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

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11 Abstract

12 The present study aimed to evaluate the antitumor activity of *Ziziphus spina-christi* leaves
 13 extract (ZSCL) against diethylnitrosamine (DENA)-induced hepatocarcinogenesis in rats. The
 14 phytochemical constituents, in vitro antioxidant and cytotoxic activities of ZSCL extract were
 15 investigated. Male Wistar rats were divided into 6 groups: normal control, ZSCL1-administered rats
 16 (100 mg/ Kg body weight “b.w.”), ZSCL2-administered rats (300 mg/ Kg b.w.), DENA-induced
 17 hepatocarcinogenesis, hepatocarcinogenesis-bearing rats treated either with ZSCL1 or ZSCL2.
 18 Serum liver function & oxidative stress tests were assayed. The expression of hepatocyte growth
 19 factor, insulin-like growth factor-1 receptor, B cell lymphoma-2, and matrix metalloproteinase-9
 20 oncogenes were quantified in liver. Histological examination of liver tissues was performed. ZSCL
 21 methanolic extract was rich in essential fatty acids, phytol, and polyphenolic flavones (luteolin and
 22 quercetin) with strong free radical & peroxide scavenging activities and cytotoxic activity.
 23 Administration of ZSCL1 and ZSCL2 to rats did not alter the studied parameters. DENA induced
 24 hepatocellular carcinoma and cholangioma by producing oxidative stress and upregulating liver
 25 oncogenes expression. Treatment of DENA-induced hepatocarcinogenesis with ZSCL2 ameliorated
 26 all the abnormalities induced by DENA except for cholangioma. In conclusion, ZSCL methanolic
 27 extract (300mg/ Kg b.w.) possessed a strong therapeutic activity against DENA-induced
 28 hepatocellular carcinoma via targeting oxidative stress and oncogenes.

29 Key Words

30 *Ziziphus spina-christi*; Leaves; Hepatocarcinogenesis; Oxidative Stress; Oncogenes

31 1-Introduction

32 Diethylnitrosamine (DENA) is a strong environmental carcinogen; present in the food chain
33 (Mittal et al. 2006). The source of DENA could be endogenously or in occupational settings, tobacco
34 smoke, processed meat, alcoholic beverages, agricultural chemicals, cosmetics, and pharmaceutical
35 substances. Furthermore, metabolism of certain drugs can produce DENA (Sivaramakrishnan et al.
36 2008). In Egypt, hepatocellular carcinoma (HCC) contributes to 14.8% of all cancer mortality and is
37 considered the second most common cancer type in Egyptian males after bladder cancer and the
38 eighth most frequent in Egyptian females (Rashad et al. 2014).

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

46 *Ziziphus spina-christi* (L.) Desf. (christ 's thorn Jujube, Rhamnaceae) is a tropical evergreen
47 tree producing small, orange-yellow fruits. It is commonly called Nabk and Sidr and widespread in
48 the Middle Eastern countries (Michel et al. 2011). Phytochemical investigations reported that
49 *Ziziphus spina-chrest*i contains tannins, essential oils, phytosterols, flavonoids, saponins, triterpenoid
50 sapogenins, and alkaloids (Kadioglu et al. 2016). Previous studies have reported the cytotoxic effects
51 of *Z. spina-Christi* crude leaves (ZSCL) extract against numerous cancer cell lines (Jafarian et al.
52 2014 and Farmani et al. 2016). ZSCL is known with its antiinflammatory, antimicrobial,
53 hypoglycemic, immunostimulatory, and hepatoprotective activities (Michel et al. 2011, Kadioglu et
54 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 Mubarak (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

79 quadrupole mass spectrometry, equipped with DB5-MS, 30m x 0.25 mm ID column (J&W
80 Scientific). ZSCL sample was dissolved in absolute methanol (Sigma, USA) to obtain a
81 concentration of 100µg/ml, filtered using a membrane discfilter (0.2 µm), and then subjected to GC-
82 MS analysis. Oven temperature was programmed from 50 °C (1 min) to 150 °C (2 min) at 10°C/min.
83 - 250°C (1 min) at 5°C/min.-270 °C (2 min) at 3.5°C/min. Ionization source EI with 70ev voltage.
84 The carrier gas was helium at a flow of 1.5 ml/min. The temperatures of the detector and injector
85 were adjusted at 300 and 250°C, respectively. The individual GC peaks were identified by mass
86 spectra using a computer search of the commercial libraries (WILEY & NIST mass spectral data).

