0.5% dimethyl sulfoxide were run for each 96 well plate as a control. After incubating for 24 h, the numbers of viable cells were determined by 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Carl Roth, Germany); MTT test. The absorbance was measured at 590
nm with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and therefore calculating the percentage of viability.

The selectivity index (SI) is the ratio between the half maximal inhibitory concentrations (IC$_{50}$) obtained from the cytotoxicity on normal cell line vs. cancer cell line. High selectivity was achieved when the value of SI $\geq$ 3 (Prayong et al. 2008). The greater the SI value is, the more selective it is. SI value $\leq$ 2 indicates general toxicity of the tested compound (Koch et al. 2005).

2.6. Animals

A total of 108 adult male Wistar rats (2-3 months old) weighing 200±20 g were used in this study and provided from the breeding unit of the Egyptian Stock Holding Company for Biological Products of Vaccines, Sera and Drugs (VACSERA). The rats were housed in steel mesh cages for one week (6 rats/ cage), under standard laboratory conditions of light/dark cycle (12/12h) and temperature 24±2ºC for acclimatization in the animal house of the Medical Research Centre, Faculty of Medicine, Ain Shams University. The protocol was approved by the Ethical Committee of National Liver Institute, Cairo, Egypt (Serial No. 9/ 2016).

2.7. Determination of Acute Median Lethal Dose (LD$_{50}$)

Forty eight male Wistar rats were divided into 8 groups (6 rats per each group) for intraperitoneal administration of ZSCL extract at doses of 20, 40, 80, 160, 360, 720, 1440, and 2880 mg/ Kg b.w. once. The animals were observed for first 2 hours and then at 6th and 24th hour for any toxic symptoms. After 24 hours, the number of deceased rats was counted in each group and percentage of mortality was calculated (Randhawa 2009).

2.8. Study Design

Sixty male Wistar rats were assigned to 6 groups (10 rats/ group); Group I (Normal Control), rats were intraperitoneally (i.p.) injected with sterile saline; Group II (ZSCL1 Extract), rats were injected i.p. with ZSCL at a dose of 100mg/ Kg b.w. thrice weekly for 6 consecutive weeks, Group III (ZSCL2 Extract), rats were injected i.p. with ZSCL at a dose of 300mg/ Kg b.w. thrice weekly for
6 consecutive weeks, Group IV (DENA, negative control), hepatocarcinogenesis was induced in rats by daily oral administration of DENA (Sigma-Aldrich, USA), at a dose of 100µl (contained 100 mg DENA)/ one liter drinking water stored in dark bottle for three consecutive months followed by a period of 5 weeks of tumor growth in which rats were left without any administrations (Yang et al. 2004), Group V (DENA+ZSCL1), hepatocarcinogenesis-bearing rats were treated i.p. with ZSCL1 thrice weekly for 6 consecutive weeks, Group VI (DENA+ZSCL2), hepatocarcinogenesis-bearing rats were treated i.p. with ZSCL2 thrice weekly for 6 consecutive weeks.

2.9. Blood Sampling and Collection of Body Organs

At the end of the experimental period, the animals were fasted for 8 hours, weighed, and the blood samples were taken from the retro-orbital venous plexus under light ether anesthesia. Serum samples were obtained by incubating blood for 30 min at 37°C followed by centrifugation at 1500x g at 4°C for 15 min. Sera were then collected, aliquoted, and stored at -80°C until analyses. After blood collection, the rats were then sacrificed by cervical dislocation. Liver, spleen, kidneys, and heart were excised, rinsed thoroughly in isotonic sterile saline containing heparin, blotted dry with a filter paper, weighed. The right lobe of liver was divided into two parts; one part was kept intact in plastic vials containing ice-cold sterile saline at -80°C until analyses. The other part was kept in 10% formalin for histological examination.

2.10. Histological Examination

Liver specimens of the different groups were fixed in 10% formalin solution for at least 3 days at 4°C for a subsequent staining by hematoxylin and eosin for histopathological examination under the electric light microscope (Olympus, Japan).

2.11. Calculation of Organs Relative Weights

The relative weights of liver, spleen, kidneys, and heart were calculated according to the formula of Yang et al. (2005).

