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Hepatoprotective Impact of Natural Products Extract and Nanoparticles Against 4-Dimethylaminoazobenzene and Phenobarbital Induced Hepatotoxicity in Rats

التأثير الوقائي للمنتجات الطبيعية والجزيئات النانونية ضد السمية الكبدية المستحدثة بواسطة 4- ثنائي ميثيل أمينواز بنزين و الفينوباربيتال في الفئران

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﴿يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ﴾



المجادلة (11)

Dedication and Acknowledgments

I see that the Master's stage has truly come to an end after a long journey filled with effort and perseverance. Today, I conclude my graduation research with pride and honor. I dedicate this success first to myself, and then to everyone who walked with me and contributed to completing this journey.

"And their final call is: Praise be to Allah, Lord of the worlds."

All praise and gratitude are due to Allah alone. It is by His grace that I was able to complete this journey; praise be to Him at the beginning and at the end.

I dedicate my graduation to the one whose name I carry with pride, to the one who removed the thorns from my path and paved the way for me toward knowledge my beloved father.

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To my companion on this journey, my life partner, who has always been and continues to be my greatest supporter at every step I dedicate this success to my husband.

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Abstract

Hepatocellular carcinoma (HCC) is one of the most prevalent and serious types of cancer worldwide, with oxidative stress playing a pivotal role in its initiation and progression. This study aimed to evaluate the hepatoprotective effect and antioxidant activity of the methanolic extract of *Saussurea costus* and its green-synthesized nanoparticles against hepatotoxicity and liver cancer induced by 4-dimethylaminoazobenzene (p-DAB) and phenobarbital (PB) in rats.

The plant extract was prepared using methanol and subsequently employed in the green synthesis of *Saussurea costus* extract-loaded chitosan nanoparticles (SC-CNPs). The prepared nanoparticles were physically characterized using dynamic light scattering, zeta potential analysis, and transmission electron microscopy. Liver cancer was experimentally induced in rats through combined treatment with p-DAB and PB. The animals were then divided into four groups: a control group, a liver cancer-induced group, a group treated with the plant extract, and a group treated with the nanoparticles.

At the end of the study period, serum liver function markers, including ALT, AST, ALP, total protein, and albumin, were evaluated. Oxidative stress markers, particularly malondialdehyde (MDA), as well as antioxidant defense parameters, including SOD, CAT, GPx, and GSH, were also assessed. In addition, the gene expression levels of selected inflammatory markers, namely TNF-α and IFN-α, were analyzed, alongside histopathological examination of liver tissues.

The results showed that both the methanolic extract and the nanoparticles produced a marked improvement in liver function, reduced oxidative stress, restored oxidative balance, and improved the histological structure of the liver compared with the liver cancer group. Furthermore, the nanoparticles exhibited greater efficacy than the methanolic extract in exerting hepatoprotective effects.

The study concludes that green-synthesized nanoparticles loaded with *Saussurea costus* extract represent a promising, safe, and eco-friendly therapeutic strategy for the prevention and management of hepatocellular carcinoma.

الملخص

يُعدّ سرطان الخلايا الكبدية (Hepatocellular Carcinoma, HCC) من أكثر أنواع السرطان انتشارًا وخطورة على مستوى العالم، حيث يلعب الإجهاد التأكسدي دورًا محوريًا في نشأته وتطوره. هدفت هذه الدراسة إلى تقييم التأثير الوقائي الكبدي والنشاط المضاد للأكسدة للمستخلص الميثانولي لنبات القسط الهندي (*Saussurea costus*) وجسيماته النانوية المحضّرة بالطريقة الخضراء، ضد السمية الكبدية وسرطان الكبد المستحث باستخدام -4ثنائي ميثيل أمينو آزوبنزين (p-DAB) والفينوباربيتال (PB) في الفئران.

تم تحضير المستخلص النباتي باستخدام الميثانول، ثم استُخدم في التخليق الأخضر لجسيمات نانوية محمّلة على الكيتوسان(SC-CNPs) ، والتي جرى توصيفها فيزيائيًا باستخدام تشتت الضوء الديناميكي، جهد زيتا، والمجهر الإلكتروني النافذ. أُحدث سرطان الكبد تجريبيًا في الفئران من خلال المعاملة المشتركة بـ p-DAB وPB، ثم قُسمت الحيوانات إلى أربع مجموعات: مجموعة ضابطة، مجموعة مصابة بسرطان الكبد، مجموعة معالجة بالمستخلص النباتي، ومجموعة معالجة بالجسيمات النانوية.

بعد انتهاء فترة الدراسة، تم تقييم مؤشرات وظائف الكبد في المصل(ALT ، AST، ALP)، البروتين الكلي، والألبومين، وعلامات الإجهاد التأكسدي (MDA) ، ونشاط إنزيمات مضادات الأكسدة (SOD ، CAT، GPx، وGSH)، بالإضافة إلى تحليل التعبير الجيني لبعض المؤشرات الالتهابية (TNF-α و-IFN α) إلى جانب الفحص النسيجي المرضي للكبد. أظهرت النتائج أن كلاً من المستخلص الميثانولي والجسيمات النانوية أدّيا إلى تحسّن ملحوظ في وظائف الكبد، وخفض الإجهاد التأكسدي، واستعادة التوازن التأكسدي، وتحسين البنية النسيجية للكبد مقارنةً بمجموعة سرطان الكبد. كما أظهرت الجسيمات النانوية كفاءة أعلى من المستخلص في التأثير الوقائي الكبدي.

تخلص الدراسة إلى أن الجسيمات النانوية الخضراء المحمّلة بمستخلص القسط الهندي تمثل استراتيجية علاجية واعدة وآمنة وصديقة للبيئة للوقاية من سرطان الخلايا الكبدية.

Contents

Chapter One 1
1.1 General Introduction 2
1.1.1 *Saussurea costus* (Indian Costus 4
1.1.2 Green Nanoparticles (GNPs) 10
1.1.3 Carcinogenic Agents Used in the Study 14
1.1.4 Combined Effect of p-DAB and Phenobarbital 17
1.1.5 Biochemical and Cellular Consequences 17
1.1.6 Experimental Justification 18
1.1.7 Antioxidant Compounds and the Role of Methanolic Extract in the Study 19
1.1.7.1 Methanol as an Extraction Solvent……..……...……19
1.1.8 Comparative Analysis of Previous Studies and the Present Research 23
1.2 Research Problem 24
1.3 Research Significance 25
1.4 Research Hypothesis 26
1.5 Research Objectives 27
1.6 Research Limitations 29
1.7 Structure of the Thesis 29
Chapter Two 31
2.1 Introduction 32
2.2 Antioxidant and Therapeutic Extracts Used in Liver Protection 33
2.3 Carcinogenic Agents Used in Experimental Hepatocellular Carcinoma 38
2.4 Previous Studies 41
2.5 Comparative Analysis and Research Gap 41
Chapter Three 49
3.1. Reagents and Instrumentation 50
3.2. Plant Material and Extract Preparation 51
3.2.1. Botanical Source and Authentication 51
3.2.2. Pre-Extraction Processing 51
3.2.3. Methanolic Extraction 52
3.3. Preparation of *S. costus*–Loaded Chitosan Nanoparticles (SC-CNPs) 53

3.3.1. Chitosan Stock Solution 55
3.3.2. Aqueous Extract Dispersion 55
3.3.3. Formation of Nanoparticles 56
3.3.4. Purification of Nanoparticles 59
3.4. Physicochemical Characterization of SC-CNPs 60
3.4.1. Dynamic Light Scattering (DLS) and Zeta Potential 60
3.4.2. Transmission Electron Microscopy (TEM) 61
3.5. In Vivo Evaluation 61
3.5.1. Ethical Approval and Experimental Animals 62
3.5.2. Experimental Design and Grouping 64
3.5.3. Induction of Hepatocellular Carcinoma (DAB + PB Model) 68
3.5.4. Treatment Protocol 68
3.6. Biochemical and Antioxidant Assays 71
3.6.1. Serum Liver Function Markers 71
3.6.2. Preparation of Liver Homogenates 73
3.6.3. Oxidative Stress Markers and Antioxidant Enzymes 73
3.7. Gene Expression Analysis by Real-Time PCR 73
3.8. Histopathological Assessment 75
3.8.1. Tissue Processing 75
3.8.2. Staining Procedure 75
3.8.3. Microscopic Evaluation and Grading Criteria 75
3.9. Statistical Analysis 75
Chapter Four 78
4.1. Formation of SC-CNPs 79
4.2. Physicochemical Profile 80
4.2.1. Particle Size Distribution 80
4.2.2. Zeta Potential 81
4.2.3. Morphology (TEM) 82
4.3. In Vivo Efficacy 89
4.3.1. Gross Liver Findings 89
4.3.2. Microscopic Findings 89

4.3.3. Histopathology Grading 90
4.4. Mechanistic Considerations 91
4.4.1. Biochemistry (Serum Liver Function Markers) 92
4.4.2. Antioxidant and Oxidative Stress Markers 99
4.4.3. PCR Analysis and Molecular Markers 106
Chapter Five 110
5.1 Conclusion 111
5.2 Medical Implications and Practical Applications 111
5.3 Recommendations and Future Research Directions 114
5.4 Overall Conclusion 115
References 116

List of Figures

Figure 1.1. Anatomical diagram of the liver. 2

Figure 1.2. Essential liver functions. 3

Figure 1.3. The Saussurea costus plant and its medicinal roots (adapted from [30]). 8

Figure 1.6 presents the proposed reaction mechanism for the biosynthesis of copper oxide nanoparticles (CuO NPs) using the leaf extract of Plumbago zeylanica. 22

Figure 3.1 Laboratory instruments used for nanoparticle preparation, purification, characterization, and histological examination. 51

Figure 3.2 *A schematic illustration of the green synthesis steps of Saussurea costus–loaded chitosan nanoparticles (SC-CNPs), including chitosan preparation, extract dispersion, nanoparticle formation, and purification.* 60

Figure 3.3 *Weighing experimental Rats prior to carcinogen administration and treatment to determine accurate body-weight–based dosing.* 64

Figure 3.4 *A schematic timeline of the in vivo experimental design showing carcinogen induction, extract/nanoparticle treatment phases, and scheduled sampling points for biochemical and histopathological evaluations.* 66

Figure 3.5 *Intraperitoneal or oral administration of carcinogenic or therapeutic agents according to the experimental grouping protocol.* 67

Figure 3.6 *Housing conditions of experimental Rats in specialized cages designed to induce mild oxidative stress, which helps accelerate the progression of DAB/PB-induced hepatocarcinogenesis.* 67

Figure 3.7 *Inhalational anesthesia of Rats using a funnel-based chamber to facilitate humane handling and sample collection.* 70

Figure 3.8 *Blood sample collection from experimental Rats for biochemical and liver function analyses.* 72

Figure 3.9 *Dissection and liver tissue harvesting for histopathological, biochemical, and oxidative stress analyses.* 74

Figure 4.1 Visual indication of Sc-CNP formation along with representative RT-qPCR amplification curves and CT values for normal, HCC, extract-treated (TK), and nanoparticle-treated (TN) groups. 80

Figure 4.2. Size distribution by intensity and DLS parameters of CNPs. 81

Figure 4.3. Zeta Potential of the prepared CNPs. 82

Figure 4.4. Representative photomicrograph of H&E-stained liver from the control group: The preserved lobular architecture, thin hepatic cords (1-2 cells thick), and uniform hepatocytes with

a low N/C ratio are characteristic of normal liver parenchyma. Scale bar = 50 µm. H= Hepatocyes, CV= Central vein. 83

Figure 4.5. Representative photomicrograph of H&E-stained liver from the control group: The preserved lobular architecture, thin hepatic cords (1-2 cells thick), and uniform hepatocytes with a low N/C ratio are characteristic of normal liver parenchyma. Scale bar = 50 µm. H= Hepatocyes, CV= Central vein. 84

Figure 4.6 Representative photomicrograph of H&E-stained liver from the control group: The preserv Figure 4.3. ed lobular architecture, thin hepatic cords (1-2 cells thick), and uniform hepatocytes with a low N/C ratio are characteristic of normal liver parenchyma. Scale bar = 50 µm. H= Hepatocyes, CV= Central vein. 84

Figure 4.7 Representative photomicrograph of H&E-stained liver from the HCC group: The trabecular growth pattern (4-5 cells thick arrow as indicated with asterisk), high N/C ratio and cellular pleomorphism (arrowhead), and prominent eosinophilic nucleoli (small arrow), are highly indicative of Hepatocellular Carcinoma, likely of a moderately differentiated grade (Grade II). Scale bar = 50 µm. 84

Figure 4.8 Representative photomicrograph of H&E-stained liver from the HCC group: The trabecular growth pattern (asterisk), high N/C ratio, cellular pleomorphism, and prominent eosinophilic nucleoli (arrow), are highly indicative of Hepatocellular Carcinoma, likely of a moderately differentiated grade (Grade II). Scale bar = 50 µm. 85

Figure 4.9 Representative photomicrograph of H&E-stained liver from the HCC group: The trabecular growth pattern (asterisk), high N/C ratio, cellular pleomorphism (large arrow), and prominent eosinophilic nucleoli (small arrow), are highly indicative of Hepatocellular Carcinoma, likely of a moderately differentiated grade (Grade II). Scale bar = 50 µm. 85

Figure 4.10 Representative photomicrograph of H&E-stained liver from the HCC+ Indian Costus group: The trabecular growth pattern (asterisk), low N/C ratio, minimal nuclear atypia (are highly indicative of Hepatocellular Carcinoma, likely of a well-differentiated grade (Grade I). Scale bar = 50 µm. H= Hepatocyes, CV= Central vein. 86

Figure 4.11. Representative photomicrograph of H&E-stained liver from HCC+ Indian Costus group: The preserved lobular architecture, thin hepatic cords (1-2 cells thick), and uniform hepatocytes with a low N/C ratio are characteristic of normal liver parenchyma. Scale bar = 50 µm. H= Hepatocyes, CV= Central vein. 86

Figure 4.12. Representative photomicrograph of H&E-stained liver from the HCC+ Indian Costus group: The trabecular growth pattern (asterisk), low N/C ratio, minimal nuclear atypia (are highly indicative of Hepatocellular Carcinoma, likely of a well-differentiated grade (Grade I). Scale bar = 50 µm. H= Hepatocyes, CV= Central vein. 87

Figure 4.13 Representative photomicrograph of H&E-stained liver from HCC+ Indian Costus (Nanoparticles) group: The preserved lobular architecture, thin hepatic cords (1-2 cells thick),

and uniform hepatocytes with a low N/C ratio are characteristic of normal liver parenchyma. Scale bar = 50 µm. H= Hepatocyes. 87

Figure 4.14 Representative photomicrograph of H&E-stained liver from HCC+ Indian Costus (Nanoparticles) group: The preserved lobular architecture, thin hepatic cords (1-2 cells thick), and uniform hepatocytes with a low N/C ratio are characteristic of normal liver parenchyma. Scale bar = 50 µm. H= Hepatocyes. 88

Figure 4.15 Representative photomicrograph of H&E-stained liver from HCC+ Indian Costus (Nanoparticles) group: The preserved lobular architecture, thin hepatic cords (1-2 cells thick), and uniform hepatocytes with a low N/C ratio are characteristic of normal liver parenchyma. Scale bar = 50 µm. H= Hepatocyes. 88

Figure 4.16 Serum ALT levels (U/L) in Normal, HCC, CS-extract, and SC-CNPs groups showing significant elevations in the HCC group and marked reductions following treatment. . 94

Figure 4.17 Serum AST concentrations (U/L) across the Normal, HCC, CS-extract, and SC-CNPs groups, showing significant elevation in the HCC group and marked reductions following treatment, particularly with the nano-form. 95

Figure 4.18 Serum AST concentrations (U/L) across the Normal, HCC, CS-extract, and SC-CNPs groups, showing significant elevation in the HCC group and marked reductions following treatment, particularly with the nano form. 96

Figure 4.19 Serum albumin (g/dl) levels across the Normal, HCC, CS-extract, and SC-CNPs groups, showing severe reduction in the HCC group and marked improvement following treatment, particularly with the nano-form. 97

Figure 4.20 Total serum protein (g/dl) across Normal, HCC, CS-extract, and SC-CNPs groups, illustrating the significant reduction in the HCC group and the marked recovery following treatment, particularly with the nano-form. 99

Figure 4. MDA concentration (nmol/g tissue) in Normal, HCC, CS-extract, and SC-CNPs groups, illustrating severe oxidative damage in the HCC group and substantial improvement following treatment, especially with the nano-formulation. 101

Figure 4.22 GPx activity (U/g tissue) in Normal, HCC, CS-extract, and SC-CNPs groups, showing severe depletion in HCC and substantial recovery following treatment, particularly in the nano-form-treated group. 102

Figure 4.23 GSH concentration (mg/g tissue) in Normal, HCC, CS-extract, and SC-CNPs groups, showing severe depletion in the HCC group and marked recovery following treatment, particularly with the nano-formulation. 104

Figure 4.24 Serum AST concentrations (U/L) across the Normal, HCC, CS-extract, and SC-CNPs groups, showing significant elevation in the HCC group and marked reductions following treatment, particularly with the nano-form.. 105

Figure 4.25 SOD activity (U/g tissue) in Normal, HCC, CS-extract, and SC-CNPs groups, illustrating severe reduction in HCC and marked recovery following treatment, particularly with the nano-formulation. 106

Figure 4.26 TNF-α / β-actin mRNA expression levels across Normal, HCC, CS-extract, and CS-NP groups, showing pronounced elevation in HCC and significant reductions following treatment, particularly with the nanoparticle formulation. 108

Figure 4.27 IFN-α / β-actin mRNA expression levels across Normal, HCC, CS-extract, and CS-NP groups, showing severe downregulation in HCC and substantial restoration following treatment, particularly with the nano-formulation. 109

List of Table

Table (2.1) Summary of Key Differences and Research Innovation 46
Table (4.1) Pathological Lesions Grading and Incidence: 89

List of Equations

Equation No.	Equation	Page
(3.1)	$\text{Chitosan repeat unit} = (C_6H_{11}NO_4)_n$	59
(3.2)	$\text{Costunolide} = C_{15}H_{20}O_2$	59
(3.3)	$\text{Dehydrocostus lactone} = C_{15}H_{18}O_2$	59
(3.4)	$CS - NH_2 + H^+ \rightleftharpoons CS - NH_3^+ \quad (pH = 4.5)$	59
(3.5)	$CS - NH_3^+ + R - COO^- \rightleftharpoons [CS - NH_3^+ \cdots R - COO^-]_{ES}$	59
(3.6)	$CS - OH + O = C - R \rightleftharpoons [CS - O - H \cdots O = C - R]_{HB}$	59
(3.7)	$CS_{hyd} + R_{terp} \rightleftharpoons [CS \cdots R_{terp}]_{HYD}$	59
(4.1)	$\text{Chitosan} + \text{S. costus extract} \xrightarrow[50°C,\ 1000\ rpm]{pH=4.5,\ 1:4} SC - CNPs$	79
(4.2)	$CS - NH_2 + H^+ \rightleftharpoons CS - NH_3^+$	79
(4.3)	$CS - NH_3^+ + R - COO^- \rightleftharpoons [CS - NH_3^+ \cdots R - COO^-]_{ES}$	79
(4.4)	$CS - OH + O = C - R \rightleftharpoons [CS - O - H \cdots O = C - R]_{HB}$	79
(4.5)	$CS_{hyd} + R_{terp} \rightleftharpoons [CS \cdots R_{terp}]_{HYD}$	79
(4.6)	$Complex_{ES} + Complex_{HB} + Complex_{HYD} \rightleftharpoons SC - CNPs$	79
(4.7)	$SC - CNPs + \text{Phytochemical} \rightleftharpoons [SC - CNPs \cdots \text{Phytochemical}]_{surface}$	79