87 2.4. *In vitro* Antioxidant Activity of ZSCL

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

91 2.5. *In vitro* Cytotoxic Activity of ZSCL

92 The cytotoxic effect of ZSCL was performed according to the methods of Mosmann (1983)
93 Elaasser and Abdel-aziz (2011). The assay was carried out on normal melanocyte cell line (HBF4)
94 and well differentiated hepatocellular cancer cell line (HepG2) that were kindly provided by
95 Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. The cell lines
96 were suspended in Roswell Park Memorial Institute-1640 (RPMI-1640) medium at concentration of
97 5×10^4 cell/well in Corning® 96-well tissue culture plates; where RPMI-1640 were supplemented
98 with 10% inactivated fetal calf serum and 50µg/ml gentamycin. The cells were then incubated for 24
99 h and ZSCL extract was added at concentration range of 15.6-500 µg/ml. Six vehicle controls with
100 media or 0.5% dimethyl sulfoxide were run for each 96 well plate as a control. After incubating for
101 24 h, the numbers of viable cells were determined by 3-(4,5-Dimethylthiazol-2-yl)-2, 5-
102 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 ≤ 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 $24 \pm 2^\circ\text{C}$ 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

128 6 consecutive weeks, Group IV (DENA, negative control), hepatocarcinogenesis was induced in rats
129 by daily oral administration of DENA (Sigma-Aldrich, USA), at a dose of 100µl (contained 100 mg
130 DENA)/ one liter drinking water stored in dark bottle for three consecutive months followed by a
131 period of 5 weeks of tumor growth in which rats were left without any administrations (Yang et al.
132 2004), Group V (DENA+ZSCL1), hepatocarcinogenesis-bearing rats were treated i.p. with ZSCL1
133 thrice weekly for 6 consecutive weeks, Group VI (DENA+ZSCL2), hepatocarcinogenesis-bearing
134 rats were treated i.p. with ZSCL2 thrice weekly for 6 consecutive weeks.

135 2.9. Blood Sampling and Collection of Body Organs

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

145 2.10. Histological Examination

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

149 2.11. Calculation of Organs Relative Weights

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

152 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 Na₂HPO₄, and 1.8 mM KH₂PO₄), 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 4°C 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 (PhotoBiomater, Eppendorf, Germany). 2µg of RNA was reverse transcribed into single-stranded complementary DNA (cDNA)

177 using QuantiTect reverse transcription kit (Qiagen, Germany). cDNA synthesis was performed using
178 Gene Amp PCR System 9700, Applied Biosystems by life technologies, USA.

179 Quantitative real time PCR was performed using SYBR green master mix (Qiagen, Germany)
180 to determine the relative expressions of the liver Hgf, Igf-1r, Bcl-2, and Mmp-9 cDNA.
181 Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for
182 normalizing mRNA expression for genes of interest. qPCR was performed in an optical 96-well plate
183 with an ABI PRISM 7500 fast sequence detection system (Applied Biosystems, Carlsbad, California)
184 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).
185 The used primers (Invitrogen, Belgium) for qPCR are listed in Table 1. Gene expression was
186 expressed in relative units ($RQ = 2^{-\Delta\Delta CT}$).