2.12. Preparation of Liver Homogenate
Ten percent of liver tissue homogenate was prepared by homogenization in ice-cold phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, and 1.8 mM KH$_2$PO$_4$), pH 7.4 using an electric homogenizer (Universal Laboratory Aid MPW-309, Poland). The whole homogenates were centrifuged at 20000x g for 20 minutes at 4ºC in a cooling microfuge Laborzentrifugen (Sigma, Germany) to obtain the cytosolic supernatants which were then collected, aliquoted, and stored at -80˚C until analyses.

2.13. Serum Analyses

The level of albumin and the activities of alanine transaminase (ALT) and gamma-glutamyl Transferase (GGT) were analyzed in sera using commercial colorimetric kits (Spectrum, Egypt). In addition, toxicity studies of the tested ZSCL extract were checked by assessment of serum creatinine using a commercial colorimetric assay kit (Spectrum, Egypt).

2.14. Hepatic Oxidative Stress Assay

Malondialdehyde (MDA) and total antioxidant capacity (TAC) were determined in the cytosolic supernatant of the whole liver homogenate using colorimetric kits provided from Biodiagnostics (Egypt).

2.15. Hepatic Total Protein Measurement

The total protein content was determined in the cytosolic supernatant of the whole liver tissue according to the method of Bradford (1976).

2.16. RNA Extraction from Liver Tissues, cDNA Synthesis and Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was extracted and purified from liver tissues using RNeasy Mini Kit according to manufacturer's instructions (Qiagen, Germany). The purity of the extracted RNA was assessed spectrophotometrically at 260/280 nm using the UV-spectrophotometer (PhotoBiometer, Eppendorf, Germany). 2\(\mu\)g of RNA was reverse transcribed into single-stranded complementary DNA (cDNA)
using QuantiTect reverse transcription kit (Qiagen, Germany). cDNA synthesis was performed using Gene Amp PCR System 9700, Applied Biosystems by life technologies, USA.

Quantitative real time PCR was performed using SYBR green master mix (Qiagen, Germany) to determine the relative expressions of the liver Hgf, Igf-1r, Bcl-2, and Mmp-9 cDNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalizing mRNA expression for genes of interest. qPCR was performed in an optical 96-well plate with an ABI PRISM 7500 fast sequence detection system (Applied Biosystems, Carlsbad, California) and universal cycling conditions (95°C 10 min, 50x at 95°C for 15 s, 60°C 60 s, and 72°C for 15 s). The used primers (Invitrogen, Belgium) for qPCR are listed in Table 1. Gene expression was expressed in relative units (RQ = 2^{ΔΔCT}).

2.17. Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Science version 20 for Windows (Chicago, USA). Individual data in the experimental groups were analyzed using one-way Analysis Of Variance followed by Duncan post hoc test for multiple comparisons between the different studied groups. P value was considered significant at \( p < 0.05 \). The percentage of change was calculated in comparison with the normal control.

3. Results

The present study reported that the crude methanolic extract of ZSCL is abundant, measured as milliequivalent “mE”, in flavonoids (710 mE rutin/g) and phenolics (500 mE gallic acid/g) compounds (Table 2). ZSCL extract possessed a strong antioxidant activity by having potent scavenging activities of \( \alpha, \bar{\alpha} \)-diphenyl-β-picrylhydrazyl (DPPH•) free radical (IC_{50} of 57 μg/ml) and hydrogen peroxide (90%). GC-MS chromatogram of ZSCL methanolic extract indicated the presence of phytol, palmitic acid, oleic acid, and palmitoleic acid methyl ester as major constituents in association with the presence of high content of polyphenolic flavones in the form of luteolin as well...
as quercetin 7,3’,4’-trimethoxy (Figure 1 and Table 3). Figure 2 reported that ZSCL exerts strongly
cytotoxic effect against HepG2 cancer cell line (IC$_{50}$ = 94.5 µg/ml) while gave IC$_{50}$ value of 426
µg/ml against normal HBF4 cell line with a high SI of 4.51.

The LD$_{50}$ results showed that ZSCL crude extract is safe upon i.p. administration to normal
rats at increasing doses up to 2880mg/ Kg b.w. Based on the high antioxidant and cytotoxic activities
of ZSCL as well as the results of LD$_{50}$, two doses of ZSCL (100 and 300mg/ Kg b.w.) were chosen
for investigating ZSCL probable therapeutic effect against hepatocarcinogenesis induced in rats by
dena.