List of Abbreviations

AFB1	Aflatoxin B1
ALB	Albumin
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
Bax	Bcl-2–Associated X Protein
Bcl-2	B-Cell Lymphoma-2
CAT	Catalase
CNPs	Chitosan Nanoparticles
CuO NPs	Copper Oxide Nanoparticles
CYP	Cytochrome P450
DAB / p-DAB	p-Dimethylaminoazobenzene
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic Acid
FTIR	Fourier Transform Infrared Spectroscopy
GGT	Gamma-Glutamyl Transferase
GNPs	Green Nanoparticles
GPx	Glutathione Peroxidase
GSH	Reduced Glutathione
HCC	Hepatocellular Carcinoma
H&E	Hematoxylin and Eosin
IL-1β	Interleukin-1 Beta

IFN-α	Interferon Alpha
LDH	Lactate Dehydrogenase
MDA	Malondialdehyde
NF-κB	Nuclear Factor Kappa-B
NO	Nitric Oxide
Nrf2	Nuclear Factor Erythroid 2-Related Factor 2
PB	Phenobarbital
PCR	Polymerase Chain Reaction
qPCR / RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
ROS	Reactive Oxygen Species
SC-CNPs	*Saussurea costus*–Loaded Chitosan Nanoparticles
SOD	Superoxide Dismutase
TEM	Transmission Electron Microscopy
TGF-β	Transforming Growth Factor Beta
TNF-α	Tumor Necrosis Factor Alpha
UV–Vis	Ultraviolet–Visible Spectroscopy
XRD	X-Ray Diffraction

Chapter One

Introduction

1.1 General Introduction

About two to three percent of a person's entire body mass is occupied by their liver, making it the largest internal organ. Seated on the right side of the abdomen, beneath the rib cage, it is around 1.3-1.5 kg (≈ three pounds) heavy and sits over the right kidney, intestines, and stomach. Liver function is vital for metabolic control, detoxification, and the maintenance of life-sustaining biochemical pathways. Venouses and bile ducts surround its four wedge-shaped lobes, as each lobe consists of 8 sections and thousands of lobules. The liver tends to be reddish brown in color, and it is shaped like a cone. The liver is divided into parenchymal (hepatocytes) and non- parenchymal (non-hepatocytes) cells [1]. Endothelial cells, hepatic stellate cells, and Kupffer cells are non-hepatocyte cells that account for 6.3% of the liver [1]. The liver is involved in a variety of processes, including fat synthesis and biotransformation, drug metabolism, hydrophobic substance removal, fluid balance and energy metabolism regulation, food digestion, and immunological and endocrine function [1]. Unlike other solid organs, liver cells have the ability to regenerate [2].

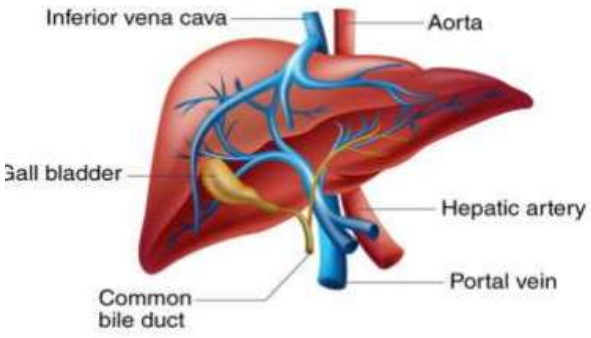


Figure (1.1). Anatomical diagram of the liver.

Metabolizing proteins, lipids, and carbs; synthesizing cholesterol, bile acids, and molecules that aid in blood clotting; and detoxifying substances and medications are among the primary roles of the liver. Figure 1.2 shows that the liver is responsible for breaking down old red blood cells and transporting bilirubin [3].

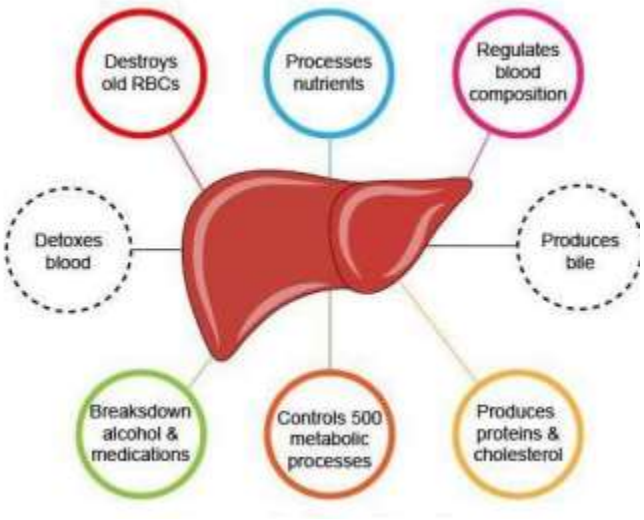


Figure (1.2). Essential liver functions.

Hepatocellular carcinoma (HCC) accounts for 80–90% of primary liver cancers worldwide, according to previous research [4,5]. Worldwide, HCC is the third leading cause of cancer-related mortality and ranks sixth among malignancies overall [4]. With an estimated 841,000 new cases and 782,000 deaths in 2018, HCC made up 4.7% of all malignancies [6]. Geographical variations in incidence are as follows: South-Central and Central/Eastern Europe have the lowest rates, while East Asia, North and West Africa, and Sub-Saharan Africa have the greatest rates [6]. According to the latest data [7], HCC ranks fifth in males for incidence and second in females for mortality globally. Aflatoxin B1 (AFB1) and other environmental and industrial hazardous substances, alcoholism, and chronic viral hepatitis are major risk factors. Forty percent of hepatocellular carcinoma (HCC) cases advance clinically from fibrosis to cirrhosis and, finally, cancer [8].

The relationship between oxidative stress and the progression of HCC. When the body's defense mechanisms are unable to adequately neutralize reactive oxygen species (ROS) produced by cells, a condition known as oxidative stress develops [9]. In both healthy and unhealthy states, reactive oxygen species (ROS) are mostly produced in the mitochondria after molecular oxygen has been transformed to hydrogen peroxide and related species [10]. Among the many significant ROS producers is the mitochondrial/cytochrome P450 catalytic cycle [10]. Reactive oxygen species (ROS) can promote carcinogenesis and tumor cell growth at high concentrations or cause cell death at low concentrations, which are seemingly contrary impacts on cancer development [11]. Reducing oxidative stress using antioxidant enzymes is viewed as a crucial tactic in the fight against cancer [12], due to the oxidative stress's central involvement in tumor biology. A number of diseases and conditions, including cancer, can be diagnosed using malondialdehyde (MDA), a famous indicator of lipid peroxidation, oxidative stress, and antioxidant status [13,14]. Researchers have found that HCC patients have higher levels of MDA [15]. Excess free radicals peroxide polyunsaturated fatty acids, leading to the formation of malondialdehyde (MDA), a very harmful by-product that destroys important macromolecules in cells [16]. Function of antioxidants in the prevention of head and neck cancer! In live cells, antioxidants prevent oxidative damage to DNA, lipids, proteins, and carbohydrates, a process widely referred to as antioxidant defense [17]. In neutralizing ROS within target tissues, they serve a vital function. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are types of enzymatic antioxidants, while vitamins, polyphenols, and flavonoids are examples of non-enzymatic antioxidants [17]. Substances that cause cancer (p-DAB and PB). One powerful azo dye that causes cellular damage and neoplastic changes in the liver is p-Dimethylaminoazobenzene, or p-DAB [18]. Many common industrial products

include it as a colorant, such as detergents, polishes, dry meals, drinks, and even some drugs [19].

Covalent adducts formed by DAB and DNA accelerate neoplastic development and induce oxidative DNA damage [20]. Some believe that DAB's hepatotoxic effects are due, in part, to the fluorine atom and its strong C-F bond [21]. As a barbiturate, phenobarbital (PB) has sedative and anticonvulsant properties [22]. Side effects including depression, unconsciousness, low blood pressure, lack of appetite, liver damage, and pain in the muscles, joints, and bones can occur with long-term use [23]. Combine PB with DAB, and it significantly increases the risk of HCC in rats and Rats [18].

Function and mechanism of action in the development of kidney cancer. After two months of treatment with DAB and PB, Rats develop HCC, which can develop into neoplastic growth [24]. The genetic abnormalities caused by these two substances, when consumed in large quantities over time, include chromosomal abnormalities and nuclear anomalies (such as micronuclei and sperm head abnormalities) [25,26]. Rats that have been treated exhibit biochemical changes such as increased hepatotoxicity, decreased superoxide dismutase and catalase, decreased glutathione and adiponectin levels, and increased mitotic indices and chromosomal abnormalities [18,20]. From a mechanistic standpoint, cytochrome P450 enzymes break down DAB, producing reactive oxygen species (ROS) and electrophiles. These byproducts then cause DNA adducts, which in turn propel the development of hepatocellular carcinoma (HCC) [19]. Inducing cytochrome P450 isoenzymes, especially CYP2B1 and CYP2B2, PB promotes carcinogenesis in the liver [27]. In the long run, these carcinogens cause oxidative damage to liver cells (and maybe other organs as well) due to their ability to deplete antioxidant defenses and sustain ROS generation [28].

Rationale and significance. Finding safe, natural ways to treat liver cancer with nanotechnology and *Saussurea costus* (Indian costus) is what prompted this study. The goal of combining traditional herbal medicine with current scientific procedures is to achieve longer-lasting and more effective therapeutic results. Discovering natural antioxidants that reduce free radical damage and improve the liver's ability to handle oxidative stress has been a growing area of research in recent years. For a long time, *saussurea costus* has been a staple in traditional medicine for digestive and liver problems. The plant is rich in bioactive chemicals such phenolic acids, sesquiterpene lactones, and flavonoids, all of which have powerful anti-inflammatory, hepatoprotective attributes. These chemicals can reduce lipid peroxidation, neutralize reactive oxygen species (ROS), and regulate important molecular processes that are involved in inflammation and cell death in the liver. Researchers nowadays are increasingly turning to nanotechnology-based sophisticated delivery systems to increase the efficacy of these naturally occurring substances. Environmentally friendly biopolymeric nanoparticles, especially those based on chitosan, enhance the effectiveness and decrease systemic negative effects of plant-derived compounds by making them more stable, absorbing them better, and releasing them under controlled circumstances. Superior antioxidant and hepatoprotective benefits can be achieved by incorporating *Saussurea costus* extract with chitosan nanoparticles, which allows for more targeted and sustained delivery to hepatic tissues. As a result, a new, sustainable, and environmentally responsible way to prevent and manage HCC is to combine herbal therapy with nanotechnology. This technique has the potential to be a better long-term solution than traditional chemotherapy, with better therapeutic safety.

The purpose of this study is to examine the antioxidant effects of a herbal extract and its nanoparticles as potential new substances in protecting Rats against

hepatocellular cancer caused by 4-dimethylaminobenzene (DAB) and phenobarbital (PB). This will be accomplished by preparing a herbal extract in methanol and using it for the green production of nanoparticles. The following step is to create HCC in 28 Rats by administering DAB and PB. With the use of a stratified sample design, the Rats will be divided into four groups of seven. The investigation will begin with the collection of blood and liver tissue samples three months later. Utilizing a spectrophotometer, real-time PCR, a cooling centrifuge, and a homogenizer, the serum biochemistry, hepatic antioxidant indicators, and histopathology will be tested.[29]

1.1.1 *Saussurea costus* (Indian Costus)

1.1.1.1 Saussurea costus

Indian Costus is officially known as *Saussurea costus* (Falc.) Lipsch in the scientific community. It has multiple uses, a pleasant aroma, and a long lifespan. It is a member of the Asteraceae family, which is often called the Compositae family. The Himalayas are the original habitats of this plant, which is native to Nepal, Bhutan, India, and Pakistan. Optimal conditions are found at elevations of 2,500 to 3,500 meters above sea level. Ayurveda, Unani medicine, and Traditional Chinese Medicine (TCM) all hold *saussurea costus* in high respect because to its many beneficial effects on health. A number of Arabic and Urdu terms refer to it, including "Kustha," "Qust al-Hindi," and "Muqassir" [30].

1.1.1.2 Morphological and Botanical Characteristics

Its wide, lance-shaped leaves and clusters of purple or bluish blooms at the tips of its stems give this plant its distinctive appearance; it can reach a height of 1 to 2 meters. The roots are the most important component of the plant when it comes to its medicinal uses. These roots are sturdy, aromatic, and thick. Their interior is

white, and their exterior is brownish. An illustration of this can be seen in Figure (1.3). Many think that the plant's many medical and health benefits stem from the substances found in its roots, which include volatile oils, resins, and a broad array of secondary metabolites.



Figure (1.3): The *Saussurea costus* plant and its medicinal roots (adapted from [30]).

1.1.1.3 Chemical Composition

Phytochemically, *Saussurea costus* is rich and diverse, including several bioactive substances. Sesquiterpene lactones, such costunolide and dehydrocostus lactone, are known to combat cancer, lessen inflammation, and shield cells from oxidative stress. In addition to quercetin, gallic acid, and chlorogenic acid, the plant also includes flavonoids and phenolic acids that aid in the neutralization of free radicals and the support of liver function. The essential oils, tannins, and alkaloids in it have antibacterial, antiulcer, and immunomodulatory properties, as mentioned in references [31,32].

1.1.1.4 Traditional and Medicinal Uses

More than sixty traditional formulas from the Ayurvedic, Unani, and Siddha medical systems have traditionally used the plant known as (*S. costus)*. Both the plant's essential oil and its root have a long history of use in alternative medicine. Hepatitis, jaundice, and cirrhosis are some of the liver-related diseases that have traditionally benefited from its usage. Gastritis, dyspepsia, cough, and asthma are among the respiratory and digestive problems that have benefited from its use. Period irregularities and various skin conditions have also found relief through the use of this plant. In addition to its anti-inflammatory properties, it has been used as a general tonic to improve general health. In addition to these applications, other studies have shown that the plant has various beneficial qualities, including as antioxidant, anticancer, antibacterial, antifungal, antiviral, and immunoprotective activities [33,34].

1.1.1.5 Pharmacological Significance in Hepatic Protection

Additional evidence points to *Saussurea costus* as a potential liver protector in recent studies. Results from research show that this plant's extracts can be useful in a variety of environments. As an example, in cases where the liver is already damaged due to toxins, they have been found to lower levels of ALT, AST, and ALP in the blood, which are indicators of hepatic injury. The antioxidant enzymes glutathione (GSH) and superoxide dismutase (SOD) can facilitate the body's detoxification processes, and these extracts can even boost their activity. Also, they help lower levels of oxidative stress indicators and hazardous byproducts such malondialdehyde (MDA). Moreover, they can help bring damaged liver tissue back to normal by removing harmful substances including paracetamol, carbon tetrachloride, and aflatoxin-B1. Additional research suggests that *Saussurea costus*

improves liver function by making the body more resistant to oxidative stress, decreasing damaging lipid reactions, and lowering the generation of inflammatory molecules like TNF-α, IL-1β, and TGF-β. Most of the protective effects are due to the plant's active ingredients, which include sesquiterpene lactones such as costunolide and dehydrocostus lactone. In addition to blocking the NF-κB pathway associated with inflammation, these chemicals are recognized for activating the Nrf2 pathway, a crucial defense mechanism against oxidative stress in the body. As a result, the liver is protected from potentially harmful oxidative stress and inflammation [35,36].

1.1.1.6 Integration with Nanotechnology

The methanolic extract of *Saussurea costus* is an excellent, all-natural material for producing biocompatible nanoparticles since it contains a wealth of bio-reductive plant-based compounds. Combining this extract with chitosan nanoparticles enhances solubility, enhances distribution to the liver, and prolongs the antioxidant benefits. A state-of-the-art nanomedical solution that can aid in liver protection and cancer prevention is created by this combination. Reference [39] supports the idea that this method of treating hepatocellular carcinoma (HCC) is safer and more eco-friendly than previous approaches by combining traditional plant-based medicine with current nanotechnology.

1.1.2 Green Nanoparticles (GNPs)

The term "green nanoparticles" (abbreviated as "GNPs") refers to materials with a nanoscale (usually between 1 and 100 nm) that are produced in an eco-friendly manner utilizing natural polymers, microbes, algae, or plants as reducing and stabilizing agents rather than harmful chemical reagents [40]. "Green nanotechnology" encompasses methods like the "green synthesis" that try to make

nanoparticles in a way that is safer, more environmentally friendly, and more biocompatible. The transformation of metal ions (such as Ag^{+}, Au^{3+}, Fe^{3+}, Zn^{+2}) into stable nanoparticles with functionalized surfaces is aided by biomolecules such flavonoids, alkaloids, terpenoids, polyphenols, proteins, and enzymes, which serve as reducing and capping agents concurrently [41].

1.1.2.1 Synthesis green nanoparticles

To synthesize green nanoparticles, the standard procedures are as follows:

- Green nanoparticle synthesis usually starts with preparing plant materials such as leaves, roots, or seeds. These materials are washed, dried, and then extracted using solvents like water, ethanol, or methanol to produce a plant extract that will be used in the synthesis process.
- The plant extract is then added to a solution containing metal salts, for example silver nitrate ($AgNO_3$), chloroauric acid ($HAuCl_4$), or ($ZnSO_4$), which serve as the source of metal ions.
- Natural compounds found in the extract convert the metal ions into nanoparticles. At the same time, plant-based molecules such as polyphenols and flavonoids help stabilize the nanoparticles and prevent them from clumping together.
- After the formation of nanoparticles, several analytical techniques are used to study their properties, including UV–Visible spectroscopy, FTIR, TEM, XRD, and DLS, in order to determine their size, structure, and shape [42].
- Compared with traditional chemical synthesis methods, green synthesis produces nanoparticles that are more environmentally friendly, relatively inexpensive, and less likely to contain toxic chemical residues.

1.1.2.2 Advantages of Green Synthesis

Compared to conventional physical and chemical synthesis methods, the green approach offers several benefits [43]:

- **Eco-friendly process:** The method avoids the use of dangerous chemicals and decreases the production of harmful waste.
- **Low-cost method:** It mainly relies on natural biological materials that are widely available and inexpensive.
- **Good biocompatibility:** The nanoparticles produced are generally less toxic, which makes them suitable for medical and pharmaceutical uses.
- **Energy saving:** The synthesis usually takes place under simple conditions such as room temperature and normal atmospheric pressure.
- **Easy to scale up:** Because of its simplicity and safety, the method can be applied in large-scale industrial or medical production.

1.2 Applications of Green Nanoparticles

The biomedical and industrial applications of green nanoparticles are diverse and rapidly expanding:

a) **Biomedical and Pharmaceutical Uses**

- **Precise drug delivery:** Nanoparticles derived from plants can carry therapeutic agents directly to certain parts of the body, such as tumors, the liver, or the brain. This targeted approach allows medications to act more effectively while reducing harmful side effects.
- **Protection against oxidative stress:** These green nanoparticles help neutralize damaging molecules known as free radicals, which can otherwise cause cell damage and inflammation.