187 2.17. Statistical Analysis

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

193 3. Results

194 The present study reported that the crude methanolic extract of ZSCL is abundant, measured
195 as milliequivalent “mE”, in flavonoids (710 mE rutin/ g) and phenolics (500 mE gallic acid/g)
196 compounds (Table 2). ZSCL extract possessed a strong antioxidant activity by having potent
197 scavenging activities of α , $\bar{\alpha}$ -diphenyl- β -picrylhydrazyl (DPPH•) free radical (IC_{50} of 57 μ g/ml) and
198 hydrogen peroxide (90%). GC-MS chromatogram of ZSCL methanolic extract indicated the presence
199 of phytol, palmitic acid, oleic acid, and palmitoleic acid methyl ester as major constituents in
200 association with the presence of high content of polyphenolic flavones in the form of luteolin as well

201 as quercetin 7,3',4'-trimethoxy (Figure 1 and Table 3). Figure 2 reported that ZSCL exerts strongly
202 cytotoxic effect against HepG2 cancer cell line ($IC_{50} = 94.5 \mu\text{g/ml}$) while gave IC_{50} value of 426
203 $\mu\text{g/ml}$ against normal HBF4 cell line with a high SI of 4.51.

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

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

219 Treatment of DENA-induced hepatocarcinogenesis with ZSCL at 100 mg/ Kg b.w. displayed
220 somehow less toxic effects in liver tissue evidenced by the presence of a focal area of dysplastic
221 hepatocytes with ballooning degeneration in hepatocytes (Figure 3, E-1). There was a focal area of
222 papillary cystic cholangioma in the bile ducts with flattened lining epithelium and basal nuclei
223 (Figure 3, E-2). Surprisingly, treatment of DENA-induced hepatocarcinogenesis with the high dose
224 of ZSCL (300 mg/ Kg b.w.) for 6 weeks displayed a high therapeutic activity revealed by the
225 reversal of HCC. Congestion in the central and portal veins associated with inflammatory cells

226 infiltration in the portal area were detected (Figure 3, F-21&F-2). In addition, cellular proliferation
227 was observed in the bile ducts forming cystic cholangioma (Figure 3, F-2).

228 Administrations of normal rats either with ZSCL1 or ZSCL2 for 6 weeks showed normal
229 relative weights of liver, heart, and kidney as well as liver function tests, liver oxidative stress
230 markers, and serum creatinine concentration (Table 4). However, ZSCL1 and ZSCL2 increased
231 significantly the relative weight of spleen (92.31 and 107.69%, respectively, $p<0.0001$), compared to
232 the normal control group. Administration of DENA caused a significant ($p<0.0001$) elevation in the
233 relative weights of liver (48.59%), spleen (161.54%), kidneys (24.56%), and heart (20.59%),
234 compared to the normal control rats. Also, DENA elevated significantly ($p<0.0001$) serum activities
235 of ALT (87.67%) and GGT (826.16%) in association with a significant reduction in serum albumin
236 concentration (29.95%, $p<0.0001$), compared to the normal control group. DENA induced imbalance
237 in oxidant/antioxidant status in the form of a significant rise in hepatic MDA content (825.56%,
238 $p<0.0001$) and a significant reduction in the hepatic TAC (45.66%, $p<0.0001$).

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

247 The relative weights of liver and heart, liver function test, and the hepatic oxidant/antioxidant
248 balance were normalized after treatment with ZSCL at the high dose (300mg/ Kg b.w.; ZSCL2).
249 Serum GGT activity was significantly decreased in comparison with DENA group, but still
250 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 2880mg/ 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 *in vivo* antitumor activity. The

276 histological examination of liver in ZSCL1 & 2-administered rats showed normal liver histological
277 findings as well as relative weights of liver, kidneys, and heart. But the relative spleen weights were
278 significantly increased, which need further investigations. In addition, administrations of ZSCL1 and
279 ZSCL2 to normal rats resulted in normal function tests of liver & kidney and balanced hepatic
280 oxidants/antioxidants status. These results are in line with the previous studies of AL-Marzooq
281 (2014) and Almeer et al. (2018).