The histological examination of all the studied groups was shown in Figure 3. Intraperitoneal
administration of ZSCL to normal rats at doses of 100 and 300 mg/ Kg b.w. showed normal
hepatocytes radiating from the normal central vein (Figure 3, B and C), similar to that of normal
control rats (Figure 3, A). On the other hand, daily oral administration of Dena caused HCC with
trabecular pattern indicating by the presence of anaplastic hepatocytes with cellular malignancy as
polarity, pleomorphism, and hyperchromasia as well as a variation in the nucleus: cytoplasm ratio
(Figure 3, D-1). Cellular proliferation was detected in the lining epithelium of the bile ducts forming
papillary projection and cysts with one cell layer and basal nuclei forming papillary cystic
cholangioma (Figure 3, D-2). In addition, nodules surrounded by fibrous tissue were detected in the
parenchyma (Figure 3, D-3).

Treatment of Dena-induced hepatocarcinogenesis with ZSCL at 100 mg/ Kg b.w. displayed
somehow less toxic effects in liver tissue evidenced by the presence of a focal area of dysplastic
hepatocytes with ballooning degeneration in hepatocytes (Figure 3, E-1). There was a focal area of
papillary cystic cholangioma in the bile ducts with flattened lining epithelium and basal nuclei
(Figure 3, E-2). Surprisingly, treatment of Dena-induced hepatocarcinogenesis with the high dose
of ZSCL (300 mg/ Kg b.w.) for 6 weeks displayed a high therapeutic activity revealed by the
reversal of HCC. Congestion in the central and portal veins associated with inflammatory cells
infiltration in the portal area were detected (Figure 3, F-21&F-2). In addition, cellular proliferation was observed in the bile ducts forming cystic cholangioma (Figure 3, F-2).

Administrations of normal rats either with ZSCL1 or ZSCL2 for 6 weeks showed normal relative weights of liver, heart, and kidney as well as liver function tests, liver oxidative stress markers, and serum creatinine concentration (Table 4). However, ZSCL1 and ZSCL2 increased significantly the relative weight of spleen (92.31 and 107.69%, respectively, \(p<0.0001\)), compared to the normal control group. Administration of DENA caused a significant (\(p<0.0001\)) elevation in the relative weights of liver (48.59%), spleen (161.54%), kidneys (24.56%), and heart (20.59%), compared to the normal control rats. Also, DENA elevated significantly (\(p<0.0001\)) serum activities of ALT (87.67%) and GGT (826.16%) in association with a significant reduction in serum albumin concentration (29.95%, \(p<0.0001\)), compared to the normal control group. DENA induced imbalance in oxidant/antioxidant status in the form of a significant rise in hepatic MDA content (825.56%, \(p<0.0001\)) and a significant reduction in the hepatic TAC (45.66%, \(p<0.0001\)).

Treatment of DENA-induced hepatocarcinogenesis with ZSCL1 decreased significantly the relative weights of liver, spleen, and kidneys, compared to DENA group, but were still higher than the normal levels. Treatment with ZSCL1 ameliorated the relative heart weight. Serum albumin level was raised significantly in DENA+ZSCL1 group, compared to DENA group, but was still less than the normal control group (7.14%, \(p<0.0001\)). Treatment with ZSCL1 caused a significant decrease in serum GGT activity, compared to DENA group, but was still more than the normal level by 210.35% (\(p<0.0001\)). No significant alterations in the hepatic oxidant/antioxidant status were observed in the DENA+ZSCL1 group.

The relative weights of liver and heart, liver function test, and the hepatic oxidant/antioxidant balance were normalized after treatment with ZSCL at the high dose (300mg/ Kg b.w.; ZSCL2). Serum GGT activity was significantly decreased in comparison with DENA group, but still significantly higher than the normal level by 93.19% (\(p<0.0001\)).
Administrations of normal rats either with ZSCL1 or ZSCL2 did not cause any alterations in the relative expression of Hgf, Igf-1r, Bcl-2, and Mmp-9 oncogenes (Figure 4). DENA induced hepatocarcinogenesis by upregulating significantly \((p<0.0001)\) the relative expressions of Hgf, Igf-1r, Bcl-2, and Mmp-9. Treatment of DENA-induced hepatocarcinogenesis with ZSCL2 returned the expression of these oncogenes to the normal level. Meanwhile, administration of ZSCL1 after DENA intoxication improved the relative expression of Igf-1r and Mmp-9 oncogenes, compared to DENA group, but the expression levels were still more than the normal. ZSCL1 therapy ameliorated the relative expression of Hgf and Bcl-2 oncogenes.