- **Potential in cancer therapy:** Some plant-based nanoparticles, including those made from *Aloe vera*, *Moringa oleifera*, and *Saussurea costus*, have been found to slow the growth of cancer cells in laboratory studies, indicating their usefulness in anticancer strategies [44].
- **Fighting infections and aiding wound healing:** Nanoparticles such as silver and zinc oxide, produced through eco-friendly methods, show strong antibacterial and antifungal activity. Their properties make them valuable in treating infections and supporting the healing of damaged tissues.
- **Applications in gene delivery and diagnostics:** The surface of these nanoparticles can be modified to carry biological molecules, which enables their use in gene transport and as tools for diagnostic testing.

b) **Environmental and Industrial Uses**

- **Wastewater treatment:** removal of heavy metals and dyes.
- **Catalysis:** serving as eco-friendly catalysts in chemical reactions.
- **Energy applications:** use in solar cells, sensors, and supercapacitors.

c) Role in Liver Protection and Antioxidant Therapy

Recent studies have shown that green nanoparticles derived from medicinal plants can strongly protect the liver. They achieve this by:

- Boosting the activity of antioxidant enzymes, including SOD, CAT, and GPx.
- Reducing harmful compounds such as MDA and reactive oxygen species.
- Preventing DNA and lipid damage.
- Helping restore liver tissue after chemical injury [45].

Chitosan-based green nanoparticles carrying plant extracts, such as *Saussurea costus*, have demonstrated better absorption and targeted delivery to liver cells. This targeted approach enhances both antioxidant and anticancer effects compared to using the plant extracts alone [46].

1.3 Green Nanoparticles and Nanomedicine

Using green nanotechnology along with herbal medicine is changing how modern medicine is handled, which is a big change in the field of nanomedicine. Green nanoparticles work like a natural link between the body's cells and molecules, allowing safe and effective interactions without causing damage. This makes them very useful for things like personalized medicine, targeted treatment delivery, and better medical imaging. Also, these nanoparticles are made from plant-based materials, which helps support environmentally friendly practices and protects the environment. Because of these advantages, green nanoparticles are becoming an important part of creating future treatments and medical breakthroughs [47].

1.1.3 Carcinogenic Agents Used in the Study

1.1.3.1 p-Dimethylaminoazobenzene (p-DAB)

Butter Yellow, or p-Dimethylaminoazobenzene, is a powerful azo dye that has been associated with the onset of liver cancer. The formula C14H15N3 represents its chemical composition. Soaps, cleaning supplies, metal polishes, dry foods, beverages, and even certain medications employed this chemical as a colorant in bygone days. It has a strong odor and takes the form of golden crystalline flakes when kept at room temperature.Research on hepatocellular carcinoma, a subtype of liver cancer, identified this compound as one among the first synthetic chemicals to be carcinogenic. Exposure to p-DAB over an extended period of time alters liver

tissue shape and function. Tumors may develop when these alterations harm cells, induce chromosomal abnormalities, and so forth. In particular, p-DAB's processing in the liver is associated with its carcinogenic mechanism. Cytochrome P450 enzymes are responsible for the compound's breakdown in the liver. As it breaks down, it releases molecules that are very reactive and may bind to DNA, such as reactive oxygen species (ROS). Dangerous DNA adducts are created when this kind of DNA damage occurs. Adducts like this have the potential to cause mutations in DNA, which may render normally functioning cells malignant. The inclusion of a fluorine atom in the molecular structure of p-DAB is another important component of its detrimental effect. Strong, almost irreversible connections are formed between this fluorine atom and carbon. Therefore, the chemical stays within the cells of the liver for a longer amount of time. An imbalance between free radical generation and the body's capacity to counteract their damaging effects is known as oxidative stress, and their presence for an extended period of time raises the body's level of this stress.Lipids, proteins, and mitochondria essential for cells to function—can be damaged by oxidative stress. Damages like this play a major role in the development of liver cancer. [48]

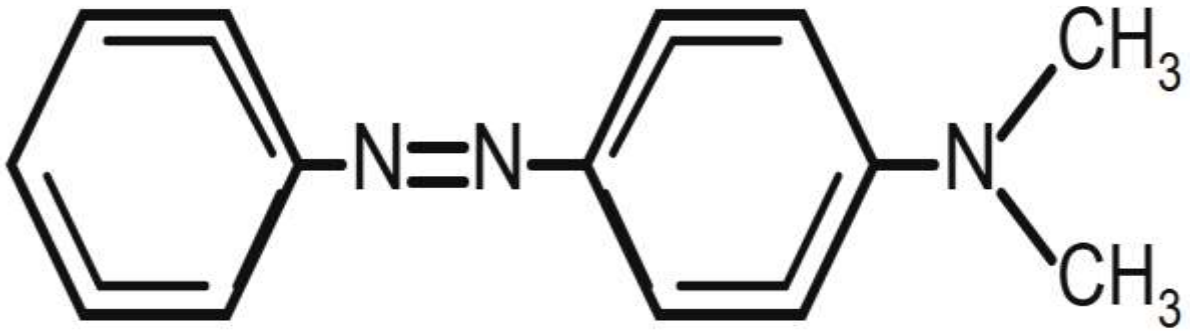


Figure (1.4): p-Dimethylaminoazobenzene (p-DAB) [48]).

1.1.3.2 Phenobarbital (PB)

One member of the class of medicines called barbiturates is phenobarbital, or PB for short. Seizures, anxiety, and insomnia are some of the most common disorders that these medications assist alleviate by calming the neurological system. Phenobarbital may be utilized as part of a medically supervised treatment protocol for managing withdrawal symptoms resulting from alcohol or benzodiazepine cessation. Phenobarbital is used under medical supervision in the management of withdrawal symptoms associated with alcohol or benzodiazepines, based on the clinical assessment of the patient's condition. The potential for liver damage and, in rare instances, tumor formation, is one of these dangers. Although phenobarbital does not induce mutations in and of itself, it may accelerate tumor growth when combined with other medications that do, including p-DAB. To exert its effects, phenobarbital stimulates the production of certain liver enzymes, most notably cytochrome P450 enzymes such as CYP2B1 and CYP2B2. Enzymes are essential for the body's chemical processes. Phenobarbital has the potential to speed up the degradation of other toxic drugs by activating these enzymes. Reactive oxygen species and other toxic byproducts may build up and cause cell damage if this happens. Phenobarbital may upset the liver's delicate equilibrium if used for an extended period of time. The buildup of dangerous chemicals in the body, known as oxidative stress, may be triggered by this. Inflammation and a decrease in the body's natural defenses are two side effects of stress. Some of the early warning symptoms of cancer include liver damage, DNA damage, and an increase in cell division brought on by these effects. Several liver enzymes, including ALT, AST, GGT, and LDH, were shown to be elevated in animal tests when phenobarbital was administered to the subjects. The presence of these enzymes suggests that the liver is either stressed out or injured. Further abnormalities included pre-cancerous

tumors, fat accumulation within the liver cells, and larger liver cells in these animals' liver tissue. According to these results, phenobarbital has the potential to induce major liver alterations that might eventually cause more severe health complications. in the text [49].

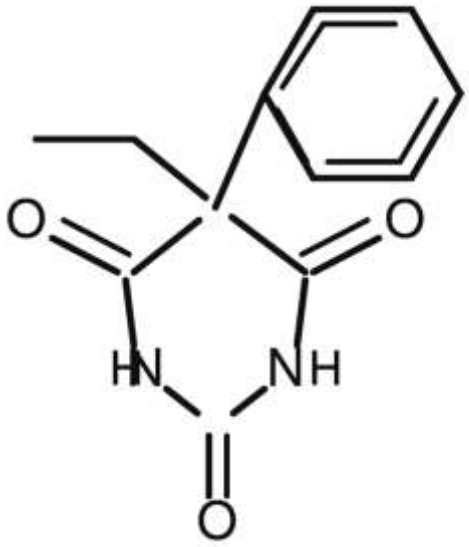


Figure (1.5): Phenobarbital (PB) [49]).

1.1.4 Combined Effect of p-DAB and Phenobarbital

As shown in the studies conducted in this research, the combination of the two substances significantly raises the risk of acquiring cancer. There are essentially two phases to this procedure. With p-DAB serving as a beginning factor, the first step is called the initiation phase. The DNA of normally functioning liver cells is altered, and dangerous chemical interactions are formed, as a result. As a result of these alterations, the liver cells begin to develop malignant tendencies. The second step, which is called the promotion phase, is when PB really comes into its own. More reactive oxygen species are produced as a result of its role in activating certain enzymes known as cytochrome P450. These chemicals are toxic and have

the potential to kill cells. An imbalance between toxic chemicals and the body's defense systems is known as oxidative stress, and when these two processes work together, it becomes much worse. Hepatocellular carcinoma, a subtype of liver cancer, develops when this imbalance damages cells and disrupts the liver's natural chemical equilibrium. Several telltale symptoms of this process may be seen when one looks at liver tissue under a microscope. Liver cell death, aberrant cell division, abnormal liver tissue shape, and abnormal alterations in cell nuclei are all examples of these. These findings provide substantial evidence in favor of the study's contention that normal cells are undergoing a metamorphosis into malignant ones. [50]

1.1.5 Biochemical and Cellular Consequences

Experimental administration of p-DAB and PB together over the course of eight weeks has been associated with a range of significant effects. One of the key observations is a noticeable rise in the levels of specific liver enzymes that are typically found in the bloodstream, such as ALT, AST, GGT, and LDH. These enzymes are usually indicators of liver health, and their increased presence suggests that the liver might be suffering from some kind of damage or stress. In addition to this, the activity of essential antioxidant enzymes in the body, like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), has been observed to decrease. These antioxidant enzymes are important because they help protect the body from harmful substances known as free radicals. A reduction in their activity implies that the body's natural defense system against these harmful molecules is becoming less effective. There is also a marked increase in chromosomal abnormalities, along with a higher-than-normal number of cells going through the process of mitosis, which is cell division. This could be a sign of genetic instability or a potential for cells to grow uncontrollably. Moreover,

the liver tissue exhibits severe histopathological changes, such as inflammation, cell death (necrosis), and the development of abnormal growths referred to as tumor nodules. These findings suggest that the structure and function of the liver are being significantly affected. Collectively, these biochemical changes imply an imbalance in the body's oxidative processes, an increase in harmful lipid peroxidation, and the occurrence of cellular mutations. All of these factors are well-documented as playing a crucial role in the progression toward liver cancer.[50]

1.1.6 Experimental Justification

Combining p-DAB with phenobarbital allows researchers to monitor the progression of chemically generated liver cancer in Rats in a controlled setting. The oxidative stress, increased levels of certain enzymes, and DNA alterations seen in actual liver cancer are all physically and chemically mirrored in this model. This model is a good tool to assess the effects of antioxidant compounds like green-synthesized nanoparticles and *Saussurea costus* extract on the liver's antioxidant defenses and on the harm that cancer-causing chemicals may produce.[51]

1.1.7 Antioxidant Compounds and the Role of Methanolic Extract in the Study

1.1.7.1 Methanol as an Extraction Solvent

Methanol is one of the most widely used solvents in biochemistry due to its high ability to dissolve polar and semi-polar compounds, such as:

- Flavonoids
- Phenolic compounds
- Terpenoids

These compounds are very important because they give *Saussurea costus* its antioxidant properties and help protect the liver, which are the main reasons this plant is often used in traditional medicine. When methanol is used as the liquid used to pull out the useful parts of the plant during the extraction process, it works well to get out the active molecules from both the leaves and the roots. This way, the resulting extract is packed with biologically active substances, which makes it a great choice for use in different kinds of biochemical experiments carried out on animals.

1.1.7.2 Chemical Properties of Methanol

Methanol (CH_3OH) is a simple alcohol characterized by:

- High polarity, allowing it to penetrate plant cell walls and extract active compounds.
- High volatility, which makes it easy to evaporate and concentrate the extract without leaving residues that could interfere with biological tests.
- Full miscibility with water, which helps in its later use during nanoparticle synthesis and formulation.[52]

1.2 Role of the Methanolic Extract in the Study

The methanolic extract of *Saussurea costus* was used for several scientific reasons:

- It contains powerful antioxidant compounds that scavenge free radicals and reduce lipid peroxidation in liver cells.
- It exhibits anti-inflammatory effects, minimizing the inflammation caused by carcinogenic substances such as DAB and PB.
- It helps maintain liver cell integrity by enhancing the activity of antioxidant enzymes such as SOD, CAT, and GPx.

1.3 Comparison Between Methanolic and Nanoparticle Forms

The study reveals that although the plain methanolic extract exhibits significant hepatoprotective properties, the nanoparticle form, which is the methanolic extract encapsulated within chitosan nanoparticles, shows even more effective results because of the following reasons:

- Improved bioavailability.
- Controlled and targeted release of active compounds into liver tissue.
- Enhanced chemical stability of the extracted molecules.[53]

1.4 Formation Mechanism of CuO Nanoparticles Using *Plumbago zeylanica* Leaf Extract

Plumbago zeylanica leaf extract is suggested to biosynthesize copper oxide nanoparticles (CuO NPs), as shown in Figure (1.6). The process begins with the interaction of copper ions with the bioactive compounds in the plant extract and ends with the creation of stable CuO nanoparticles. There are numerous critical processes involved in this procedure.The procedure begins with the presence of many bioactive components, such as phenols and flavonoids, in the leaf extract of *Plumbago zeylanica*. These substances may attach to copper ions due to reactive functional groups like hydroxyl (-OH) and carbonyl (C=O). Copper ions often come from a solution of copper sulfate ($CuSO_4$). Throughout this process, the chemicals derived from plants do a double duty as both reducing agents and stabilizing agents.Figure 7 shows that the nucleation stage is where the reaction starts. During this stage, tiny copper nuclei are formed when hydrated Cu^{2+} ions interact with the functional groups of plant macromolecules. The next stage is

reduction, when the plant biomolecules' electron-donating abilities cause Cu^{2+} to be transformed into Cu^0 metal. At this point, nanoparticle creation has begun.The next step after reduction is for the nanoparticles to start growing. During this stage, smaller nanostructures are formed as copper nuclei combine with one another. At the same time, the biomolecules in the vicinity serve as both stabilizers and caps. These biomolecules guarantee that the nanoparticles are homogeneous in size and structure and aid in preventing them from clumping.The next step, calcination, involves heating the nanoparticles to 400°C once they have partly formed. By applying heat, the metallic copper (Cu^0) is transformed into copper oxide (CuO) and any remaining organic matter is eliminated. The end product is CuO nanoparticles (CuO NPs) that are structurally distinct and have excellent chemical stability; they are also very stable. [54]

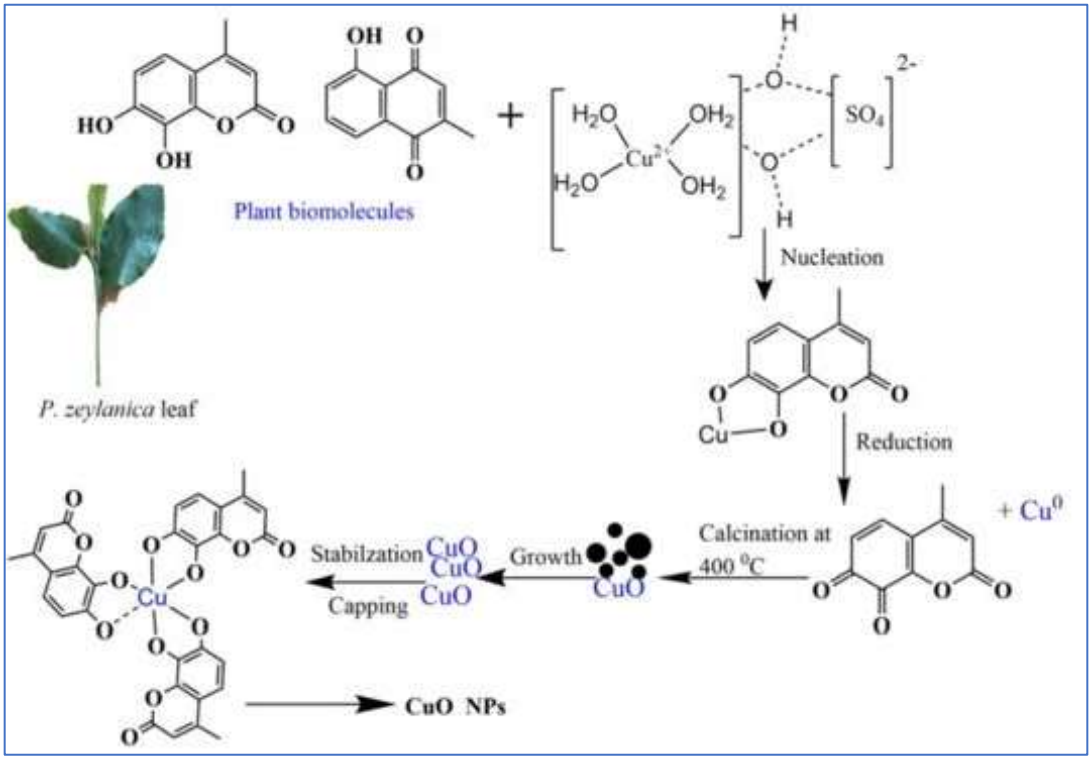


Figure (1.6) presents the proposed reaction mechanism for the biosynthesis of copper oxide nanoparticles (CuO NPs) using the leaf extract of Plumbago zeylanica.