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

291 Treatment of hepatocarcinogenesis-induced rats with ZSCL1 improved the hepatic alterations
292 induced by DENA but did not return the liver to its normal status, since a focal area of dysplastic
293 hepatocytes with ballooning degeneration was noticed in association with papillary cystic
294 cholangioma in the bile ducts. On the other hand, no hepatocellular carcinoma could be seen in
295 DENA+ZSCL2 group but the cystic cholangioma could not be treated. Treatments either with
296 ZSCL1 or 2 normalized serum liver and kidney function tests and maintained the balance between
297 oxidants and antioxidants. Both therapeutic modalities improved serum GGT activity. Hafiz and
298 Mubarak (2016) reported that treatment of *Plasmodium berghei*-induced liver and spleen injury with
299 ZSCL extract at a dose of 300mg/ Kg b.w. for 9 days improved the histological picture of liver and
300 spleen *via* normalizing the balance between oxidants and antioxidants.

301 There are many cell signaling pathways involved in liver cancer, including HGF/c-Met
 302 (Goyal et al. 2013), IGF/IGFR (Sprinzl et al. 2015), BCL-2-mediating cell death (Guo et al. 2002),
 303 MMP-9 (Hidalgo and Eckhardt 2001). HGF is one of the most potent growth factors for hepatocytes
 304 since it has vital roles in proliferation, migration, cell survival, morphogenesis, angiogenesis, and
 305 tissue regeneration (Efimova et al. 2004). HCC has been shown to release tumor cell products to
 306 stimulate stellate cells and myofibroblasts to secrete HGF. The increased HGF from these cells in
 307 turn enhances the invasiveness of tumor cells (Guirouilh et al. 2001). IGF-1R is a transmembrane
 308 tyrosine kinase receptor expressed ubiquitously. Upon IGF-1R activation, differentiation or increased
 309 cell proliferation and migration occur (Enguita-Germán and Fortes 2014). The tumorigenic role of
 310 IGF-1R signaling pathway is documented by promoting proliferation, protecting from apoptosis, and
 311 potentiating cell migration (Baserga 1995) making it one of the hallmarks of HCC (Aleem et al.
 312 2012). MMPs are transmembrane proteins that digest the extracellular matrix and basement
 313 membrane (Ordoñez et al. 2014). Expression of MMP-9 in HCC has been closely associated with
 314 tumor invasion (Libra et al. 2009 and Chen et al. 2012). Moreover, Apoptosis is tightly associated
 315 with the efficiency of cancer therapy (Ferreira et al. 2002). It has been reported that BCL-2 plays an
 316 important role as antiapoptosis (Marquez and Xu 2012). Targeting reduction of BCL-2 oncogene
 317 expression can significantly promote tumor cell death (Kang and Reynolds 2009).

318 In the current study, DENA upregulated significantly the expressions of Hgf, Igf-1r, Mmp-9
 319 and Bcl-2 oncogenes which agreed with the results of Okubo et al. (2002) and Mitchell et al. (2016).
 320 Hepatocarcinogenesis process involves genetic and epigenetic alterations of various oncogenes.
 321 DNA methylation, an epigenetic process, controls gene transcription, histone acetylation, and
 322 chromosomal stability and therefore affecting differentiation, genomic imprinting, DNA mutation,
 323 and DNA repair (Tsujiuchi et al. 1999, Zuo 2005, and Beer et al. 2008). Hypomethylation of the
 324 oncogenes at CpG island due to DENA carcinogen leads to overexpression of oncogenes in
 325 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-chrestis* 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.

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523 Table 1: Primers list for qPCR

Gene	Primer sequence	Product	Start	Stop	GC%	Self	Self-3'
Accession No.		Length				Complementary	Complementary
GAPDH	Forward: 5'- TGTGAACGGATTTGGCCGTA		90	109	50	4	2
NM_017008.4	Reverse: 5'-ACCAGCTTCCCATTCTCAGC	183	272	253	55	4	2
HGF	Forward: 5'-ACAGCTTTTTGCCTTCGAGC		624	643	50	4	2
NC_005103.4	Reverse: 5'- TAGCTTTCACCGTTGCAGGT	178	801	782	50	4	2
IGF-1R	Forward: 5'- GACCTGCTGATCCTCGACCC		4149	4168	65	4	0
NM_052807.2	Reverse: 5'- TCCACTGAGATACAGGAGGCC	142	4290	4270	52.38	3	3
BCI-2	Forward: 5'-TGGGATACTGGAGATGAAGACT		88	109	45.45	3	3
AC_000081.1	Reverse: 5'-TCTCAGGCTGGAAGGAGAAGAT	76	163	142	50	5	2
MMP-9	Forward: 5'-ATAAACACGGATCCCCCAACC		2301	2321	52.38	6	0
AC_000071.1	Reverse: 5'- CTTCTGACCAACCACAGGCTT	92	2392	2372	52.9	3	2