4. Discussion

Genomic studies reported that phytochemicals can modulate the expression of a broad range of tumor suppressor genes and/or oncogenes to stop cancer development (Niedzwiecki et al. 2016). The current study revealed the presence of high amount of polyphenolic flavones in the crude methanolic extract of ZSCL, which agreed with the results of Elaloui et al. (2017). GC-MS reported that phytol, luteolin, and quercetin 7,3',4'-trimethoxy are the abundant phytochemical compounds in ZSCL methanolic extract. Furthermore, ZSCL extract contained palmitic and oleic fatty acids and their esters. These data are in parallel with the study of Asgarpanah (2012). ZSCL crude methanolic extract exerted strong free radical and peroxide scavenging activities which agree with Setorki (2016) and Alnahdi et al. (2017) reports.

The crude methanolic extract ZSCL possessed a strong cytotoxic effect against HepG2 cell line with a high selectivity index due to the known anticancer properties of ZSCL ingredient; phytols (Sheeja et al. 2016). Flavonoids may inhibit cancer progression through interfering with invasion, metastasis, angiogenesis, apoptotic mechanisms and cell cycle arrest (George et al. 2017). The current research revealed that i.p. administration of ZSCL to normal rats once at increasing doses up to 2880 mg/ Kg b.w. did not cause any mortality. So, we chose using two different doses; 100 and 300 mg/ Kg b.w. (ZSCL1 and ZSCL2, respectively) to evaluate their \textit{in vivo} antitumor activity. The
histological examination of liver in ZSCL1 & 2-administered rats showed normal liver histological findings as well as relative weights of liver, kidneys, and heart. But the relative spleen weights were significantly increased, which need further investigations. In addition, administrations of ZSCL1 and ZSCL2 to normal rats resulted in normal function tests of liver & kidney and balanced hepatic oxidants/antioxidants status. These results are in line with the previous studies of AL-Marzooq (2014) and Almeer et al. (2018).

Intoxication of rats with DENA induces well-differentiated HCC similar to that in human (Di Stefano et al. 2005). In the present work, DENA caused HCC with trabecular pattern and papillary cystic cholangioma in the lining epithelium of the bile ducts with an increase in the relative weight of liver, alterations in liver function tests, and oxidant/antioxidant balance. These data are paralleled with the findings of Du et al. (2009) and Govindaraj and Perumal (2017). DENA is converted to highly reactive molecule by cytochrome P450 (CYP450) dependent oxygenases generating reactive oxygen species (ROS) resulting in oxidative stress and DNA alkylation that induces hepatic chromosomal aberration, micronuclei and chromatid exchanges responsible for liver cancer development (Mandal et al. 2008).

