



**Biochemical Studies on the Antitumor Activity of  
*Ziziphus spina-christi* Extracts on Experimentally-  
Induced Hepatocellular Carcinoma in Rats**

*Submitted By*

**Manar Salah Eldin Abdel Aziz**

(B. Sc. in Biochemistry, 2012)

Submitted for fulfillment of Master's degree in Biochemistry

*Under Supervision of*

**Dr. Ahmed M. Salem**  
Professor of Biochemistry  
Faculty of Science  
Ain Shams University

**Dr. Ahmed Abdel Aziz Sayed**  
Professor of Biochemistry  
Faculty of Science  
Ain Shams University

**Dr. AlShaimaa Mohamed Taha**  
Lecturer of Biochemistry  
Faculty of Science  
Ain Shams University

**2019**

# List of Contents

<b>ABSTRACT</b> .....	1
<b>CHAPTER I: INTRODUCTION</b> .....	2
<b>AIM OF THE WORK</b> .....	5
<b>CHAPTER II: REVIEW OF LITERATURE</b> .....	6
<b>Stages of Carcinogenesis</b> .....	6
<b>Initiation</b> .....	6
<b>Promotion</b> .....	7
<b>Progression</b> .....	8
<b>Genes-Regulated Carcinogenesis</b> .....	8
<b>Proto-oncogenes</b> .....	9
<b>Tumor Suppressor Genes</b> .....	10
<b>DNA Repair Genes</b> .....	10
<b>Epidemiology of Hepatocellular Carcinoma</b> .....	13
<b>Risk Factors Involved in the Mechanisms of HCC</b> .....	14
<b>Environmental Risk Factors</b> .....	15
<b>Host – Related Risk Factors for HCC</b> .....	19
<b>Diethylnitrosamine: A Chemical Carcinogen</b> .....	20
<b>Correlation between Oxidative Stress and Hepatocarcinogenesis</b> .....	23
<b>Role of Hepatocyte Growth Factor/ Cellular-Mesenchymal Epithelial Transition Factor (HGF/c-MET) Pathway in HCC</b> .....	24
<b>Role of Insulin-like Growth Factor I Receptor (IGF-IR) Pathway in HCC Proliferation</b>	27
<b>B-Cell Lymphoma 2 (BCL-2) Role as an Apoptotic Marker in HCC</b> .....	30
<b>The Currently Therapeutic Modalities of HCC</b> .....	32
<b>The Role of Herbal Medicine as Efficient Treatment</b> .....	35
<b>Phenolic Compounds</b> .....	36
<b>Flavonoids Classification and Basic Structure</b> .....	38

Anti-Cancer Activity of Phenolic-Flavones .....	39
Ziziphus spina-christi .....	43
Identification and History .....	43
Religious Value .....	44
Medical Value .....	44
Characterization and Phytochemical Constituents of ZSCL .....	45
<b>CHAPTER III: MATERIALS AND METHODS .....</b>	<b>47</b>
<b>Part I: Plant Studies .....</b>	<b>47</b>
Sample Collection .....	47
Extraction of the active ingredients present in ZSCL .....	47
Analysis of the phytochemical Constituents in ZSCL Extract.....	48
<i>In vitro</i> Studies of ZSCL Extract.....	55
<b>Part II: Evaluating the Therapeutic Effect of <i>Ziziphus spina-christi</i> Leaves Extract against Diethylnitrosamine-Induced Hepatocellular Carcinoma in Rats.....</b>	<b>63</b>
Animals .....	63
Preparation of ZSCL Extract, freshly prepared .....	63
Study Design.....	63
Collection of Blood and Some Body Organs.....	65
Toxicity Studies of ZSCL Extract .....	65
Blood Analysis.....	67
Liver Function Tests.....	67
Liver Studies .....	72
Oxidative Stress Markers.....	75
Molecular Studies .....	79
Determination of the relative expression of some genes by quantitative real time polymerase chain reaction (qRT-PCR).....	79
Histological Studies.....	92
Statistical Analysis .....	93
<b>CHAPTER IV: RESULTS .....</b>	<b>95</b>
<b>Part II: <i>In vivo</i> Studies .....</b>	<b>101</b>
<b>CHAPTER V: DISCUSSION .....</b>	<b>132</b>
<b>CONCLUSIONS .....</b>	<b>158</b>
<b>RECOMMENDATIONS .....</b>	<b>159</b>
<b>ENGLISH SUMMARY .....</b>	<b>160</b>

<b>REFERENCES .....</b>	<b>166</b>
<b>APPENDIX.....</b>	<b>224</b>
<b>Ethical Approval .....</b>	<b>224</b>
<b>Published Manuscript.....</b>	<b>225</b>
<b>المستخلص العربي.....</b>	<b>أ</b>
<b>الملخص العربي .....</b>	<b>ب</b>

## **Acknowledgements**

I would first like to thank my thesis advisor *Dr. Ahmed Mohamed Salem*, professor of Biochemistry, Faculty of Science, Ain Shams University.