524

Draft

525 Table 2. Contents of total flavonoids, phenolics, *in vitro*
526 free radicals and peroxide scavenging activities of ZSCL
527 methanolic extract

	ZSCL
Total Flavonoids (Rutin mE/g extract)	710±28.5
Total Phenolics Content (Gallic acid mE /g extract)	500±10.5
Free Radicals Scavenging IC ₅₀ (µg/ ml) ^a	57.8±2.3
Peroxide Scavenging Activity (%) ^a	90±13.3

528 -The data are expressed as means ± SD.
529 -mE = mg equivalent.
530 -^a: The activity is expressed as equivalent of trolox (µg trolox/g extract).

Draft

531 Table 3. GC-MS Report for the major components in ZSCL

Rt	Formula	Compounds	Area [%] ^a	M.Wt
7.71	C ₁₈ H ₂₆ O	11-Octadecenal (CAS)	2.16	266
8.98	C ₁₂ H ₂₂ O ₁₁	α-D-Glucopyranose,4-O-α-D-galactopyranosyl	0.22	342
9.02	C ₁₄ H ₂₄ O ₃ Si ₂	Benzeneacetic acid, 3-[(trimethylsilyl)oxy]-, trimethylsilyl ester	0.36	296
11.03	C ₂₀ H ₄₀ O ₃ Si	2-Trimethylsiloxy-6-hexadecenoic acid, methyl ester	0.75	356
13.47	C ₁₈ H ₁₆ O ₇	Quercetin 7,3',4'-Trimethoxy "4H-1-Benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy- (CAS)"	0.7	344
15.10	C ₁₈ H ₃₄ O ₂	9-Octadecenoic acid (Z)-(CAS) "Oleic Acid"	0.32	282
15.25	C ₁₈ H ₃₆ O	2-Pentadecanone, 6,10,14-trimethyl- (CAS)	0.93	268
16.25	C ₂₇ H ₅₂ O ₄ Si ₂	9,12,15-Octadecatrienoic acid "alpha-Linolenic acid; ω3"	0.78	496
16.54	C₂₀H₄₀O	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (CAS)	5.39	296
		"Phytol"		
16.68	C ₁₇ H ₃₄ O ₂	Hexadecanoic acid, methyl ester	2.01	270
17.69	C₁₆H₃₂O₂	n-Hexadecanoic acid (CAS) "Palmitic Acid"	24.66	256
19.02	C ₁₈ H ₁₆ O ₇	Quercetin 7,3',4'-Trimethoxy "4H-1-Benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy- (CAS)"	0.58	344
19.70	C ₁₈ H ₃₄ O ₂	9-Octadecenoic acid "Oleic Acid"	1.06	282
19.91	C₂₀H₄₀O	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-[R-[R*,R*-(E)]]- (CAS) "Phytol"	16.17	296
20.15	C ₁₉ H ₃₈ O ₂	Octadecanoic acid, methyl ester	0.29	298
20.66	C₁₇H₃₀O₂	Hexadecadienoic acid, methyl ester " Palmitoleic acid, methyl ester"	4.84	266
20.80	C ₁₆ H ₃₀ O ₂	9-Hexadecenoic acid, methyl ester " Palmitoleic acid, methyl ester"	2.47	254
21.1	C₁₈H₃₄O₂	9-Octadecenoic acid (Z)-(CAS) "Oleic Acid; ω9"	11.12	282
21.65	C ₁₈ H ₁₆ O ₇	Quercetin 7,3',4'-Trimethoxy*	0.59	344
22.43	C ₁₈ H ₃₄ O ₂	9-Octadecenoic acid (Z) (CAS) "Oleic Acid; ω9"	1.07	282
24.09	C ₁₈ H ₁₆ O ₇	Quercetin 7,3',4'-Trimethoxy "4H-1-Benzopyran-4-one,2-(3,4-dimethoxyphenyl)-5,7-dihydroxy- (CAS)"	0.39	344
26.82	C ₂₄ H ₃₈ O ₄	1,2-Benzenedicarboxylic acid, dioctyl ester	2.84	390
29.39	C ₂₇ H ₃₀ O ₁₆	4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy- (CAS) "Luteolin"	0.51	610
29.47	C ₂₇ H ₃₀ O ₁₆	4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy- (CAS) "Luteolin"	0.69	610
29.67	C ₂₇ H ₃₀ O ₁₆	4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy- (CAS) "Luteolin"	1.27	610
29.83	C ₂₆ H ₄₂ O ₄	1,2-Benzenedicarboxylic acid, diisononyl ester	1.87	418
29.97	C ₂₇ H ₃₀ O ₁₆	4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy- (CAS) "Luteolin"	0.50	610
30.02	C ₂₇ H ₃₀ O ₁₆	4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy- (CAS) "Luteolin"	0.28	610
30.20	C ₂₆ H ₄₂ O ₄	-1,2Benzenedicarboxylic acid, diisononyl ester	1.4	418
30.31	C ₂₇ H ₃₀ O ₁₆	4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy- (CAS) "Luteolin"	0.92	610
30.45	C ₂₇ H ₃₀ O ₁₆	4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy- (CAS) "Luteolin"	0.85	610
30.53	C ₂₇ H ₃₀ O ₁₆	4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy- (CAS) "Luteolin"	0.54	610
30.70	C ₂₇ H ₃₀ O ₁₆	4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy- (CAS) "Luteolin"	2.71	610
30.89	C ₂₇ H ₃₀ O ₁₆	4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy- (CAS) "Luteolin"	0.2	610
31.01	C ₂₇ H ₃₀ O ₁₆	4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy- (CAS) "Luteolin"	0.31	610
31.30	C ₂₇ H ₃₀ O ₁₆	4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy- (CAS) "Luteolin"	0.84	610
31.76	C ₁₇ H ₃₆ O	1-Hexadecanol, 2-methyl	2.86	256
34.77	C ₂₇ H ₃₀ O ₁₆	4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-6,8-di-α-D-glucopyranosyl-5,7-dihydroxy- (CAS) "Luteolin"	0.58	610