1.1.8 Comparative Analysis of Previous Studies and the Present Research

Plumbago zeylanica leaf extract is suggested to biosynthesize copper oxide nanoparticles (CuO NPs), as shown in Figure (1.6). The process begins with the interaction of copper ions with the bioactive compounds in the plant extract and ends with the creation of stable CuO nanoparticles. There are numerous critical processes involved in this procedure.The procedure begins with the presence of many bioactive components, such as phenols and flavonoids, in the leaf extract of *Plumbago zeylanica*. These substances may attach to copper ions due to reactive functional groups like hydroxyl (-OH) and carbonyl (C=O). Copper ions often come from a solution of copper sulfate ($CuSO_4$). Throughout this process, the chemicals derived from plants do a double duty as both reducing agents and stabilizing agents.Figure 7 shows that the nucleation stage is where the reaction starts. During this stage, tiny copper nuclei are formed when hydrated Cu^{2+} ions interact with the functional groups of plant macromolecules. The next stage is reduction, when the plant biomolecules' electron-donating abilities cause Cu^{2+} to be transformed into Cu^0 metal. At this point, nanoparticle creation has begun.The next step after reduction is for the nanoparticles to start growing. During this stage, smaller nanostructures are formed as copper nuclei combine with one another. At the same time, the biomolecules in the vicinity serve as both stabilizers and caps. These biomolecules guarantee that the nanoparticles are homogeneous in size and structure and aid in preventing them from clumping.The next step, calcination, involves heating the nanoparticles to 400°C once they have partly formed. By applying heat, the metallic copper (Cu^0) is transformed into copper oxide (CuO) and any remaining organic matter is eliminated. The end product is CuO nanoparticles (CuO NPs) that are structurally distinct and have excellent chemical stability; they are also very stable. [54]

In contrast, Davarpanah and their team in 2015 chose a different approach, focusing on chemical methods instead of biological ones. They used a technique called microemulsion to create copper oxide nanoparticles, which had a size between 50 and 60 nanometers. Their main interest was in using these nanoparticles for gas sensor applications, and they did not look into any potential uses related to biology or medicine.[59]

In the collection known as Sahih al-Bukhari, there is a narration reported by Umm Qays bint Mihsan, may Allah be pleased with her. She said that she heard the Prophet Muhammad, peace be upon him, say the following:

"I advise you to use this Indian aloeswood, which is called Qust al-Hindi. It has seven kinds of healing properties. It can be used as a nasal treatment for throat problems and also as a drink for people suffering from pleurisy." This hadith is found in the Book of Medicine of Sahih al-Bukhari, with the reference number 5692.Another narration, this time from Anas ibn Malik, may Allah be pleased with him, mentions:

"The most effective remedies you can use are cupping therapy and Qust al-Bahri, which is the marine costus."This hadith is also included in the Book of Medicine of Sahih al-Bukhari, with the reference number 5696. [60]

In contrast to previous research in the same field, this one used a methanolic extract from the plant *Saussurea costus*, which is famous for its high concentrations of flavonoid and phenolic chemicals. The potent antioxidant properties of these chemicals have made them renowned for their role in warding off free radical damage to the body. The scientific community investigated the potential medicinal and biological uses of *Saussurea costus*-derived copper oxide

nanoparticles (CuO NPs) in this study. Prior research mostly concentrated on producing and evaluating the nanoparticles; this study expands upon that. Rather, it investigated the nanoparticles' chemical and physical properties as well as their interactions with biological things. Researchers used a liver cancer model using the chemicals dimethylnitrosamine (DAB) and phenylhydrazine (PB) to evaluate the efficacy of these nanoparticles. After that, they looked at the liver effects of *Saussurea costus's* methanolic extract and copper oxide nanoparticles. The results demonstrated that both *Saussurea costus* types increased the activity of key antioxidant enzymes including catalase, glutathione peroxidase (GPx), and superoxide dismutase (SOD). Malondialdehyde (MDA) is a measure of oxidative stress, and they decreased its levels simultaneously. These findings provide compelling evidence that both plant varieties possess substantial antioxidant characteristics and may protect the liver. This work stands out because it provides the first evidence that copper oxide nanoparticles derived from *Saussurea costus* may combat oxidative stress and hence aid in the prevention of liver cancer. To summarize, this work stands out due to its thorough examination of the nanoparticles, assessments of their medicinal applications, and green manufacturing methodologies all in one. Copper oxide nanoparticles' antibacterial and catalytic activities, as well as their physical characteristics, have been the primary foci of most prior study. This work adds to the growing body of research on medicinal applications of plant-based nanomaterials by establishing a connection between the physical and chemical properties of the nanoparticles and their efficacy in preventing oxidative stress and liver cancer.

1.2 Research Problem

Hepatocellular carcinoma, or HCC for short, is one of the main international causes of cancer-related mortality. In terms of overall cancer-related deaths, HCC

is third; when all cancers are included, it ranks sixth. Despite advancements in early detection and therapy, HCC remains a challenging condition to manage. This is due, in large part, to the fact that there are currently no effective treatments for HCC and, even after treatment, the disease often returns. Possible treatments for HCC that might improve survival rates and lengths of time patients spend alive include liver transplants, surgical removal of tumors, and local medicines that destroy cancer cells. However, factors such as the patient's general liver health, the stage of the malignancy, and the availability of organ donors may limit the effectiveness of these treatments. Natural substances and herbal remedies have recently attracted a lot of attention from cancer experts who are looking for new ways to prevent and cure the disease. Some of the many beneficial health impacts of these plant-based compounds include reducing inflammation and protecting healthy cells from harmful free radicals. In addition to preventing damage to the liver, these positive benefits may also slow or stop the development of cancer. Herbal and nano-herbal therapies, especially those made from *Saussurea costus* (Indian costus) and related plants, have therefore been the subject of increased investigation. These approaches may provide safer, more successful, and less invasive alternatives to current procedures for treating HCC, according to the study's findings.

1.3 Research Significance

This thesis is focused on exploring and creating safe, affordable strategies for protecting the liver and preventing cancer, utilizing green-synthesized nanoparticles and natural herbal extracts. Certain plant-derived compounds, including flavonoids, terpenoids, and polyphenols, have been widely recognized for their strong antioxidant, anti-inflammatory, and protective effects on cells. These properties are particularly important because oxidative stress is a key factor

in the development of liver cancers like hepatocellular carcinoma (HCC) and other types of liver cancer. These natural compounds help counteract oxidative stress by neutralizing harmful free radicals, protecting cellular lipids, maintaining stable cell membranes, and supporting overall cellular health. One of the plants under study is *Saussurea costus*, commonly known as Indian costus, which has been used for centuries in traditional herbal medicine due to its anti-inflammatory and liver-protective qualities. It is frequently used to treat various digestive and liver-related conditions. Through scientific research, both the methanolic extract and nanoparticle form of this plant can offer deeper insights into its numerous health benefits and therapeutic potential. Additionally, this work contributes to the broader field of green nanotechnology by developing an eco-friendly method to produce chitosan nanoparticles (CNPs). This method uses plant-based extracts as natural components to reduce and stabilize the nanoparticles, which helps the active ingredients remain effective during the process. This results in better absorption and a more controlled release of the compounds over time. The process also eliminates the need for harmful chemicals and harsh solvents, making it not only more environmentally friendly but also safer. By combining traditional herbal medicine with green nanotechnology, this study is paving the way for new, safe, and sustainable approaches to liver protection and cancer treatment. These approaches aim to improve the effectiveness of therapies while promoting both medical and environmental safety.

1.4 Research Hypothesis

In this study, we investigate the notion that methanolic Saussurea costus extract and green-synthesized chitosan nanoparticles are effective liver protectors and powerful antioxidants. Pesticides phenobarbital (PB) and 4-dimethylaminoazobenzene (p-DAB) are toxic to mouse livers. As a result of these

traits, these impacts could be lessened. Others have speculated that the nanoparticle form of the extract may be much more powerful than the original extract alone. Because nanoparticles are able to target the liver precisely, stay stable for a long period, and do not cause any gastrointestinal side effects, this is the case. For this reason, they have the potential to lower the raw extract's performance in a battery of chemical tests while simultaneously raising antioxidant activity, triggering positive molecular changes, and enhancing the appearance of liver tissue.

1.5 Research Objectives

The main goal of this study is to accomplish the following tasks:

1. To check how well the liver is working by looking at certain chemicals in the blood, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and albumin, in different groups of animals or samples.

2. To look at how much damage is being done to the liver due to free radicals and how well the body is fighting this damage by measuring levels of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx) .

3. To study the activity of certain genes that are linked to cell death, inflammation, and self-cleaning processes in the body. This involves checking how much the genes for Bax, TNF-α, INF-α, TGF-β, a method called RT-qPCR.

4. To examine the liver tissues under a microscope and use special staining techniques to find any changes in the structure, check for cell damage, and assess how severe any issues are in each group.

5. To see which is better at helping the liver heal from damage caused by DAB and PB using the methanolic extract of *Saussurea costus* or its nanoparticle form.

1.6 Research Limitations

Rats, which are commonly used as small laboratory animals, were the main focus of this research. A variety of techniques that relied on these animals were also part of the study. To gain a better understanding of the possible effects on the body from a specific extract of the *Saussurea costus* plant and nanoparticles made from environmentally safe materials, A total of 28 Rats were randomly divided into four separate groups.These Rats were kept in a controlled setting, allowing us to compare the impacts of various treatments. The main aim of the study was to explore how these compounds might help reduce oxidative stress and improve liver function. It's important to understand how oxidative stress affects the body because it is known to cause harm and has been linked to several health issues. Since this research was not meant to involve human subjects, no clinical trials or other human-related testing were carried out. The experiments took place between the years 2024 and 2025, and the findings suggested that combining nanotechnology with herbal extracts could be a promising approach for protecting the liver. However, it's important to keep in mind that these results are based only on animal studies and that the procedures are still in the early stages of development. To fully understand the potential medical applications of these technologies, more research is needed. This includes toxicological studies, investigations into how chemicals are absorbed and distributed within the body, and similar types of research. Before human trials can be considered, several important conditions must be met.

1.7 Structure of the Thesis

This thesis is divided into five main chapters, each focusing on a different part of the research:

1. Chapter One – Introduction: This chapter gives a general idea of the study's background, the problem being studied, why the research is important, the main idea or guess being tested, what the study aims to do, its limits, and sets up the basic ideas or concepts that guide the whole research.

2. Chapter Two – Literature Review: This chapter looks at previous studies about hepatocellular carcinoma (HCC), how oxidative stress works, and how natural products and nanoparticles can protect the liver and act as antioxidants. It also points out where previous research has not been enough, which makes this study necessary.

3. Chapter Three – Materials and Methods: This chapter describes all the materials used in the study, how plant extracts were made, how nanoparticles were made using a green method, the animal models used, the scientific tests done, and the ways the tissue samples were examined under a microscope.

4. Chapter Four – Results and Discussion: This chapter shares the findings from the experiments, including results from biochemical tests, antioxidant activity, molecular studies, and tissue analysis. It also explains what these results mean, compares them with previous research, and talks about what they tell us about the science behind the study.

5. Chapter Five – Conclusion and Recommendations: This chapter summarizes what was learned from the research, explains how the findings could be used in

medicine, and suggests what future studies could look into, as well as how the results might be used in real medical situations.

Chapter Two

Literature Review

2.1 Introduction

An important part of the study's scientific foundation, this chapter provides a systematic and comprehensive review of the most important studies and research on the topic at hand. Hepatocellular carcinoma (HCC) is a deadly cancer of the liver. In this part, Its biology and chemistry will be examined, along with whether antioxidants and plant extracts might help with prevention and treatment.. This chapter's strength is in the theoretical and conceptual groundwork it provides for the ongoing research, which in turn draws attention to cutting-edge developments in the area that combine conventional plant-based medicine with environmentally friendly nanotechnology. According to [61], this combination shows great promise as a new approach to creating safe and effective therapies for a range of liver-related diseases.

Oxidative stress, chronic inflammation, and exposure to carcinogens are the three main causes of liver cancer, a devastating disease. These compounds are known carcinogens, and they are poisonous and may harm cells in the body. Both the incidence of the disease and the number of deaths it causes rank liver carcinoma among the top cancers globally. How oxidative stress affects liver cells is a topic that has been the subject of much research. A buildup of reactive oxygen species (ROS) occurs in response to prolonged or extreme oxidative stress, and these molecules are toxic to many cellular components, including DNA, lipids, and proteins. These dangerous substances may do serious damage to the body if they accumulate to high enough levels.Researchers have started to pay close attention to natural plant extracts because of the wealth of beneficial compounds they contain, including flavonoids and phenolics. By bolstering essential antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD), these plant-based chemicals are believed to aid in the body's inherent

defensive mechanism against dangerous free radicals. Indian costus, formally known as *Saussurea costus*, is one plant whose potential medical uses are the subject of intensive research. The plant's active ingredients may provide substantial protection for the liver, decrease inflammation, and strengthen the antioxidant defenses of the body (reference [62]). Natural medicines have always been there, but because to developments in environmentally friendly nanotechnology, they are now even more powerful. By altering their physical and chemical characteristics, scientists are turning these compounds into nanoparticles. Increased stability, better absorption, and more precise distribution of the active components are just a few of the benefits of this method. Despite the scarcity of treatments for progressive and chronic illnesses like cancer, a novel strategy that combines nanotechnology with conventional herbal therapy has enormous promise. Healthcare and sustainable development might both benefit greatly from this integration of traditional medicines and contemporary science. This section delves into the following subjects:

1. The role of plant extracts and antioxidants in protecting liver tissues against oxidative damage.
2. The chemical carcinogenic agents commonly used in experimental liver cancer models and their biochemical mechanisms of action.
3. The latest research developments concerning the use of plant-based extracts and green nanotechnology in combating hepatocellular carcinoma.

By thoroughly analyzing this topic, the chapter seeks to clearly define the specific area of research that this study is intended to investigate. This helps establish a solid scientific foundation for examining the antioxidant and liver-protective effects of the methanolic extract of *Saussurea costus* and its green-synthesized nanoparticles. The main goal of this research is to understand how these substances

can provide protection to the liver from damage caused by harmful chemicals such as p-Dimethylaminoazobenzene (p-DAB) and Phenobarbital (PB) in laboratory Rats [63].

2.2 Antioxidant and Therapeutic Extracts Used in Liver Protection

In order to provide a solid scientific foundation for investigating the antioxidant and liver-protective properties of the methanolic extract of *Saussurea costus* and its green-synthesized nanoparticles, this analysis seeks to emphasize the particular research topic that this study is focused on [64].

2.2.1 Methanolic Extract:

For this study, the main method used to prepare the sample was the methanolic extraction of *Saussurea costus*. Methanol is a very effective solvent for extracting phytochemicals because of its special chemical and physical features. This solvent helps dissolve and extract various bioactive components from the aerial parts and roots of medicinal plants, such as phenolic acids, flavonoids, terpenoids, and alkaloids. Methanol is a polar protic solvent, which means it can interact with both polar and non-polar substances. These bioactive compounds are known for their powerful antioxidant, anti-inflammatory, and liver-protective properties, which have made them widely recognized and studied.[65]

As the extraction process begins, methanol starts to penetrate the walls of the plant cells, helping to release antioxidant molecules and other compounds that are normally trapped inside. The medicinal benefits of *Saussurea costus* are largely attributed to the combination of secondary metabolites present in its final methanolic extract. Research has demonstrated that this extract is very effective at neutralizing free radicals, especially reactive oxygen species like hydrogen

peroxide, hydroxyl radicals, and superoxide anions. These harmful chemicals are closely linked with oxidative stress in the liver, and their presence can contribute to the development of cancer.The methanolic extract of *Saussurea costus* has been proven in multiple studies to boost the activity of important enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase. These enzymes are vital in reducing lipid peroxidation and protecting cells from oxidative damage by maintaining a balance between oxidation and reduction in the cell. Moreover, the extract may also influence the expression of genes related to oxidative stress [66]. Methanol's quick evaporation and good solubility in water make it an excellent choice for extraction. This property allows the extract to be more concentrated without leaving behind harmful residues that could interfere with future experiments. Additionally, the active compounds are consistently and reliably produced in methanol, which means that the results of various studies can be trusted and replicated [67] .

The methanolic extract of *Saussurea costus* is a stable and strong antioxidant preparation, rich in bioactive compounds. In the liver, it can help reduce oxidative stress caused by carcinogens. Because of its high polarity and comprehensive biochemical profile, it shows great potential as a natural treatment for managing and preventing liver cancer, and it is effective in recovering a wide range of phenolic and flavonoid compounds.

2.2.2 Nanoparticle Form (Green-Synthesized Chitosan Nanoparticles):

"Green nanotechnology" is a relatively young field of research that has lately generated a lot of interest. The fundamental objective of this area of research is to develop environmentally and socially safe nanoparticles. These developments have made the production of these nanoparticles quite easy. These nanoparticles have a

wide range of potential medical applications and are entirely safe for human usage. As stabilizers and reducing agents, these nanoparticles are made using a broad range of natural substances, the majority of which come from plants. This research mainly focused on nanoparticle combinations using chitosan and a methanolic *Saussurea costus* extract. The main objectives of this technique were to enhance the stability, bioavailability, and efficiency of the extract's transport to the liver. The nanoparticles were transported using chitosan, a natural polymer. Shrimp and crab, along with other crustaceans, contain chitin, the primary component of chitosan. Chitosan was chosen because it can attach to mucosal membranes, is naturally biodegradable, and is safe for humans to use. When produced in an acidic environment, nanoparticles made of chitosan may find it simpler to infiltrate cells because the positively charged chitosan may link to the negatively charged surfaces of the cells. Ionic gelation and electrostatic interactions are two methods by which chitosan may create solid nanostructures, which in turn allow for the controlled and gradual release of active compounds. One feature of traditional methods for making nanoparticles is the use of chemicals, which may be harmful and drain resources quickly [68].

However, our work avoided these issues by using a green synthesis method. This was replaced by the use of proteins, phenolic acids, flavonoids, and terpenoids found in the methanolic extract of *Saussurea costus*. The production method was less detrimental to the environment due to the use of these natural compounds as reducing and capping agents. Scientists combined these plant-based ingredients with chitosan to create nanoparticles that have strong biological activity and strong structural features. Potentially potent therapeutic benefits of a combination of chitosan nanoparticles and *Saussurea costus* extract have been suggested [69].

Targeted Liver Delivery: Due to the nanoscale size (typically between 50–150 nm) and surface charge of chitosan nanoparticles, they can efficiently accumulate in hepatic tissue, allowing for direct interaction with hepatocytes. From a pharmacological perspective, the incorporation of S. costus extract into chitosan nanoparticles provides several key benefits:

1. Controlled and Sustained Release: The polymeric matrix of chitosan enables gradual diffusion of the encapsulated extract, maintaining a steady therapeutic concentration and reducing dosing frequency.
2. Reduced Toxicity: The green synthesis route eliminates residual solvents and toxic reagents, ensuring biosafety and compatibility with biological systems.[70]

Recent studies have shown that chitosan-based green nanoparticles can significantly boost the activities of antioxidant enzymes such as Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione Peroxidase (GPx). These nanoparticles also help in lowering the levels of lipid peroxidation markers, like Malondialdehyde (MDA), and support the recovery of liver tissue that has been damaged by chemical substances. The positive outcomes are linked to the better absorption and distribution of active components within the body, as well as their continuous engagement with the body's natural antioxidant systems in the liver. Furthermore, the use of natural materials like chitosan supports the development of sustainable nanomedicine, which is not only focused on creating effective treatments but also on being kind to the environment. In conclusion, the chitosan nanoparticles created through a green method and combined with the methanolic extract of *Saussurea costus* offer a new, safe, and effective way to treat liver diseases. These nanoparticles are powerful in their antioxidant effects, are made in an eco-friendly manner, and are able to target the liver efficiently. All these

qualities make them a strong and promising alternative to traditional chemotherapy drugs for the prevention and treatment of hepatocellular carcinoma [71].

2.3 Carcinogenic Agents Used in Experimental Hepatocellular Carcinoma

It has been noticed that some harmful chemicals can act like real diseases, changing how the body works and causing features similar to human liver cancer. Because of this, they are useful for studying a type of liver cancer called hepatocellular carcinoma (HCC) in animals. This research used two chemicals, phenobarbital (PB) and p-Dimethylaminoazobenzene (p-DAB), which are known to cause liver cancer. These chemicals were chosen because they create oxidative stress, a type of stress that can mess up the body's normal balance and turn healthy liver cells into cancerous ones. By using both of these chemicals together, scientists can create a strong model to test how well certain substances that protect the liver and reduce oxidative stress can help prevent or treat liver cancer. This method helps scientists understand more about this serious illness and find ways to control or stop it. [72]

2.3.1 p-Dimethylaminoazobenzene (p-DAB):

P-Dimethylaminoazobenzene, or Butter Yellow as it was more often known, was an azo dye that found widespread application in many different consumer goods for quite some time. It was a trailblazer in this field since it was one of the first synthetic chemicals linked to an elevated risk of liver cancer. The existence of strong chemical connections. Its chemical formula is C14H15N3.Metabolic activation is the primary mechanism by which p-DAB causes cancer. This reaction mostly takes place in the liver and is mediated by the cytochrome P450 system, namely the CYP1A2 enzyme. Electrophilic intermediates, which are very reactive and dangerous compounds, are formed when p-DAB goes through chemical modifications throughout this process. Important molecules like DNA and proteins

may be bound to these intermediates by covalent bonding. The establishment of these bonds has the potential to produce mutations, chromosomal abnormalities, and alterations in gene function all of which may contribute to the development of liver cancer [73].