Treatment of hepatocarcinogenesis-induced rats with ZSCL1 improved the hepatic alterations induced by DENA but did not return the liver to its normal status, since a focal area of dysplastic hepatocytes with ballooning degeneration was noticed in association with papillary cystic cholangioma in the bile ducts. On the other hand, no hepatocellular carcinoma could be seen in DENA+ZSCL2 group but the cystic cholangioma could not be treated. Treatments either with ZSCL1 or 2 normalized serum liver and kidney function tests and maintained the balance between oxidants and antioxidants. Both therapeutic modalities improved serum GGT activity. Hafiz and Mubaraki (2016) reported that treatment of *Plasmodium berghei*-induced liver and spleen injury with ZSCL extract at a dose of 300mg/ Kg b.w. for 9 days improved the histological picture of liver and spleen *via* normalizing the balance between oxidants and antioxidants.
There are many cell signaling pathways involved in liver cancer, including HGF/c-Met (Goyal et al. 2013), IGF/IGFR (Sprinzl et al. 2015), Bcl-2-mediating cell death (Guo et al. 2002), MMP-9 (Hidalgo and Eckhardt 2001). HGF is one of the most potent growth factors for hepatocytes since it has vital roles in proliferation, migration, cell survival, morphogenesis, angiogenesis, and tissue regeneration (Efimova et al. 2004). HCC has been shown to release tumor cell products to stimulate stellate cells and myofibroblasts to secrete HGF. The increased HGF from these cells in turn enhances the invasiveness of tumor cells (Guirouilh et al. 2001). IGF-1R is a transmembrane tyrosine kinase receptor expressed ubiquitously. Upon IGF-1R activation, differentiation or increased cell proliferation and migration occur (Enguita-Germán and Fortes 2014). The tumorigenic role of IGF-1R signaling pathway is documented by promoting proliferation, protecting from apoptosis, and potentiating cell migration (Baserga 1995) making it one of the hallmarks of HCC (Aleem et al. 2012). MMPs are transmembrane proteins that digest the extracellular matrix and basement membrane (Ordoñez et al. 2014). Expression of MMP-9 in HCC has been closely associated with tumor invasion (Libra et al. 2009 and Chen et al. 2012). Moreover, Apoptosis is tightly associated with the efficiency of cancer therapy (Ferreira et al. 2002). It has been reported that Bcl-2 plays an important role as antiapoptosis (Marquez and Xu 2012). Targeting reduction of Bcl-2 oncogene expression can significantly promote tumor cell death (Kang and Reynolds 2009).

In the current study, DENA upregulated significantly the expressions of Hgf, Igf-1r, Mmp-9 and Bcl-2 oncogenes which agreed with the results of Okubo et al. (2002) and Mitchell et al. (2016). Hepatocarcinogenesis process involves genetic and epigenetic alterations of various oncogenes. DNA methylation, an epigenetic process, controls gene transcription, histone acetylation, and chromosomal stability and therefore affecting differentiation, genomic imprinting, DNA mutation, and DNA repair (Tsujiiuchi et al. 1999, Zuo 2005, and Beer et al. 2008). Hypomethylation of the oncogenes at CpG island due to DENA carcinogen leads to overexpression of oncogenes in hepatocarcinogenesis (Du et al. 2009).
Treatment of DENA-intoxicated rats with ZSCL2 ameliorated the relative expression of the studied genes. The high hepatotherapeutic activity of ZSCL2 against hepatocellular carcinoma could be attributed to its high contents of phytols and polyphenolic flavons “luteolin and quercetin” with their known anticancer and antioxidant activities. The molecular mechanisms by which flavonoids possess their anticancer effects include: induction of apoptosis (Vásquez-Garzón et al. 2013), cell cycle arrest (Lee et al. 2015), inhibition of reactive oxygen species formation (Sun et al. 1998), inhibition of vascular endothelial growth factor- and basic fibroblast growth factor-mediated angiogenesis (Fotsis et al. 1997 and Schindler 2006), decreasing proliferation and removing the tumor-initiating cells (Carrasco-Torres et al. 2017), and having antiangiogenic effects by regulating the expression of MMPs (Mojzis et al. 2008).

5. Conclusion

Results obtained from the present investigation report that *Ziziphus spina-chresti* leaves methanolic extract at a dose of 300mg/ Kg b.w. possesses an excellent therapeutic activity against diethylnitrosamine-induced hepatocellular carcinoma through ameliorating oxidants/antioxidants status and the expression of oncogenes.

Acknowledgment

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6. References


Table 1: Primers list for qPCR

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<td></td>
<td>4290</td>
<td>4270</td>
<td>52.38</td>
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<td>3</td>
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<tr>
<td>BCl-2</td>
<td>AC_000081.1</td>
<td>Forward: 5’-TGGGATACCTGGAGATGAAGACT</td>
<td>76</td>
<td>88</td>
<td>109</td>
<td>45.45</td>
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<td>3</td>
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<tr>
<td></td>
<td></td>
<td>Reverse: 5’-TCTCAGGTGGAAAGAGAT</td>
<td></td>
<td>163</td>
<td>142</td>
<td>50</td>
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<tr>
<td>MMP-9</td>
<td>AC_000071.1</td>
<td>Forward: 5’-ATAAACACGGATCCCCCAACC</td>
<td>92</td>
<td>2301</td>
<td>2321</td>
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<td>6</td>
<td>0</td>
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<tr>
<td></td>
<td></td>
<td>Reverse: 5’-CTCTGACCAACCACAGGCTT</td>
<td></td>
<td>2392</td>
<td>2372</td>
<td>52.9</td>
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<td>2</td>
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</table>
Table 2. Contents of total flavonoids, phenolics, *in vitro* free radicals and peroxide scavenging activities of ZSCL methanolic extract