532 -a: Values are expressed as relative area percentage to the total identified components.

533 -The major components are highlighted in bold.

534 Table 4. Relative organs weights, liver function tests and oxidative stress markers in the tested groups

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

535 -Results are Mean±SD of 10 values.
536 -NC= normal control, ZSCL1 = Ziziphus spina-christi leaves dose 1 (100mg/ Kg b.w.), ZSCL2 = Ziziphus spina-christi
537 leaves dose 2 (300mg/ Kg b.w.), DENA = diethylnitrosamine-induced hepatocarcinogenesis, DENA+ZSCL1 = treatment
538 of DENA with ZSCL1, and DENA+ZSCL2 = treatment of DENA with ZSCL2.
539 - Relative Organ Weight = (Organ Weight/ Body Weight) x 100
540 -Similar characters Denote insignificance between groups.
541 -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.

546 Figure 2. Cytotoxic activities of ZSCL methanolic extract against HBF4 (A) and HepG2 (B) cell
547 lines. A: The methanolic extract of ZSCL showed IC₅₀ value of 426 µg/ml against HBF4. B: The
548 same extract produced cytotoxic activity against HepG2 with IC₅₀ of 94.5 µg/ml and SI value of
549 4.51. Selectivity index (SI) = IC₅₀ against HBF4/ IC₅₀ against HepG2.

A

B

C

D-1

D-2

D-3

E-1

E-2

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 $\pm$ SD of 6 values/group. * and # denote significance at $p < 0.0001$. The mean difference is significant at $p < 0.05$.