In spite of its metabolic role, p-DAB produces ROS that may directly damage DNA, including superoxide and hydrogen peroxide. Additionally, these ROS may cause increased oxidative stress, which is detrimental, and damage to the lipids in the cells of the liver. Inflammation, mitochondrial malfunction, and programmed cell death are some of the bad outcomes that may arise from this kind of damage. Unchecked, these side effects might hasten the onset of liver cancer [74].

Symptoms of liver injury, such as tumor or abnormal growth development, enlarged cells, aberrant nuclei, and cell death, have been seen in animals exposed to p-DAB in studies. Significant damage to the liver cells is indicated by elevated levels of certain enzymes, including gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Because of these features, p-DAB is a promising material for use in developing animal models of liver cancer [75].

2.3.2 Phenobarbital (PB):

Barbiturates like phenobarbital (PB) are often prescribed by doctors to individuals with epilepsy so that they may relax and have fewer seizures. Even while PB may not alter DNA on its own, it can raise the risk of cancer when given after being exposed to p-DAB and other chemicals known to cause cancer. This is due to PB's immense capacity to boost cytochrome P450 enzyme activity, especially that of CYP2B1 and CYP2B2. These types of enzymes are essential for the elimination of xenobiotics, or harmful compounds, from the body[76]

Because PB accelerates the digestion and modification of harmful compounds, it is possible that an excess of reactive oxygen species (ROS) will be created when these enzymes are activated. Because of their instability, reactive oxygen species (ROS) pose a threat to cells, the liver's defenses, and oxidative stress. Since PB affects fat accumulation, cell activity, and liver growth, its use over an extended period of time may raise the risk of liver cancer.

Programed cell death, or apoptosis, is the body's normal way of getting rid of unhealthy or damaged cells; however, PB could stop this. Consequently, abnormal liver cells may not be able to die off properly and might ultimately multiply uncontrolled. Animals given PB often have higher blood levels of certain enzymes, including ALT, AST, GGT, and LDH [77].

If the liver isn't working properly, these levels will show it. Precursors to the fatal hepatocellular carcinoma may be found in histological examinations of liver tissue, which can also indicate abnormally big liver cells and abnormally fast cell divisions.

2.3.3 Combined Toxic Effect:

The concurrent administration of p-DAB and PB produces a synergistic carcinogenic effect that closely replicates the multistage process of human liver cancer development. This process involves two key phases:

2.3.4 Initiation Phase (p-DAB):

p-DAB acts as the initiating agent by inducing genetic mutations, DNA adduct formation, and chromosomal instability. These changes set the foundation for neoplastic transformation.

2.3.5 Promotion Phase (PB):

PB helps in the growth of liver cells that have already started to multiply by activating certain enzymes called cytochrome P450 and creating reactive oxygen species. These reactive oxygen species cause ongoing oxidative stress and inflammation, which damage the liver cells. When these harmful effects happen together, they throw off the balance between the body's ability to handle oxidative stress and its antioxidant defenses. This leads to a decrease in glutathione, which is a key antioxidant in the liver, and an increase in markers like malondialdehyde (MDA), which indicate damage to fats in the liver. These changes result in widespread death of liver cells, scarring of the liver tissue, and the development of abnormal growths called tumor nodules. This process shows how liver cancer develops gradually over time.[78]

Therefore, the model where liver cancer is induced using p-DAB and PB is a good setup to test how well certain treatments, such as the methanolic extract and green-synthesized nanoparticles from *Saussurea costus*, can help protect the liver and reduce oxidative damage in cases of chemically induced liver cancer.

2.4 Previous Studies

A tremendous rise in both funds and personnel has been seen in the past twenty years for research into the possible use of medicinal plants in the treatment and prevention of liver disease. Among the many impressive characteristics about these plants is the abundance of anti-inflammatory and free radical-fighting flavonoids and phenolics. Studies have only focused on one Saussurea costus plant. Among the several useful compounds found in the plant are dehydrocostus lactone and the anti-inflammatory costunolide, which may shield the liver from damage and delay the development of hepatocellular carcinoma (HCC), a kind of liver cancer. [79]

In 2016, Alnahdi and colleagues used a water-based Saussurea lappa root extract to study the effects of the herbicide deltamethrin on rats' livers. Their study indicated that rats administered deltamethrin had elevated levels of liver enzymes (ALT, AST, ALP, and GGT), which might be a sign of liver stress or injury. Reduced amounts of antioxidant enzymes (SOD, CAT, and GST) provide further evidence that the organism's ability to withstand oxidative stress has been impaired. Microscopically, the exposed liver tissues revealed obvious evidence of injury. On the other hand, rats given 300 mg/kg of the plant extract had much better liver enzyme levels and less tissue damage. It is clear from this that the positive benefits of the extract are due to its antioxidant activity. Because it was based on an acute toxicity model, the research neglected to include the pesticide's biological processes that produce long-term effects, such as cancer.[80]

The Researchers Al-Duais and colleagues (2017) looked at how well a water-based extract of Saussurea costus roots protected guinea pig livers against carbon tetrachloride. Centrilobular necrosis, a kind of liver cell death, was one outcome of carbon tetrachloride exposure. The researchers also found increased blood levels of certain enzymes, which are often signs of liver injury. The liver tissue of the guinea pigs showed improvement and these enzyme levels were drastically lowered when they were given the S. costus extract. The fact that the results were similar to those of the well-known medicine Liv-52 indicates that the extract might be helpful in avoiding liver damage. Scientists hypothesised that the extract's protective benefits stemmed from its high levels of antioxidants, which help the body ward off harmful substances. But they didn't use any fancy techniques to figure out what was in the extract chemically or which components were responsible for these benefits.[81]

In 2020 study, Tousson and colleagues set out to find out if a Saussurea lappa root extract might protect rats' livers from the damaging effects of the toxin ethephon. Damage, including increased transaminase levels, was seen in liver function tests conducted on rats exposed to Ethephon. The liver also showed obvious structural damage. However, the liver's structure was conserved and normal levels of these enzymes were restored when Ethephon and Saussurea lappa extract were combined. The presence of therapeutic chemicals, including antioxidant flavonoids and lactones, was believed to be responsible for this medical effect. Although the results were promising, the study did not reveal anything new about the molecular processes by which these medications work. This mostly impacted important pathways like Nrf2 or NF- ʁB. That is why it is still unclear the specific chemical pathways the extract uses to provide its protective effects.[82]

In 2021, Ansari and colleagues investigated the several advantages of extracts in depth, concentrating on their ability to fight cancer, protect cells from oxidative stress, and enhance liver function. The DPPH findings showed that the alcohol extract greatly decreased the number of harmful free radicals. Although carbon tetrachloride is known to cause liver damage, its negative effects were mitigated. There was hope that the extract may inhibit cancer cell growth in a controlled laboratory setting. The findings suggest that Saussurea lappa may have therapeutic potential, even though the experiments were carried out in a controlled laboratory environment and did not include real patients with liver cancer. On top of that, the researchers didn't use any complex delivery mechanisms, such as nanocarriers, to enhance the extract's efficacy.[83]

Elshaer and colleagues conducted study in 2022 to examine the possible preventive properties of Saussurea costus roots against sodium nitrite ($NaNO_2$)-induced liver and renal damage in rats. Sodium nitrite caused a lot of bad side effects in the rats

that the researchers tested. Among these problems were low levels of protective proteins like IL-4 and Bcl-2, elevated levels of inflammatory markers like TNF-α, and impaired liver and kidney function. When rats were administered different dosages of the *Saussurea costus* extract, the protective effects were shown to be dose-dependent; nonetheless, higher doses provided more protection. The medication had the same positive impact on the liver and kidneys when examined under a microscope. Although the study did not examine the plant extract's capacity to help with liver cancer or its transport via nanoparticles, the results are encouraging and may suggest that it has a protective effect, since the research only examined its overall toxicity. Elshaer and colleagues analyzed the chemical components found in S. costus roots in 2024 in order to find a remedy to this problem [84].

Based on models of chronic poisoning, the roots were discovered to contain phenolic and flavonoid compounds that exhibited strong antioxidant and anti-inflammatory properties. According to their findings, supplementation with S. costus enhanced overall physiological function and reduced damage caused by inflammation and oxidative stress. The researchers neglected to use nanotechnology to boost the plant extract's effectiveness and omitted a hepatocellular carcinoma (HCC) model from their study.There are additional researchers that are considering the use of eco-friendly chitosan nanoparticles to treat liver ailments.

In 2020, Bonferoni and colleagues put their results from a literature review on chitosan nanoparticles for HCC detection and treatment into publication. In particular, the assessment emphasized chitosan's biodegradability, compatibility with biological tissues, and positive electrical charge as distinguishing features. Some of the benefits of using nanoparticles include the capacity to control the rate

of medication release, reduce systemic side effects, and specifically target the liver. In spite of the limited research, the authors highlighted the advantages of combining chitosan nanocarriers with plant extracts.[85]

In the year 2021, Sun and colleagues created chitosan nanoparticles that were attached to galactose. The goal of this design is to zero in on the hepatocellular carcinoma cells' galactose receptors. According to their findings, the nanoparticles were able to inhibit the multiplication of cancer cells by binding to and absorbing them. The researchers also found that this technique improved several biochemical markers of liver function. However, synthetic drugs were used in the study rather for natural substances. Antioxidants found in plants, like those in S. costus, cannot yet be transported by similar nanocarriers.[86]

In 2024, Wang and colleagues sought to cure hepatocellular cancer by researching compounds based on chitosan. Their findings point to the possibility that these compounds may slow the formation of new blood vessels, lower levels of harmful molecules in the liver, and make controlled cell death easier. There may be cancer-fighting potential in chitosan-based medicines, according to their results. One possible drawback of the treatment that they did mention is the lack of study on mixing chitosan with natural antioxidants extracted from whole plants.[86] In 2025, Gebrie and colleagues successfully green synthesized CuO NPs using an extract from Plumbago zeylanica leaves. The generated nanoparticles' moderate antioxidant properties and high antibacterial activity were confirmed by a battery of microscopic and spectroscopic exploratory techniques. Since the study did not include testing in animal models or hepatic models, more research into *Saussurea costus* as a natural source for CuO NPs is necessary regardless of these findings. This might lead to new applications in the fight against cancer and liver disease.[87]

Past research suggests that chemically-induced liver malignancies, such as p-DAB and PB, have received less attention than acute liver injury models. In addition, research into creating effective nanocarrier systems using (*S. costus)* extracts has stalled. Moreover, extensive research on crucial molecular processes, including gene expression and pathways exhibiting indicators of oxidative stress, including Nrf2 and NF-κB, has been in short supply. By combining a methanolic *Saussurea costus* extract with eco-friendly chitosan nanoparticles, our study aims to address these problems. That combination will be tested in an animal model of liver cancer caused by p-DAB and PB. To discover the composition's antioxidant and liver-protecting mechanisms, we will undertake comprehensive biochemical, molecular, and histopathological investigations.[88]

2.5 Comparative Analysis and Research Gap

That is why the chemical induction mechanism employed in this work is a combination of p-DAB and PB. Potentially useful for gauging the success of liver cancer treatments and preventative measures, this approach is more in line with the disease's normal progression in humans. Historical research has limitations due to its reliance on traditional extraction methods for S. costus, such as ethanolic or water-based extraction [90].

While it's possible that these technologies can extract some bioactive compounds, they don't consider other distribution possibilities. The compounds' lack of medicinal efficacy might be due to their fast metabolism, poor solubility, or quick disintegration. In order to get around this, the current study used eco-friendly chitosan nanoparticles produced from a methanolic extract of S. costus. This not only makes the molecules more stable, but it also makes it easier for them to be delivered directly and gradually to the liver. Combining nanotechnology with

plant-based medicines is an innovative and intriguing step beyond traditional extraction techniques.

Ansari et al. (2021) and Elshaer et al. (2022, 2024) found that S. costus extracts exhibit antioxidant properties, but they didn't look into various ways of administering the extracts. The use of chitosan nanoparticles for medication administration in HCC has been investigated by Bonferoni et al. (2020), Sun et al. (2021), and Wang et al. (2024). Research on natural antioxidants was underrepresented in these studies compared to manufactured chemotherapeutic medications. None of the studies cited above even remotely addressed the possibility that nanocarriers' natural chemical interactions may have an impact on cancer.[91]

The Contrarily, our research integrates chitosan nanoparticles' biocompatibility and tailored delivery capabilities with the effective antioxidant characteristics of S. costus. This opens the door to a novel therapeutic path that may one day shield cells from free radicals and put a stop to cancer's progression.

Table (2.1) Summary of Key Differences and Research Innovation

Aspect	Previous Studies	Current Research
Model Used	Acute hepatotoxicity (CCl_4, $NaNO_2$, pesticides)	Chemically induced hepatocellular carcinoma (p-DAB + PB)
Plant Material	Aqueous / ethanolic extract of *S. lappa* or *S. costus*	Methanolic extract of *S. costus*
Technology Applied	Conventional extraction methods	Green synthesis of chitosan-based nanoparticles
Mechanistic Focus	Limited to enzyme and oxidative markers	Includes molecular signaling (Nrf2, NF-κB, TNF-α, Bcl-2)
Therapeutic Strategy	Phytotherapy only	Combined phytotherapy–nanotechnology approach

Aspect	Previous Studies	Current Research
Outcome Evaluation	Partial biochemical and histological recovery	Comprehensive biochemical, molecular, and histopathological evaluation

In short, this thesis makes a unique and important contribution by bringing together different areas of study, such as natural antioxidants and green nanotechnology, within a realistic experimental setup that closely mirrors the actual conditions of hepatocellular carcinoma. It tackles some of the key shortcomings in previous research, like the lack of effective nanoparticle delivery methods, insufficient molecular-level analysis, and the use of models that aren't cancerous. By doing so, this study introduces a new and environmentally friendly approach to treating hepatocellular carcinoma, using bio-nanomedicine based on *Saussurea costus*. This represents a fresh and promising direction in the fight against this type of cancer.

Chapter Three

Materials and Methods

3.1. Reagents and Instrumentation

The main material used to make the nanoparticles was medium-molecular-weight chitosan, which has a viscosity between 60 and 300 cP. This chitosan was bought from Bio Basic Inc. in Canada and has a degree of deacetylation higher than 90%. The chitosan was dissolved, and the pH was adjusted using sodium hydroxide and glacial acetic acid, both of which were obtained from El-Nasr Pharmaceutical Co. in Egypt. To extract the plant material, technical-grade methanol was used, which was purchased from Sigma-Aldrich in the U.S. Transmission electron microscopy (TEM) was done using a negative stain made from 2% (weight/volume) phosphotungstic acid in water. To prevent contamination and ensure consistent results, all water used for making solutions was double-distilled or of HPLC grade. The chitosan was dissolved in a controlled temperature environment using a magnetic stirrer, and the plant extract was added. For mixing, a Branson 450 Digital Sonifier was used for probe sonication, and an IKA T25 Ultra-Turrax homogenizer was used for high-shear mixing. A Büchi R-300 rotary evaporator was used to remove the solvent from the methanolic extract under low pressure. An Hitachi Himac CP 100WX ultracentrifuge was used to perform ultracentrifugation. A TECNAI G2 Spirit BioTwin TEM was used to look at the shape of the nanoparticles, while a Malvern Zetasizer Nano ZS was used to measure their charge and size. An Olympus BX53 light microscope was used to view prepared slides after tissue was cut using a Leica RM2235 microtome for histological analysis. Figure (3.1) shows the instruments used in this study, including those for making the nanoparticles, purifying them, and analyzing their properties.[92]

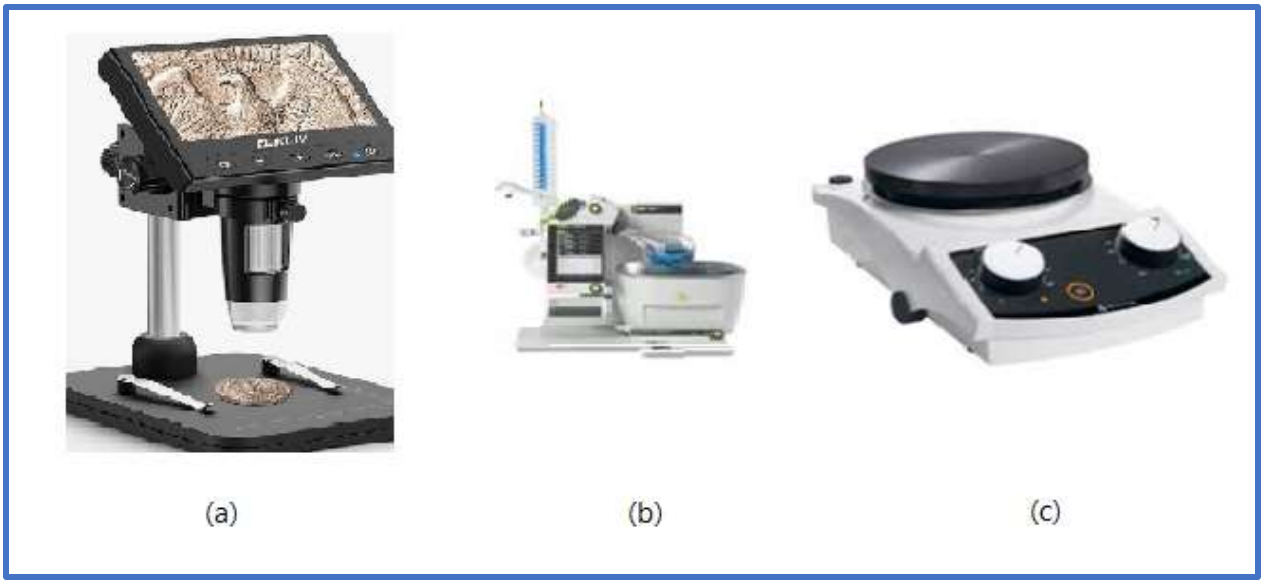


Figure (3.1) Laboratory instruments used for nanoparticle preparation, purification, characterization, and histological examination.