I would like to express my thanks to *Dr. Ahmed Abdel-Aziz Sayed*, professor of Biochemistry, Faculty of Science, Ain Shams University, for his help and support.

I would like to express my gratitude to my supervisor *Dr. AlShaimaa M.Taha* for the useful comments, remarks and engagement through the learning process of this master thesis. She consistently allowed this paper to be my own work, but steered me in the right direction whenever she thought I needed it.

I would also like to thank *Dr. Sayed Abdel Raheem*, Prof. Ass. of Histopathology at The Faculty of Medicine, Al Azhar university, Cairo, who were involved in the histopathological studies for this research project.

## List of Abbreviations

Abbreviation	Meaning
A	Absorbance
AIH	Autoimmune Hepatitis
AKT	Protein kinase B
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
b.w.	Body weight
BC	Before christ
Bcl-2	B cell lymphoma-2
BSA	Bovine serum albumin
CD44	Cell differentiation molecule 44
CDC42	Cell division control protein 42 homolog
cDNA	Complementary deoxyribonucleic acid
COX	cyclooxygenase
CYP2E1	cytochrome P450
DEAE	Diethyl aminoethyl
DENA	Diethylnitrosamine
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl Sulfoxide
dNTPs	Deoxyribonucleotide triphosphate
DPPH·	free radical, $\alpha$ , $\bar{\alpha}$ -diphenyl- $\beta$ picrylhydrazyl

ds-DNA	Double stranded DNA
<i>E. coli</i>	<i>Escherichia coli</i> (bacteria)
EDTA	Ethylenediamine tetraacetic acid
ERK	Extracellular-signal-regulated kinase
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC-MS	Gas Chromatography- Mass Spectroscopy
GFR	Glomerular filtrate rate
GGT	Gamma Glutamyl transferase
GSH	Glutathione
GST	Glutathione S-transferases
H&E	Hematoxylin & Eosin stain
H <sub>2</sub> O	Water molecule
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HBF4	Normal melanocyte cell line
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HepG2	well-differentiated hepatocellular carcinoma cell line
HGF	Hepatocyte growth factor
HGF/c-MET	Hepatocyte Growth Factor/ Cellular-Mesenchymal Epithelial Transition Factor

IC <sub>50</sub>	The half maximal inhibitory concentration
IGFBPs	IGF high affinity binding proteins
IGF-IR (Igf-1r)	Insulin-like Growth Factor I Receptor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRS	insulin receptor substrate
kDa	Kilo Dalton
LD <sub>50</sub>	Acute Median Lethal Dose
LOX55	Lipoxygenase
MAPK	mitogen-activated protein kinase
MCF-7	Michigan cancer foundation- 7
MDA	Malondialdehyde
mE	milliEquivalent
mM	Millimole
MMP-9	Matrix metalloproteinase -9
MMPs	Matrix metalloproteinases
mTOR	mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor
NADPH	Reduced “Nicotinamide adenine dinucleotide phosphate”
NASH	Nonalcoholic steatohepatitis
NC	Normal control

nm	nanometer
NO	Nitric oxide
OH	Hydroxide group
OS	Oxidative stress
p53	Tumor protein p53
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
PKB	protein kinase B
Px	Peroxidase
qRT- PCR	Quantitative real time- polymerase chain reaction
Raf	Rapidly Accelerated Fibrosarcoma
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal ribonucleic acid
RTK	Receptor tyrosine kinase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
TAC	Total antioxidant capacity
TBA	Thiobarbituric acid

TGF- $\beta$	Transforming growth factor beta
TNF- $\alpha$	Tumor necrosis factor alpha
tRNA	Transfer ribonucleic acid
UDP-GT	UDP-Glucuronosyl transferases
VEGFR1-3	Vascular Endothelial Growth Factor
ZSCL	<i>Ziziphus spina-christi</i> leaves

## List of Tables

<b>Table No.</b>	<b>Heading</b>	<b>Page No.</b>
<b>2.1</b>	Characteristics of proto-oncogenes, cellular oncogenes, and tumor-suppressor genes	<b>9</b>
<b>3.1</b>	Primers list for qPCR	<b>88</b>
<b>4.1</b>	Phytochemical analysis and <i>in vitro</i> antioxidant activity of ZSCL extract	<b>95</b>
<b>4.2</b>	GC-MS Report for the major components in ZSCL	<b>97</b>
<b>4.3</b>	Toxicity studies of ZSCL extract on some relative organs weights as well as serum creatinine level in all the studied groups.	<b>102</b>
<b>4.4</b>	Relative liver weight and liver function tests in all the studied groups	<b>106</b>
<b>4.5</b>	Hepatic oxidative stress markers in all the studied groups	<b>109</b>