<table>
<thead>
<tr>
<th></th>
<th>ZSCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Flavonoids (Rutin mE/g extract)</td>
<td>710±28.5</td>
</tr>
<tr>
<td>Total Phenolics Content (Gallic acid mE /g extract)</td>
<td>500±10.5</td>
</tr>
<tr>
<td>Free Radicals Scavenging IC$_{50}$ (µg/ml)$^a$</td>
<td>57.8±2.3</td>
</tr>
<tr>
<td>Peroxide Scavenging Activity (%)$^a$</td>
<td>90±13.3</td>
</tr>
</tbody>
</table>

*The data are expressed as means ± SD.*

*mE = mg equivalent.*

*$^a$: The activity is expressed as equivalent of trolox (µg trolox/g extract).*
<table>
<thead>
<tr>
<th>Rt</th>
<th>Formula</th>
<th>Compounds</th>
<th>Area [%]*</th>
<th>M.Wt</th>
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</thead>
<tbody>
<tr>
<td>7.71</td>
<td>C₁₀H₁₈O₂</td>
<td>11-Octadecenal (CAS)</td>
<td>2.16</td>
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<tr>
<td>8.98</td>
<td>C₁₂H₂₄O₁₁</td>
<td>á-D-Glucopyranose,4-O-á-D-galactopyranosyl</td>
<td>0.22</td>
<td>342</td>
</tr>
<tr>
<td>9.02</td>
<td>C₁₄H₂₄O₃Si₂</td>
<td>Benzenacetic acid, 3-[(trimethylsilyl)oxy], trimethylsilyl ester</td>
<td>0.36</td>
<td>296</td>
</tr>
<tr>
<td>11.03</td>
<td>C₂₀H₃₂O₄Si</td>
<td>2-Trimethylsiloxy-6-hexadecenoic acid, methyl ester</td>
<td>0.75</td>
<td>356</td>
</tr>
<tr>
<td>13.47</td>
<td>C₁₃H₂₁O₇</td>
<td>Quercetin 7,3',4'-Trimethoxy “4H-1-Benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy-(CAS)”</td>
<td>0.7</td>
<td>344</td>
</tr>
<tr>
<td>15.10</td>
<td>C₁₈H₃₂O₂</td>
<td>9-Octadecenoic acid (Z)-(CAS) “Oleic Acid”</td>
<td>0.32</td>
<td>282</td>
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<tr>
<td>15.25</td>
<td>C₂₀H₄₀O₃Si₂</td>
<td>2-Pentadecanone, 6,10,14-trimethyl-(CAS)</td>
<td>0.93</td>
<td>268</td>
</tr>
<tr>
<td>16.25</td>
<td>C₂₁H₄₂O₅Si₂</td>
<td>9,12,15-Octadecatrienoic acid “alpha-Linolenic acid; σ3”</td>
<td>0.78</td>
<td>496</td>
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<td>16.54</td>
<td>C₂₀H₃₂O₄</td>
<td>2-Hexadecene-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (CAS) “Phytol”</td>
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<td>16.68</td>
<td>C₁₇H₃₄O₂</td>
<td>Hexadecanoic acid, methyl ester</td>
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<td>17.69</td>
<td>C₁₆H₃₂O₃</td>
<td>n-Hexadecanoic acid (CAS) “Palmitic Acid”</td>
<td>24.66</td>
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<tr>
<td>19.02</td>
<td>C₁₈H₃₆O₂</td>
<td>Quercetin 7,3',4'-Trimethoxy “4H-1-Benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy-(CAS)”</td>
<td>0.58</td>
<td>344</td>
</tr>
<tr>
<td>19.70</td>
<td>C₁₈H₃₂O₂</td>
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<td>20.15</td>
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<td>Octadecanoic acid, methyl ester</td>
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<tr>
<td>20.66</td>
<td>C₁₇H₃₄O₂</td>
<td>Hexadecadienoic acid, methyl ester “Palmitoleic acid, methyl ester”</td>
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<td>20.80</td>
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<td>21.1</td>
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<td>Quercetin 7,3',4'-Trimethoxy</td>
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<td>24.09</td>
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<td>Quercitin 7,3',4'-Trimethoxy “4H-1-Benzopyran-4-one,2-(3,4-dimethoxyphenyl)-5,7-dihydroxy-(CAS)”</td>
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<td>26.82</td>
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<td>1,2-Benzenedicarboxylic acid, diisooctyl ester</td>
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<tr>
<td>29.39</td>
<td>C₂₇H₅₄O₁₆</td>
<td>4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy-(CAS) “Luteolin”</td>
<td>0.51</td>
<td>610</td>
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<td>29.47</td>
<td>C₂₇H₅₆O₁₆</td>
<td>4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy-(CAS) “Luteolin”</td>
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<td>29.67</td>
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<td>29.83</td>
<td>C₂₆H₄₂O₄</td>
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<td>29.97</td>
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<td>30.53</td>
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<td>0.54</td>
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<tr>
<td>30.70</td>
<td>C₂₇H₅₆O₁₆</td>
<td>4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy-(CAS) “Luteolin”</td>
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<tr>
<td>30.89</td>
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<td>0.31</td>
<td>610</td>
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<tr>
<td>31.30</td>
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<td>4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy-(CAS) “Luteolin”</td>
<td>0.84</td>
<td>610</td>
</tr>
<tr>
<td>31.76</td>
<td>C₁₇H₃₂O</td>
<td>1-Hexadecanol, 2-methyl</td>
<td>2.86</td>
<td>256</td>
</tr>
<tr>
<td>34.77</td>
<td>C₂₇H₅₄O₁₆</td>
<td>4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy-(CAS) “Luteolin”</td>
<td>0.58</td>
<td>610</td>
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</tbody>
</table>
Table 4. Relative organs weights, liver function tests and oxidative stress markers in the tested groups