3.2. Plant Material and Extract Preparation

3.2.1. Botanical Source and Authentication

In November 2023, dried roots from the plant **Saussurea costus** (Falc.) Lipsch., which belongs to the Asteraceae family, were received from a supplier who is officially recognized for doing botanical work in Cairo, Egypt. The decision to use these roots for chemical extraction and to test their biological effects was based on the fact that they were in good condition, had a strong and noticeable smell, and showed no signs of mold or other harmful growth.It was important to check where the plant came from and how it was handled during transportation to make sure that its chemical makeup remained consistent. This involved getting documents from the supplier. The plant's identity was checked by a taxonomist at the Environmental Studies and Research Institute (ESRI), which is part of the University of Sadat City. The roots were examined under a microscope to confirm that they were genuine. The taxonomist looked at several important features, such

as the thickness of the roots, the texture on the outside, the pattern of the inside cross-section, and the strong scent.Using the microscope also helped in identifying the species by looking at specific internal characteristics, such as how the blood vessels were arranged, the presence of small channels that carry resin, and the way the cells were arranged in the tissue. These features were compared with information from reliable taxonomic references and descriptions from well-known sources, like the Flora of Egypt, to be absolutely certain about the correct identification.Once the identity of the plant was confirmed, a sample (EN-USC-21) was cleaned, labeled, and stored in the herbarium at ESRI for use in the future. This sample acts as a reference for any future verification. Keeping accurate records of this kind is very important in botanical research because plants can vary depending on where they are found, and there is a risk of confusing or even using fake medicinal plants. [93]

3.2.2. Pre-Extraction Processing

First and foremost, our main job was to make sure there was absolutely no contamination in the raw root material. took our time looking closely at the roots to check for any visible signs of dirt, mold, or damage. It was really important to confirm that the root material was completely free from any impurities before moved on to the next steps. Once was done with the initial visual check, carefully washed the roots using distilled water. If there was any dust, dirt, or plant debris stuck to them, made sure to remove it completely. These impurities could be really harmful because they might bring in bad bacteria or mess up the extraction process. To make sure got the natural active ingredients from *Saussurea costus* in the end product, this cleaning step very thoroughly. After washing, placed the roots in a cool, shaded spot with good airflow, and kept the temperature between 25 and 30 C^0. didn't leave them in direct sunlight; instead, dried them in a shaded area for

about two weeks. This was because several important plant compounds, like essential oils, polyphenols, and sesquiterpene lactones, are very sensitive to both heat and light. If these components were lost during drying, the medicinal properties of the plant would decrease, and the final product wouldn't be as effective. To protect these key phytochemicals and stop bacterial growth, dried the roots at the right temperature with proper air circulation. When the roots were fully dry and brittle, it meant they were ready for the next processing step. The next step was to grind the dried roots into a fine powder using a Retsch SM 200 grinder. A machine was needed to grind the fibrous plant material consistently and effectively. Grind the roots into a fine powder increased the surface area, which made it much easier to extract the active compounds using solvents in the next step. Once the grinding was done, the powder was then passed through a 60-mesh stainless steel sieve with a mesh size of 250 micrometers. This helped to break down any large particles that might not dissolve completely, leading to a more uniform powder. Keeping the particle size consistent ensured better results, improved extraction efficiency, and even mixing with the solvents used. Finally, the powdered material was stored in dark-colored glass canisters. This was a very important step because powders can break down or get contaminated by bacteria if they come into contact with moisture. The canisters not only stored the powder but also helped to block out light, which could cause some of the compounds to break down. The substance was kept in a controlled environment until it was needed for the methanolic extraction process. [94]

3.2.3. Methanolic Extraction

First and foremost, our primary responsibility was to ensure that there were absolutely no signs of contamination in the raw root material. As part of this process, carefully examined the roots for any obvious signs of dirt, mold, or

physical damage. It was extremely important to confirm that the medicine was entirely free from any impurities before moving forward with further steps. Once had finished our initial visual inspection, gently cleaned the roots using distilled water. If there was any dust, dirt, or plant debris stuck to the roots, thoroughly removed it. These impurities could pose a serious threat to the quality of the final product because they might introduce harmful bacteria or interfere with the extraction process. In order to accurately capture the natural active compounds, present in *Saussurea costus* in the end product, it was essential to follow this cleaning procedure thoroughly. Once the washing was complete, the roots were placed in a cool, shaded area with proper ventilation, and the temperature was kept between 25 and 30 C^0. The roots were not left in direct sunlight but were instead dried in a shaded location for about two weeks. This choice was made because several significant plant compounds, such as essential oils, polyphenols, and sesquiterpene lactones, are sensitive to both heat and light. If these components were lost during the drying process, the medicinal properties of the plant would diminish, and the final product would not be as effective. To maintain the integrity of these vital phytochemicals and prevent bacterial growth, the roots were dried at an appropriate temperature with good air circulation. When the roots were completely dry and brittle, it indicated that they were ready for the next phase of processing. The next step in the process was to grind the dried roots into a fine powder using a Retsch SM 200 grinder. A machine was necessary to consistently and effectively grind the fibrous plant material. Grinding the material into a fine powder increased its surface area, which made the subsequent After grinding, the plant material was converted into a fine powder to enhance the efficiency of the subsequent solvent extraction of active compounds. The resulting powder was then passed through a 60-mesh stainless steel sieve with a pore size of 250 µm to remove larger particles that might not dissolve completely. This sieving step

helped obtain a more homogeneous powder with a consistent particle size, thereby improving extraction efficiency and ensuring more uniform contact between the powdered material and the solvents. The prepared powder was finally stored in dark-colored glass containers to protect it from light exposure, moisture, and possible microbial contamination. This storage condition was essential to preserve the stability of the bioactive compounds until the material was used for methanolic extraction. [95]

3.3. Preparation of *S. costus*–Loaded Chitosan Nanoparticles (SC-CNPs)

3.3.1. Chitosan Stock Solution

At a temperature of 40 C^0, a 1% (w/v) aqueous acetic acid solution containing chitosan was prepared by gradually incorporating the polymer into the solution while rapidly mixing it at 1500 rpm for four hours. This naturally occurring polysaccharide will not dissolve entirely in either mildly acidic or neutral environments. Dissolving it properly calls for an acidic environment. Chitosan becomes much more soluble when its amino groups change from a neutral ($-NH_2$) to a positively charged ($-NH_3^+$) form under these conditions. In order to avoid clumping and provide thorough particle coverage before equal dissolution, the chitosan was added extremely slowly. In the end, the result of carefully controlling the heat and keeping the combination in constant motion was a viscous, clear, smooth, and evenly mixed solution. All of the polymer chains exhibited uniform hydration.

By gradually adding 0.1M NaOH while stirring constantly, the pH of the produced chitosan solution was brought to a range of 4.5 to 5.0. chose this specific pH range to compensate for some of the excess acetic acid without completely neutralizing the positive charge of the chitosan amino groups. As the nanoparticles' negatively

charged plant compounds react strongly with the chitosan molecules' positively charged groups, maintaining an adequate protonation level is of the utmost importance. By controlling the pH, the chitosan solution may be made into stable and homogeneous nanoparticles without losing its outstanding solubility, reactivity, or viscosity.

Changing the pH of the solution gradually during the process was essential to avoid it from getting too alkaline too quickly. This might cause the chitosan to start precipitating out too quickly or to agglomerate into clusters of varying sizes. The physical and chemical properties of the (*S. costus)*-loaded chitosan nanoparticles were monitored by using this final chitosan solution as a starting material for their production.[96]

3.3.2. Aqueous Extract Dispersion

The dried methanolic extract (CME) was weighed precisely to 1 (1g), and (10 ml) of deionized water were carefully mixed again to create a concentrated aqueous solution for making nanoparticles. To prevent any problems with nanoparticle formation or changes to chitosan's electrical properties later on, deionized water was used for this step. High shear pressure was applied for 5 minutes to blend the mixture thoroughly. This process helps break apart bigger chunks of the dried extract, allowing the liquid to better dissolve the plant chemicals. Homogenization also helps create a more even and consistent mixture, which is important for making nanoparticles with uniform size and properties. The next step was combining the ingredients and then placing them in an ice-cold ultrasonic bath for 15 minutes of sonication. The sonication was done in short bursts—30 seconds on and 10 seconds off to prevent the mixture from getting too hot. The ultrasonic waves create a powerful effect called cavitation, which helps break up any leftover

clumps in the extract and spreads the material evenly. Some compounds, like sesquiterpene lactones, flavonoids, and essential oils, are sensitive to heat, so using ice in the sonication bath was necessary to keep the temperature low. After the homogenization and sonication were done, the mixture was filtered through a 0.45 (µm) membrane filter. This step removed any large, undissolved particles and made the solution smooth and uniform. Removing these particles is important because they can affect the stability of the nanoparticles, make size measurements unreliable, or result in an uneven final product. Adding the filtered water extract to the nanoparticle production process was straightforward.[97]

3.3.3. Formation of Nanoparticles

The pre-filtered aqueous S. costus extract dispersion was carefully introduced into the chitosan solution in a ratio of 1:4, meaning that for every part of the extract dispersion, four parts of the chitosan solution were used. This slow addition process was very important because it helped prevent areas in the mixture from having too much of the extract at once, which could cause the extract molecules to clump together too soon or form particles that weren't uniform in size. To keep everything well mixed and ensure that all the components interacted properly, a magnetic stirrer was used at a speed of 1000 (rpm) throughout the process. This continuous mixing was key to making sure the active ingredients from the plant extract and the chitosan chains in the solution mixed thoroughly and evenly.

The temperature of the reaction mixture was kept at 50 degrees Celsius. This temperature was chosen because it allowed the chitosan to move around more easily, which is important for forming nanoparticles, but it also helped to protect the sensitive plant compounds from being damaged by high heat.

The pH of the solution was closely monitored and held constant at 4.5 throughout the entire nanoparticle formation process. At this pH level, the amino groups in the chitosan remained positively charged, which is vital because it allows for electrostatic interactions with negatively charged or electron-rich molecules found in the plant extract. These interactions help bring the different components together, encouraging them to form stable structures. Keeping the pH at 4.5 also helps maintain a good balance between how well the chitosan dissolves, how reactive it is, and how strong the structure of the resulting complex becomes.

Under these carefully controlled conditions, the various compounds found in the S. costus extract acted as natural binders and stabilizers. Several of these compounds like phenolic compounds, flavonoids, and sesquiterpenes contain functional groups that can form hydrogen bonds or electrostatic attractions with the protonated amino groups of the chitosan. These kinds of non-covalent bonds are what drive the assembly of the nanoparticles, allowing them to naturally come together and form stable, chitosan-based nanoparticles that contain the extract inside them. This method of making the nanoparticles uses natural ingredients instead of harmful chemical cross-linkers that are commonly used in other nanoparticle manufacturing processes, which makes it a safer and more environmentally friendly way to create nanoparticles that can be used in biomedical applications.

The entire process of making the nanoparticles including dissolving the chitosan, mixing in the extract dispersion, creating the nanoparticles, cleaning them up, and collecting them was shown in a schematic diagram in Figure (3.2). This diagram provides a clear visual of the steps involved in the nanoparticle formation process and emphasizes the principles of green chemistry that were used in the synthesis of these SC-CNPs.[98]

3.3.4. Purification of Nanoparticles

At pH 4.5, the primary amino groups of chitosan are protonated, converting CS-NH2 into positively charged CS-NH3+ sites. The S. costus extract contains phenolic, flavonoid, and sesquiterpene-lactone compounds. Representative constituents include costunolide (C15H20O2) and dehydrocostus lactone (C15H18O2), while flavonoids have the general C6-C3-C6 skeleton. These functional groups associate with chitosan through the non-covalent equilibria below [35], [49], [68].

$$\text{Chitosan repeat unit} = (C_6H_{11}NO_4)_n \tag{3.1}$$

$$\text{Costunolide} = C_{15}H_{20}O_2 \tag{3.2}$$

$$\text{Dehydrocostus lactone} = C_{15}H_{18}O_2 \tag{3.3}$$

$$CS - NH_2 + H^+ \rightleftharpoons CS - NH_3^+ \quad (pH = 4.5) \tag{3.4}$$

$$CS - NH_3^+ + R - COO^- \rightleftharpoons \left[CS - NH_3^+ \cdots R - COO^-\right]_{ES} \tag{3.5}$$

$$CS - OH + O = C - R \rightleftharpoons [CS - O - H \cdots O = C - R]_{HB} \tag{3.6}$$

$$CS_{hyd} + R_{terp} \rightleftharpoons [CS \cdots R_{terp}]_{HYD} \tag{3.7}$$

Here, ES, HB, and HYD denote electrostatic association, hydrogen bonding, and hydrophobic association, respectively. These are physical interactions rather than newly formed covalent bonds. Together, they promote phytochemical loading, self-assembly, and colloidal stabilization of the SC-CNP dispersion [40], [47], [68].



Figure (3.2) *illustration of the green synthesis steps of Saussurea costus–loaded chitosan nanoparticles (SC-CNPs), including chitosan preparation, extract dispersion, nanoparticle formation, and purification.* [99]

3.4. Physicochemical Characterization of SC-CNPs

3.4.1. Dynamic Light Scattering (DLS) and Zeta Potential

Before the particle size study could begin, a small amount of the freshly made nanoparticle solution was diluted ten times using deionized water. This step was taken to reduce any issues caused by light scattering, which can interfere with accurate results. Temperature has a direct effect on Brownian motion, which is the random movement of particles in a fluid. Since Brownian motion affects how particle size is estimated, the diluted samples were allowed to cool down to a stable temperature of 25°C before any analysis was carried out. The measurements were performed using a Zetasizer Nano ZS from Malvern. This instrument uses dynamic

light scattering (DLS) to measure the hydrodynamic particle size and the polydispersity index (PdI), while electrophoretic light scattering is used to measure the zeta potential. To make sure that the results were both accurate and consistent, each measurement was repeated three times. DLS is a technique used to estimate the hydrodynamic diameter of particles, which represents their actual size when suspended in a liquid, including the layer of water molecules surrounding them. A lower (PdI) value indicates that the particles are more uniform in size, which is important for their predictable and consistent behavior in biological systems. This uniformity was assessed through the (PdI) value. The zeta potential measurements provided information about the surface charge of the nanoparticles. This charge plays a key role in determining how stable the nanoparticles are in solution, whether they tend to clump together, and how they interact with each other. A high zeta potential, either positive or negative, suggests strong electrostatic repulsion, which helps keep the nanoparticles suspended in the solution. When considering the behavior of nanoparticles in living organisms, both their surface charge and particle size are important factors. These characteristics influence how the nanoparticles are taken up by cells, how they spread throughout the body, how they interact with proteins to form a protein corona, and ultimately how effective they are in delivering treatment. Figure (3.2) shows the main results, with a peak in the nanometer range for the SC-CNPs. The nanoparticles were produced with well-defined and consistent properties that are suitable for biomedical applications, as evidenced by the narrow size distribution visible in the image .[100]

3.4.2. Transmission Electron Microscopy (TEM)

A small amount of Sc-CNP suspension that had been properly diluted was carefully added to carbon-coated copper grids to closely examine the shape of the nanoparticles. It was important to dilute the sample because if too many particles

were on the grid, they would crowd together and it would be hard to see individual ones clearly. After placing the suspension on the grids, the sample was left for a few minutes to let the nanoparticles settle and attach themselves to the carbon layer evenly.

To avoid disturbing the arrangement of the particles, filter paper was used to gently remove any extra liquid. Then, right away, a 2% (w/v) solution of phosphotungstic acid at pH 7 was added as a negative stain. This technique surrounds the nanoparticles instead of piercing through them, which helps to show their outer shape while keeping their inner structure intact, making the images easier to see.To keep the nanoparticles stable and avoid any unwanted changes caused by heat, the grids were allowed to dry naturally at room temperature. The TECNAI G2 Spirit BioTwin transmission electron microscope, which operates at 200 kV, was used to look at the prepared grids. This high voltage allows for better resolution and deeper penetration into the sample, making it possible to see fine details like the smoothness of the particle edges, their boundaries, and variations in their internal density.The images produced gave high-resolution information about the shape and distribution of the nanoparticles, which couldn't be obtained using DLS alone. This method provided a more detailed view of the nanoparticles' structure and how they are spread out.[101]

3.5. In Vivo Evaluation

3.5.1. Ethical Approval and Experimental Animals

Under the approval number MU-BCH-2024-11, the Institutional Animal Ethics Committee (IAEC) of Misurata University thoroughly reviewed and officially approved the in vivo experimental procedure. Throughout all stages of the research, the National Institutes of Health (NIH) Guide for the Care and Use of

Laboratory Animals was strictly followed, ensuring that all activities were conducted in line with internationally recognized ethical standards for animal welfare, humane treatment, and the reduction of animal suffering. All individuals involved in handling animals before the start of the study were adequately trained to meet both institutional and federal regulations. To eliminate any potential complications arising from sex-based hormonal differences, adult male Rats that were within the appropriate weight range for the hepatocellular carcinoma (HCC) model were selected. In order to accurately determine the appropriate dose of the carcinogen and subsequent treatments based on body weight, each mouse's weight was measured at the beginning of the study, as detailed in Figure (3.3). The animals were housed in clean polypropylene cages with sterilized bedding under controlled environmental conditions, including a temperature range of 22–25°C, a relative humidity level of 50–60%, and a regular 12-hour light/12-hour dark cycle. These controlled conditions were essential for minimizing physiological stress and maintaining consistent metabolic and behavioral patterns throughout the study. To support normal physiological functioning and avoid any complications caused by varying nutritional factors that could influence liver metabolism or tumor development, the animals had unrestricted access to standard mouse chow and filtered water. Before the experiment started, the Rats were allowed to acclimate to the laboratory environment for at least one week to help stabilize their endocrine and immune responses. Based on the experimental design and statistical power calculations outlined in the study plan, a total of 28 Rats were used in this investigation. During the approximately three-month observation period, routine monitoring of clinical symptoms, body weight, behavioral changes, and overall health was conducted. [102].



Figure (3.3) *Weighing experimental Rats prior to carcinogen administration and treatment to determine accurate body-weight–based dosing.*

3.5.2. Experimental Design and Grouping

To reduce the chance of choosing animals based on personal preference and to make sure that each group starts off as similar as possible, the animals were divided into four groups of equal size, with seven animals in each group (n = 7 per group). This random approach helped spread out any natural differences between animals so that any changes seen in their responses could more likely be because of the treatments they received, rather than because they were different to begin with.

3.5.2.1 G1 – Control Group

For the duration of the trial, the animals in this group were given merely drinking water and a typical mouse diet. Neither therapeutic drugs nor carcinogens were

present. When evaluating normal liver anatomy, function, and biochemical indicators, this group was used as the baseline reference.

3.5.2.2 G2 – DAB + PB Group (HCC Model)

In this group, the Rats were given 4-dimethylaminoazobenzene (DAB) first, and then they were treated with phenobarbital (PB), following a well-known method that is commonly used to start and promote the development of hepatocellular carcinoma (HCC). DAB starts the process of liver cancer formation by creating harmful chemical attachments to DNA, which can lead to mutations. PB, on the other hand, helps in making the liver cells divide more quickly, which in turn supports the growth of tumors. This group of Rats serves as a model for the disease, allowing researchers to compare and assess how effective the extract and nanoparticles are in treating HCC.

3.5.2.3 G3 – DAB + PB + Extract (CME) Group

Animals in this group were given the crude methanolic extract (CME) of *Saussurea costus* at a predetermined therapeutic oral dose (mg/kg) after tumor formation with DAB and PB equal to G2. This group's objective was to assess the crude extract's possible anti-HCC and protective properties in order to provide a standard by which the performance of nanoparticles could be evaluated.

- **G4 – DAB + PB + Nano-extract (SC-CNPs) Group**

After the same cancer-inducing procedure as Groups G2 and G3, this particular group of Rats were given *Saussurea costus*-loaded chitosan nanoparticles (SC-CNPs). In order to provide a fair and accurate comparison of the treatments' efficacy, the dosage of nanoparticles was adjusted so that they supplied the same quantity of plant-based compounds as the crude methanolic extract (CME) used in

Group 3. Being smaller, more stable, and able to release their active elements more gradually should allow SC-CNPs reach cells better and have a greater impact, which is why it was believed that they would perform better than the crude extract. Detailed timing information on the administration of the cancer-causing drug, administration of the therapy, and sampling for testing is provided in Figure (3.4), which depicts the whole procedure that occurred in the live Rats. In addition to depicting the course of the illness and its therapy throughout the course of the three months of the research, this graphic provides a comprehensive chronology of both.