## List of Figures

<b>Figure No.</b>	<b>Legend</b>	<b>Page No.</b>
<b>2.1</b>	Multistage model of carcinogenesis	<b>6</b>
<b>2.2</b>	DNA damage and repair mechanisms	<b>12</b>
<b>2.3</b>	Mechanisms of hepatocarcinogenesis	<b>15</b>
<b>2.4</b>	Possible mechanisms of DENA-induced hepatocarcinogenesis	<b>22</b>
<b>2.5</b>	c-MET activation signaling pathways	<b>26</b>
<b>2.6</b>	Simplified schematic diagram of insulin-like growth factor (IGF) signaling	<b>29</b>
<b>2.7</b>	Mechanisms of BCL-2 family deregulation in human cancer	<b>31</b>
<b>2.8</b>	Barcelona clinic liver cancer treatment strategy. Staging is linked to treatment indication according to evidence-based data	<b>33</b>
<b>2.9</b>	Biosynthesis of plant phenolic compounds	<b>37</b>
<b>2.10</b>	Classifications of the plant phenolics, based on their structure	<b>38</b>
<b>2.11</b>	Hypothesis of inhibition of carcinogenesis by flavonoids	<b>42</b>

<b>3.1</b>	Standard curve of gallic acid by Ciocalteau phenol method	<b>51</b>
<b>3.2</b>	Standard curve of rutin	<b>53</b>
<b>3.3</b>	Standard curve of bovine serum albumin by the binding method of Bradford	<b>75</b>
<b>3.4</b>	Melting curves of the studied genes obtained from the real time polymerase chain reaction	<b>90</b>
<b>3.5</b>	Amplification curves of Gapdh (A), Bcl-2 (B), Mmp-9 (C), Igf-1r (D), and Hgf (E) genes obtained from the real time polymerase chain reaction	<b>91</b>
<b>4.1</b>	Gas chromatography-mass spectrometry (GC-MS) chromatogram of ZSCL methanol extract.	<b>96</b>
<b>4.2</b>	Cytotoxic activities ZSCL extract against normal melanocyte cell line (HBF4, A) and a well-differentiated hepatocellular carcinoma cell line (HepG2, B)	<b>99</b>
<b>4.3</b>	Serum level of creatinine in all the studied groups Serum level of creatinine in all the studied groups	<b>103</b>
<b>4.4</b>	Relative spleen, kidneys and heart weights in all the studied groups	<b>104</b>

<b>4.5</b>	Serum albumin level in all the studied groups	<b>107</b>
<b>4.6</b>	Activities of ALT and GGT in sera of all the studied groups	<b>106</b>
<b>4.7</b>	The content of liver MDA in all the studied groups	<b>110</b>
<b>4.8</b>	Liver total antioxidant capacity in all the studied groups	<b>110</b>
<b>4.9</b>	The expression of Hgf oncogene in all studied groups	<b>112</b>
<b>4.10</b>	The expression of Igf-1r oncogene in all studied	<b>114</b>
<b>4.11</b>	The expression of Bcl-2 oncogene in all studied groups	<b>116</b>
<b>4.12</b>	The expression of Mmp-9 oncogene in all studied groups	<b>118</b>
<b>4.13</b>	Photomicrograph of liver section of normal control group stained with hematoxylin and eosin	<b>122</b>
<b>4.14</b>	Photomicrograph of liver section of normal control group stained with hematoxylin and eosin	<b>122</b>
<b>4.15</b>	Photomicrograph of liver section of ZSCL1 group stained with hematoxylin	<b>123</b>

	and eosin	
<b>4.16</b>	Photomicrograph of liver section of ZSCL2 stained with hematoxylin and eosin	<b>123</b>
<b>4.17</b>	Photomicrograph of liver section of DENA group stained with hematoxylin and eosin	<b>124</b>
<b>4.18</b>	Photomicrograph of liver section of DENA group stained with hematoxylin and eosin	<b>125</b>
<b>4.19</b>	Photomicrograph of liver section of DENA group stained with hematoxylin and eosin	<b>125</b>
<b>4.20</b>	Photomicrograph of liver section of DENA group stained with hematoxylin and eosin	<b>126</b>
<b>4.21</b>	Photomicrograph of liver section of DENA group stained with hematoxylin and eosin	<b>126</b>
<b>4.22</b>	Photomicrograph of liver section of DENA+ZSCL1 group stained with hematoxylin and eosin	<b>127</b>
<b>4.23</b>	Photomicrograph of liver section of DENA+ZSCL1 group stained with	<b>127</b>