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>ZSCL1</th>
<th>ZSCL2</th>
<th>DENA</th>
<th>DENA+ZSCL1</th>
<th>DENA+ZSCL2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (g/100 gm)</td>
<td>2.84±0.48a</td>
<td>2.79±0.38a</td>
<td>2.73±0.44a</td>
<td>4.22±1.00b</td>
<td>3.44±0.30c</td>
<td>2.91±0.42ac</td>
</tr>
<tr>
<td>Spleen (g/100 gm)</td>
<td>0.26±0.07a</td>
<td>0.5±0.11b</td>
<td>0.54±0.13c</td>
<td>0.68±0.25b</td>
<td>0.42±0.07b</td>
<td>0.44±0.12b</td>
</tr>
<tr>
<td>Kidneys (g/100 gm)</td>
<td>0.57±0.05a</td>
<td>0.56±0.06a</td>
<td>0.52±0.06a</td>
<td>0.71±0.05b</td>
<td>0.68±0.04b</td>
<td>0.6±0.01c</td>
</tr>
<tr>
<td>Heart (g/100 gm)</td>
<td>0.34±0.03a</td>
<td>0.34±0.03a</td>
<td>0.36±0.05a</td>
<td>0.41±0.06b</td>
<td>0.35±0.05a</td>
<td>0.38±0.04ab</td>
</tr>
<tr>
<td>Albumin (g%)</td>
<td>3.64±0.27a</td>
<td>3.67±0.34a</td>
<td>3.81±0.28a</td>
<td>2.55±0.11b</td>
<td>3.38±0.24c</td>
<td>3.89±0.19a</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>11.63±1.85a</td>
<td>7.02±1.66a</td>
<td>15.75±2.31a</td>
<td>31.21±9.23b</td>
<td>13.94±6.40a</td>
<td>16.50±3.38a</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>3.67±0.76a</td>
<td>3.19±0.88a</td>
<td>3.36±1.00a</td>
<td>33.99±6.14b</td>
<td>11.39±3.39c</td>
<td>7.09±0.67d</td>
</tr>
<tr>
<td>MDA (nmol/ mg protein)</td>
<td>0.45±0.04a</td>
<td>0.45±0.06a</td>
<td>0.45±0.05a</td>
<td>4.30±0.25b</td>
<td>0.46±0.05a</td>
<td>0.43±0.05a</td>
</tr>
<tr>
<td>TAC (nmol/ mg protein)</td>
<td>96.18±8.93a</td>
<td>90.27±5.96a</td>
<td>92.53±11.67a</td>
<td>52.26±6.29b</td>
<td>93.31±10.23a</td>
<td>91.29±4.86a</td>
</tr>
<tr>
<td>Creatinine (mg%)</td>
<td>0.36±0.07a</td>
<td>0.38±0.05a</td>
<td>0.36±0.06a</td>
<td>0.47±0.05b</td>
<td>0.33±0.03a</td>
<td>0.36±0.05a</td>
</tr>
</tbody>
</table>