The carcinogens and therapies, including the SC-CNPs and crude extract, were administered via conventional ways, such as intravenous injections into the belly or oral administration, as shown in Figure (3.4). All the animals received the same quantity of each chemical, adjusted for their body weight, thanks to these methods of administering the medications. This made the findings more reliable by reducing variations in the amount of the chemical given to each animal. [103]



Figure (3.4) *A schematic timeline of the in vivo experimental design showing carcinogen induction, extract/nanoparticle treatment phases, and scheduled sampling points for biochemical and histopathological evaluations.*



Figure (3.5) *Intraperitoneal or oral administration of carcinogenic or therapeutic agents according to the experimental grouping protocol.*



Figure (3.6) *Housing conditions of experimental Rats in specialized cages designed to induce mild oxidative stress, which helps accelerate the progression of DAB/PB-induced hepatocarcinogenesis.*

3.5.3. Induction of Hepatocellular Carcinoma (DAB + PB Model)

A traditional two-stage initiation-promotion model was used to develop hepatocellular carcinoma (HCC). In this model, phenobarbital (PB) acts as the tumor-promoting agent, while 4-dimethylaminoazobenzene (DAB) serves as the initial chemical that starts the process. This model closely mimics the gradual changes in liver tissue that occur during human liver cancer and has been widely studied in experimental research related to liver cancer development.

During the initiation phase, DAB, which is an aromatic azo-dye known to cause liver cancer, is added to the animals' food and given consistently for a specific time period. This helps in starting the process of cancer development. The liver enzymes called cytochrome P450 play a key role in converting DAB into harmful chemicals that are reactive and can bind to DNA, forming lasting DNA adducts. These changes in the DNA can lead to the formation of pre-cancerous liver cells and genetic alterations. Instead of causing immediate harm, the method of delivering DAB through food ensures a slow and steady absorption, which leads to long-term damage in the liver.

In the promotion phase, phenobarbital is administered either directly into the abdomen or mixed into the drinking water, following the standard laboratory procedures. PB is known to activate the constitutive androstane receptors (CAR), which increases oxidative stress in the liver and encourages the growth of abnormal liver cells. This stimulation leads to the expansion of the early liver lesions that were started during the initiation phase. This growth eventually leads to the formation of cancerous nodules and, finally, hepatocellular carcinoma.

The entire study spanned three months and involved both DAB and PB treatments. The duration and dosage of each drug were selected based on established protocols

from previous scientific literature, ensuring that the process reliably induces chronic liver damage, including fibrosis, pre-cancerous areas, and the growth of cancerous tissue. At the same time, it avoids causing excessive death or harmful side effects throughout the body. This approach provides a stable and repeatable model for testing the potential of an extract from the plant *Saussurea costus* and its nano-sized form. [104]

3.5.4. Treatment Protocol

Therapeutic treatments were commenced according to the experimental strategy after the carcinogen exposure phase was over and the induction period had elapsed. Oral dosages of either the crude methanolic extract (CME) in Group 3 or *Saussurea costus*-loaded chitosan nanoparticles (SC-CNPs) in Group 4 were given to the animals every day as part of the treatment groups. The oral delivery route was used to replicate the physiological pathways of exposure and to guarantee uniform and regulated doses throughout the study.

According to the research, the therapy phase lasted for the last three months of the trial, either concurrently with or immediately after the carcinogen exposure's final stages. The compounds' therapeutic and preventative effects on the development of DAB/PB-induced hepatocarcinogenesis may be evaluated at this time. The dosages of CME and SC-CNPs were standardized to have an identical quantity of phytochemical content in order to allow for a direct comparison between the crude extract and its nanoformulation.

The animals were observed extensively throughout the study to gauge their overall health and treatment tolerance. We monitored the Rats's weight, food and water consumption, and looked for symptoms of liver malfunction or systemic toxicity, including lethargy, changes in grooming behavior, jaundice, or enlargement of the

belly, once a week. Early identification of adverse events and improved interpretation of therapy outcomes were both made possible by continuous clinical monitoring.

Following a night of fasting, the animals were put to sleep using a recognized inhalational technique to guarantee humane treatment, as seen in Figure (3.7). The goal was to reduce the potential for post-meal fluctuations in metabolic rate as the trial came to a close. Sedation was followed by compassionate euthanasia, and blood samples were obtained for molecular and biochemical studies by puncturing the animals' hearts. Detailed histological investigation, gene expression profiling, and oxidative stress tests were part of the subsequent testing that followed the rapid removal and cleaning of the liver tissues with cold saline. These approaches laid forth a thorough plan for evaluating CME and SC-CNPs as prospective therapeutics for lowering DAB/PB-induced hepatocellular carcinoma.[105]



Figure (3.7) *Inhalational anesthesia of Rats using a funnel-based chamber to facilitate humane handling and sample collection.*

3.6. Biochemical and Antioxidant Assays

3.6.1. Serum Liver Function Markers

Cardiac puncture is a method that ensures a good amount of blood is collected and helps prevent the breakdown of red blood cells, which can make the sample less reliable. This technique was used to take blood samples right after the animals were sacrificed. It allows for a fast and efficient way to collect whole blood under clean, sterile conditions, as shown in Figure (3.8). To separate the liquid part of the blood (called serum) from the cells, the collected samples were placed into plain tubes that do not contain any substances that cause clotting. These tubes were left at room temperature so that the blood could naturally form clots. Once the clot had formed, the tubes were spun in a centrifuge at the correct speed to get clear serum. The serum was then divided into smaller portions and stored at -20 degrees Celsius until it was ready for biochemical testing. Storing it at this temperature helps keep the biomarkers stable and prevents them from breaking down, which is important for accurate results. Before carrying out the tests, the samples are kept at this temperature to ensure that the biomarkers remain intact, both those that are affected by enzymes and those that are not. To measure the liver function in the serum, standard colorimetric tests were used. These tests are part of diagnostic kits that have been tested and approved for use in clinical settings, ensuring that the results are consistent and meet the accepted standards in medicine. The specific biomarkers that were measured are listed in reference [106].

• Alanine aminotransferase (ALT)

• Aspartate aminotransferase (AST)

These cytosolic transaminases are sensitive indicators of hepatocellular injury. Elevated serum levels reflect compromised membrane integrity and leakage of intracellular enzymes into the bloodstream.

• Alkaline phosphatase (ALP)

• γ-glutamyl transferase (GGT)

These enzymes are associated with cholestasis and biliary dysfunction. Elevated concentrations indicate obstruction or morphological alterations in bile ducts.

• Total bilirubin, a marker of hepatobiliary clearance and hemoglobin turnover.
• Albumin, a major plasma protein reflecting hepatic synthetic capacity.
• Total protein, indicative of overall nutritional and hepatic status.

To ensure methodological correctness and analytical consistency, all measurements were carried out in compliance with the guidelines provided by the manufacturers for each particular assay kit. A calibrated UV-visible spectrophotometer was used to measure absorbance, and the results were reported in standard clinical units to make comparison with reference values easier. When taken as a whole, these biochemical markers offered a thorough picture of hepatocellular integrity, biliary function, and liver damage in response to DAB/PB induction and subsequent treatment.[107]



Figure (3.8) *Blood sample collection from experimental Rats for biochemical and liver function analyses.*

3.6.2. Preparation of Liver Homogenates

Each animal's liver tissue was weighed, cleaned of blood with ice-cold saline, and homogenized in the proper buffer (such as 0.1 M phosphate buffer, pH 7.4) using a glass-Teflon homogenizer on ice. The supernatants from centrifuging homogenates at 10,000 × g for 15–20 minutes at 4 °C were utilized for antioxidant and oxidative stress tests. To enable data to be normalized, the protein content of each sample was ascertained (e.g., using the Lowry and Bradford method).

3.6.3. Oxidative Stress Markers and Antioxidant Enzymes

- **Lipid peroxidation (MDA):** Malondialdehyde (MDA) levels were quantified as thiobarbituric acid reactive substances (TBARS) and expressed as nmol MDA/mg protein.
- **Glutathione (GSH):** Reduced glutathione content was measured using a colorimetric method based on reaction with Ellman’s reagent (DTNB) and expressed as µmol GSH/g tissue.
- **Superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities:** Enzymatic activities of SOD and GPx were determined by standard kinetic spectrophotometric methods and expressed as units/mg protein.

3.7. Gene Expression Analysis by Real-Time PCR

Employed either a column-based extraction kit and the traditional phenol-chloroform method to extract total RNA from frozen liver tissue samples, adhering to the manufacturer's instructions. To maintain RNA integrity, liver samples were collected immediately after removal from the body, as illustrated in Figure (3.9). RNA concentration and purity were assessed using a spectrophotometer by

measuring the A260/A280 absorbance ratio. Agarose gel electrophoresis was also utilized to confirm RNA integrity. From 1-2 micrograms of total RNA, complementary DNA (cDNA) was synthesized using a commercial reverse transcription kit. Subsequently, quantitative real-time PCR (qPCR) was conducted using SYBR Green chemistry on a calibrated real-time PCR machine. analyzed several markers associated with apoptosis, inflammation, and fibrosis, including Bax , Bcl-2, TNF-α , IL-1β , TGF-β, and genes related to autophagy. β-actin was used as a reference gene for comparative analysis. To compare different groups and account for inter-sample variability, the $2^{\Delta Ct}$ method was applied to determine relative gene expression levels. Figure (3.9) illustrates the molecular effects of CME and Sc-CNP treatments on DAB/PB-induced liver cancer, providing a detailed visual depiction of gene expression patterns. The number [108].



Figure (3.9) *Dissection and liver tissue harvesting for histopathological, biochemical, and oxidative stress analyses.*

3.8. Histopathological Assessment

3.8.1. Tissue Processing

The livers of all the animals were carefully tested after slaughter to make sure they were in perfect shape. For at least two days, the samples were submerged in a 10% formalin solution after being washed with cold salt water. The samples were then immersed in paraffin, rinsed with xylene, and finally dried with alcohol, as is traditionally done. Leica RM2235 microtome slices of 4 to 5 micrometers in thickness were used to slice the liver tissue. Before staining, these slices were placed on sterile glass slides. Section 3.82: Staining Method Hematoxylin and eosin (H&E) staining follows a standard protocol that involves deparaffinizing and rehydrating liver tissue on slides that have been treated with different grades of alcohol. Next, hematoxylin was applied to the sections, followed by rinsing, blueing, and differentiation. Sections were stained with eosin to give contrast, and that was the last step. Staining was followed by further dehydration of the slides to maintain tissue integrity, cleaning with xylene, and sealing with a permanent synthetic mounting media to allow for microscopic analysis. Examination Under the Microscope and Standards for Grading (3.8.3) Applying a 400× magnification with an Olympus BX53 light microscope, histopathological evaluation was carried out. In order to ensure that there was no bias, two seasoned pathologists conducted each examination separately while keeping themselves anonymous about the experimental grouping. Figure 4.2 displays the findings of the semi-quantitative grading of lesions as either absence (-), mild (+), moderate (++), or severe (+++). All of the experimental groups were defined by their unique morphology: • Group 1 (control): Standard lobular architecture, thin hepatic cords (1-2 cells thick), homogeneous hepatocytes, and low nuclear-to-cytoplasmic (N/C) ratio were seen in liver slices from this group. Liver morphology was characterized by normal

appearance of the central veins and hepatic plates, free of necrosis and cellular atypia. In the HCC group (DAB + PB; G2), sections revealed hepatocellular carcinoma hallmarks such as greatly thicker hepatic cords (4-5 cells), an increased N/C ratio, cellular pleomorphism, nucleoli that stood out, sinusoidal dilatation, and spots of localized necrosis. This research confirms the presence of Grade II, moderately differentiated HCC. G3: Animals given a crude extract of *Saussurea costus* showed significant improvement over the control group that did not receive any treatment for HCC. Liver cords showed less nuclear atypia and a thinner appearance, with lower N/C ratios. Gradation I pattern shift and partial restoration of normal lobular structure are indicated by these findings. Significant histological recovery was seen in the HCC + nano-extract group (G4) after treatment with SC-CNPs. There was a low N/C ratio in the hepatocytes, a thin hepatic cord (1-2 cells thick), and a lobular architecture that was almost normal in the liver sections. Pleomorphism, trabecular thickening, and conspicuous nucleoli all hallmarks of cancer were almost completely absent, suggesting that the nanoformulation had potent hepatoprotective and restorative effects. [109].

3.9. Statistical Analysis

The mean ± standard deviation (SD) is used to express all data. Because of the small sample size for the conference pilot study, statistical comparisons were mostly descriptive. One-way analysis of variance (ANOVA) and Tukey's post-hoc test for multiple comparisons will be used to examine inter-group variations in biochemical, antioxidant, and gene expression parameters in the present expanded master's thesis dataset (n = 7 per group). Non-parametric tests such the Kruskal–Walli test will be employed for ordinal histopathology scores, followed by the proper post-hoc analysis. A statistically significant p-value is one that is less than 0.05.

Chapter Four

Results and Discussion

4.1. Formation of SC-CNPs

SC-CNP formation occurred when S. costus extract was added to chitosan at an extract-to-chitosan ratio of 1:4 while stirring at 1000 rpm and 50 degrees C, with the pH maintained at 4.5. Protonation created a positively charged chitosan surface, whereas carboxylate and oxygen-containing phytochemical groups formed electrostatic and hydrogen-bonded complexes. Non-polar terpenoid regions also contributed through hydrophobic association [40], [49], [68].

$$\text{Chitosan} + \text{S. costus extract} \xrightarrow[50°\text{C, 1000 rpm}]{\text{pH=4.5, 1:4}} \text{SC} - \text{CNPs} \tag{4.1}$$

$$\text{CS} - \text{NH}_2 + \text{H}^+ \rightleftharpoons \text{CS} - \text{NH}_3^+ \tag{4.2}$$

$$\text{CS} - \text{NH}_3^+ + \text{R} - \text{COO}^- \rightleftharpoons \left[\text{CS} - \text{NH}_3^+ \cdots \text{R} - \text{COO}^-\right]_{\text{ES}} \tag{4.3}$$

$$\text{CS} - \text{OH} + \text{O} = \text{C} - \text{R} \rightleftharpoons [\text{CS} - \text{O} - \text{H} \cdots \text{O} = \text{C} - \text{R}]_{\text{HB}} \tag{4.4}$$

$$\text{CS}_{\text{hyd}} + \text{R}_{\text{terp}} \rightleftharpoons [\text{CS} \cdots \text{R}_{\text{terp}}]_{\text{HYD}} \tag{4.5}$$

$$\text{Complex}_{\text{ES}} + \text{Complex}_{\text{HB}} + \text{Complex}_{\text{HYD}} \rightleftharpoons \text{SC} - \text{CNPs} \tag{4.6}$$

$$\text{SC} - \text{CNPs} + \text{Phytochemical} \rightleftharpoons [\text{SC} - \text{CNPs} \cdots \text{Phytochemical}]_{\text{surface}} \tag{4.7}$$

The combined non-covalent complexes assemble into a phytochemical-loaded core surrounded by a chitosan-rich surface. Surface-associated phytochemicals act as capping and stabilizing species, which limits aggregation and visible settling. The yellow-to-yellow-brown color change supports colloid formation, while DLS, zeta-potential, and TEM measurements provide the confirmatory evidence.

sample	CT.value
Normal 1	31,1262
Normal 2	32,344
Normal 3	31,424
Normal 4	32,4102
HCC1	32,3722
HCC2	32,976
HCC3	32,9289
HCC4	30,6694
TK 1	29,3164
TK2	28,1103
TK3	31,4689
TK4	29,7435
TN1	29,2668
TN2	29,3995
TN3	31,149
TN4	31,891

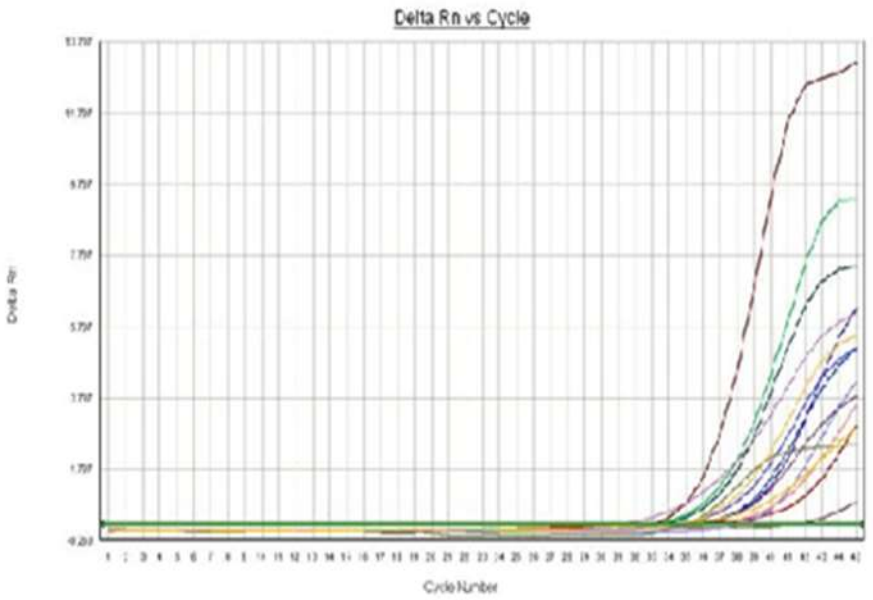


Figure (4.1) shows a visual representation of the formation of Sc-CNP, along with example RT-qPCR amplification curves and the corresponding CT values for the different groups, including normal, HCC, extract-treated (TK), and nanoparticle-treated (TN) groups.

4.2. Physicochemical Profile

4.2.1. Particle Size Distribution

The SC-CNPs had an average hydrodynamic diameter of 98.0 ± 0.2 nanometers and a polydispersity index (PdI) of 0.436 ± 0.004, as determined by dynamic light scattering (DLS) analysis. More than 85% of the nanoparticle population was represented by a major peak located within the 96–97 nm range of the DLS intensity profile. This narrow size distribution strongly suggests that the nanoparticles were primarily formed as a single, uniform size group, indicating that the synthesis process had good control over both the initial formation and the growth of the nanoparticles.

In the field of biomedical applications, nanoparticles that measure between 50 and 150 nanometers are often considered ideal for spreading throughout the body because they can take advantage of the enhanced permeability and retention (EPR) effect, which allows them to accumulate more effectively in diseased tissues. Unlike free phytochemicals, which may be quickly removed from the bloodstream by the kidneys, the small size of SC-CNPs makes them more likely to remain in the circulatory system longer, allowing them to reach target tissues more efficiently and concentrate in unhealthy liver areas. This characteristic enhances their potential for improved tissue penetration and targeted delivery in therapeutic applications.