- Results are Mean±SD of 10 values.
- NC = normal control, ZSCL1 = Ziziphus spina–christi leaves dose 1 (100mg/ Kg b.w.), ZSCL2 = Ziziphus spina–christi leaves dose 2 (300mg/ Kg b.w.), DENA = diethylnitrosamine-induced hepatocarcinogenesis, DENA+ZSCL1 = treatment of DENA with ZSCL1, and DENA+ZSCL2 = treatment of DENA with ZSCL2.
- Relative Organ Weight = (Organ Weight/ Body Weight) x 100
- Similar characters Denote insignificance between groups.
- The mean difference is significant at p<0.05.
Figure 1. Gas chromatography-mass spectrometry (GC-MS) chromatogram of ZSCL methanol extract. Peaks assignment was listed in Table 2.
Figure 2. Cytotoxic activities of ZSCL methanolic extract against HBF4 (A) and HepG2 (B) cell lines. A: The methanolic extract of ZSCL showed IC$_{50}$ value of 426 µg/ml against HBF4. B: The same extract produced cytotoxic activity against HepG2 with IC$_{50}$ of 94.5 µg/ml and SI value of 4.51. Selectivity index (SI) = IC$_{50}$ against HBF4/IC$_{50}$ against HepG2.
Figure 3. Photomicrographs of liver sections stained with hematoxylin and eosin staining (H&E, 400x). Normal control (A), *Ziziphus spina−christi* leaves extract “ZSCL1”, 100 mg/ Kg b.w. (B), *Ziziphus spina−christi* leaves extract “ZSCL2”, 300 mg/ Kg b.w. (C), Diethylnitrosamine “DENA”-induced hepatocarcinogenesis (D), DENA+ZSCL1 (E), and DENA+ZSCL2 (F). Normal control rats as well as rats administered ZSCL at 100 and 300 mg/ Kg b.w. showed normal histological structure of the central vein (CV) with normal radiating hepatocytes (H) and average intervening blood sinusoids (S). DENA caused hepatocellular carcinoma (HCC) characterized by mildly pleomorphic hepatocytes (P) arranged in trabecular (T) and sinusoidal patterns (S) with the presence of areas of necrosis (N) (D-1). In addition, DENA produced bile duct cystification (BC) with papillary formation covered by single cell layer (cholangioma, D-2). D-3 illustrated nodular formation surrounded completely by fibrous tissue (F). In DENA+ZSCL1, hydropic degeneration of hepatocytes (D) with mild dysplastic changes could be seen (E-1). Furthermore, bile duct cystification with flat epithelial lining was observed (E-2). Dilated congested central vein was detected in DENA+ZSCL2 group with no evidence for HCC (F-1). Furthermore, dilated congested portal vein (PV) with inflammatory cell infiltration in association with a proliferation in the lining epithelium of bile ducts was noticed forming cystic cholangioma (F-2).
Figure 4. Relative gene expression of hepatic oncogenes. Hgf (A), Igf-1r (B), Bcl-2 (C), and Mmp-9 (D) in all the studied groups. The data of the qPCR were normalized by GAPDH as a housekeeping gene. Data are presented as Mean ± SD of 6 values/group. * and # denote significance at $p<0.0001$. The mean difference is significant at $p<0.05$. 