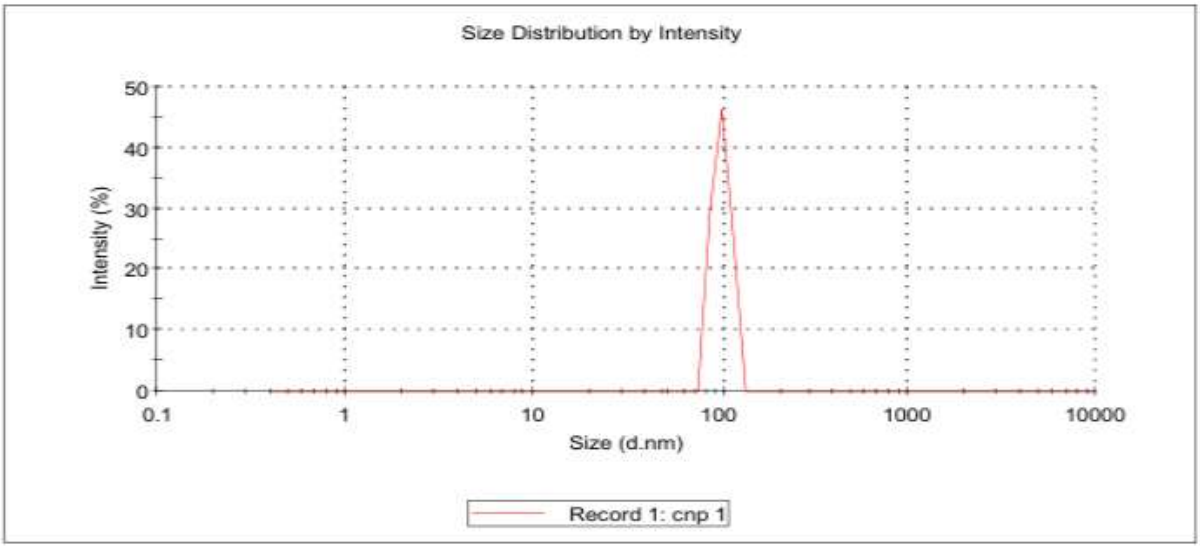


Figure 4.2. Size distribution by intensity and DLS parameters of CNPs.

4.2.2. Zeta Potential

The SC-CNPs had a very strong positive charge on their surface, about +50.7 mV with a small variation, as measured by zeta potential. In slightly acidic conditions, chitosan has a large positive charge because the amino groups on its structure become protonated. In colloidal systems, good electrostatic stability is usually seen when the zeta potential is above +30 mV or below -30 mV. This is because

charged particles repel each other and don't stick together. The high positive zeta potential of the Sc-CNP solution means it has excellent stability. This makes the solution less likely to form clumps when it is diluted or stored for a long time. The positively charged surface of the S. costus compounds inside the nanoparticles interacts with negatively charged membranes and proteins in the body, which might help them be better absorbed and taken up by cells.

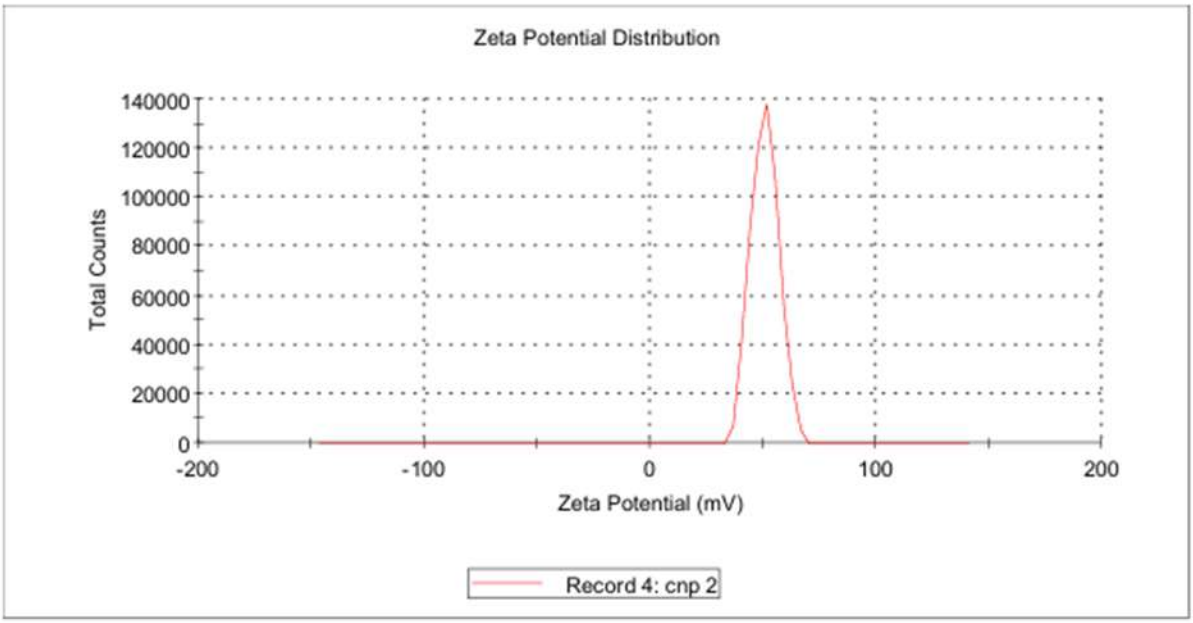


Figure 4.3. Zeta Potential of the prepared CNPs.

4.2.3. Morphology (TEM)

By using transmission electron microscopy (TEM), the shape and size of the SC-CNPs' cores were clearly observed. The core diameters ranged from 50 to 68 nanometers, and the nanoparticles had smooth and well-defined shapes. TEM photomicrographs confirmed these findings. This is consistent with the strong electrostatic stability indicated by the zeta potential data, which resulted in minimal aggregation of the nanoparticles.

It's typical for nanoparticle systems to have hydrodynamic diameters that are a bit larger than the core sizes measured by TEM. The difference between TEM and DLS is that DLS measures the hydrodynamic size, which includes the hydration layer and any loosely bound molecules, whereas TEM measures the dry, projected core size. In addition to confirming the accuracy of the physicochemical characterization, the close agreement between the two methods supports the conclusion that the generated SC-CNPs have a consistent, nanoscale, and pharmaceutically acceptable morphology.

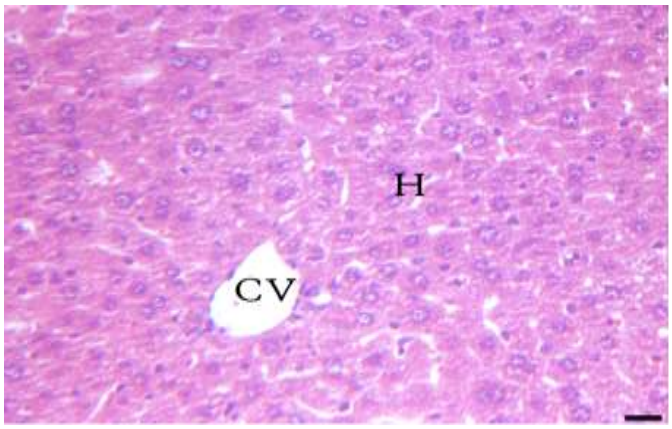


Figure 4.4. Representative photomicrograph of H&E-stained liver from the control group: The preserved lobular architecture, thin hepatic cords (1-2 cells thick), and uniform hepatocytes with a low N/C ratio are characteristic of normal liver parenchyma. Scale bar = 50 μm. H= Hepatocyes, CV= Central vein.

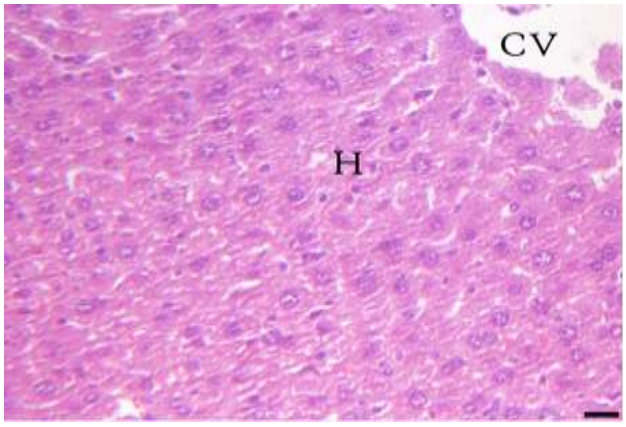


Figure 4.5. Representative photomicrograph of H&E-stained liver from the control group: The preserved lobular architecture, thin hepatic cords (1-2 cells thick), and uniform hepatocytes with a low N/C ratio are characteristic of normal liver parenchyma. Scale bar = 50 μm. H= Hepatocyes, CV= Central vein.

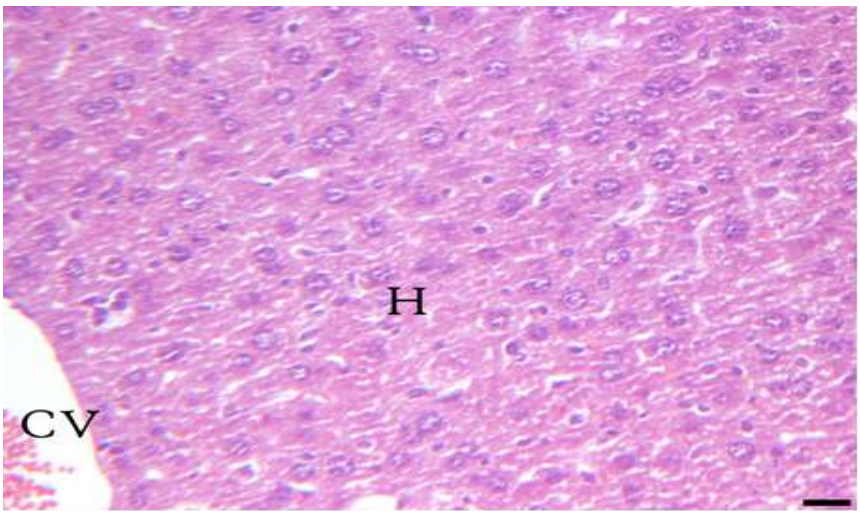


Figure 4.6 Representative photomicrograph of H&E-stained liver from the control group: The preserv Figure 4.3. ed lobular architecture, thin hepatic cords (1-2 cells thick), and uniform hepatocytes with a low N/C ratio are characteristic of normal liver parenchyma. Scale bar = 50 μm. H= Hepatocyes, CV= Central vein.

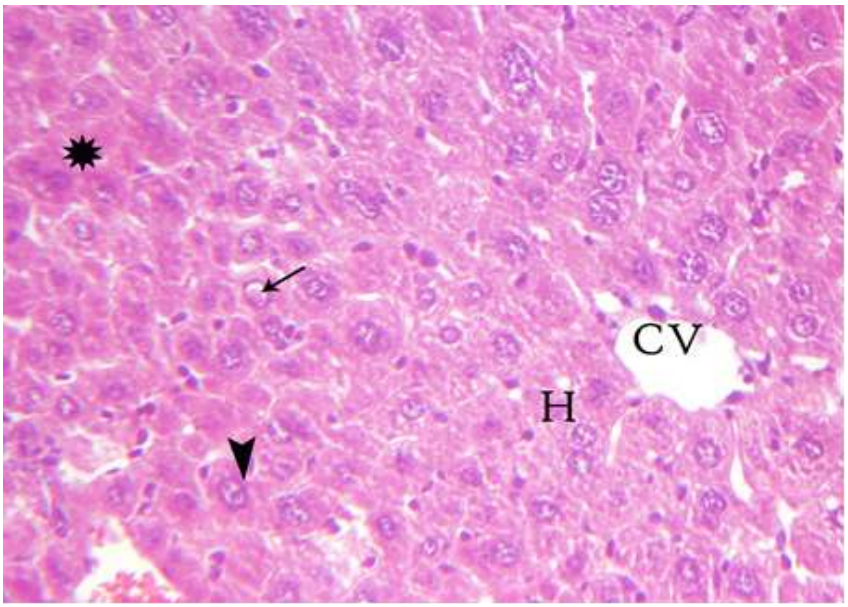


Figure 4.7 Representative photomicrograph of H&E-stained liver from the HCC group: The trabecular growth pattern (4-5 cells thick arrow as indicated with asterisk), high N/C ratio and cellular pleomorphism (arrowhead), and prominent eosinophilic nucleoli (small arrow), are

highly indicative of Hepatocellular Carcinoma, likely of a moderately differentiated grade (Grade II). Scale bar = 50 μm.

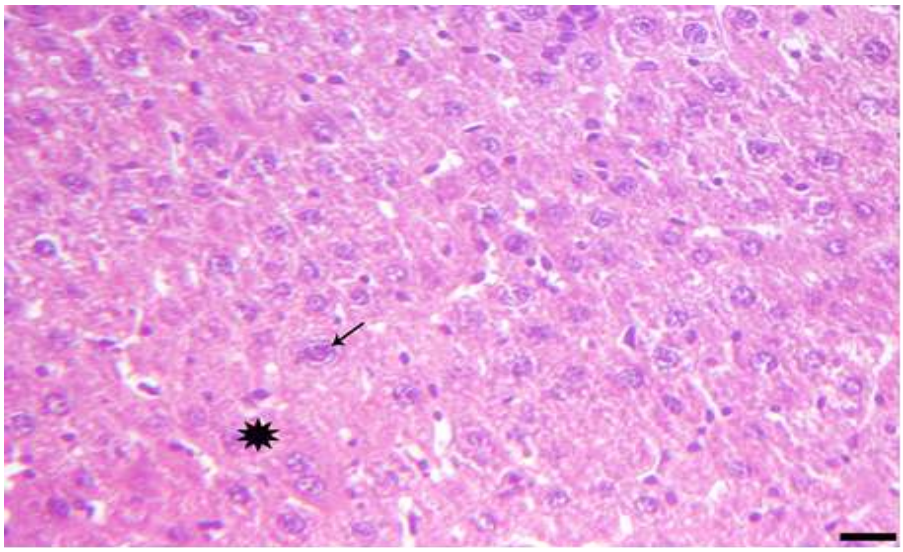


Figure 4.8 Representative photomicrograph of H&E-stained liver from the HCC group: The trabecular growth pattern (asterisk), high N/C ratio, cellular pleomorphism, and prominent eosinophilic nucleoli (arrow), are highly indicative of Hepatocellular Carcinoma**,** likely of a moderately differentiated grade (Grade II). Scale bar = 50 μm.

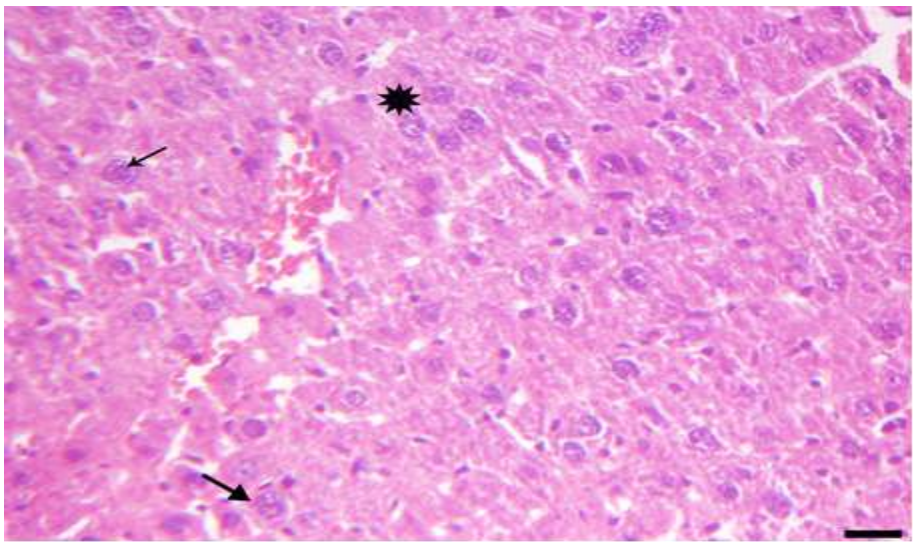


Figure 4.9 Representative photomicrograph of H&E-stained liver from the HCC group: The trabecular growth pattern (asterisk), high N/C ratio, cellular pleomorphism (large arrow), and prominent eosinophilic nucleoli (small arrow), are highly indicative of Hepatocellular Carcinoma**,** likely of a moderately differentiated grade (Grade II). Scale bar = 50 μm.

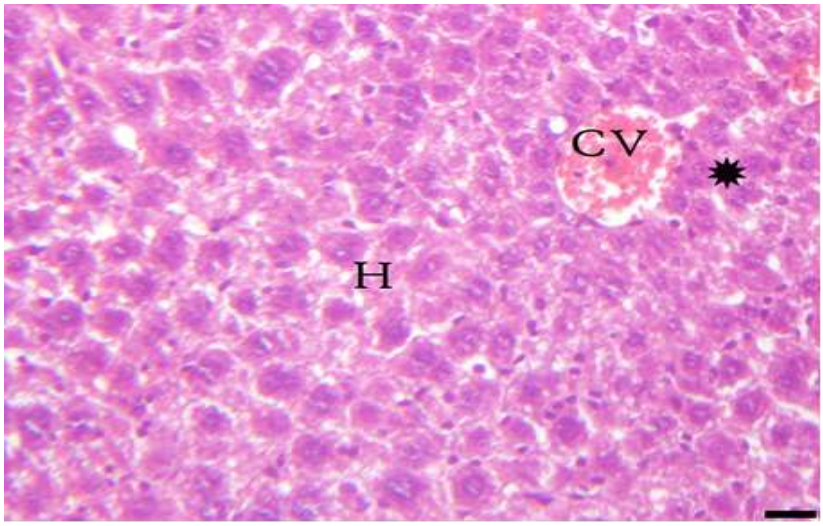


Figure 4.10 Representative photomicrograph of H&E-stained liver from the HCC+ Indian Costus group: The trabecular growth pattern (asterisk), low N/C ratio, minimal nuclear atypia (are highly indicative of Hepatocellular Carcinoma**,** likely of a well-differentiated grade (Grade I). Scale bar = 50 μm. H= Hepatocyes, CV= Central vein.

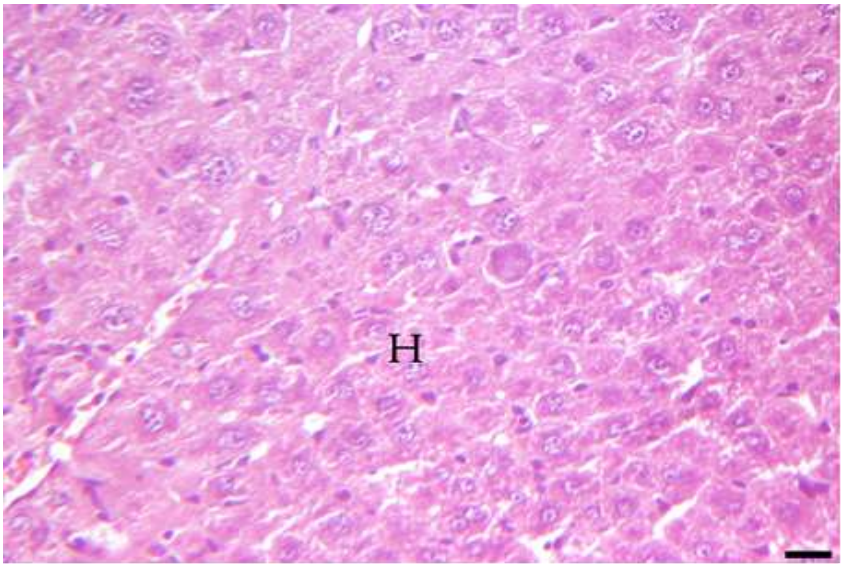


Figure 4.11. Representative photomicrograph of H&E-stained liver from HCC+ Indian Costus group: The preserved lobular architecture, thin hepatic cords (1-2 cells thick), and uniform hepatocytes with a low N/C ratio are characteristic of normal liver parenchyma. Scale bar = 50 μm. H= Hepatocyes, CV= Central vein.

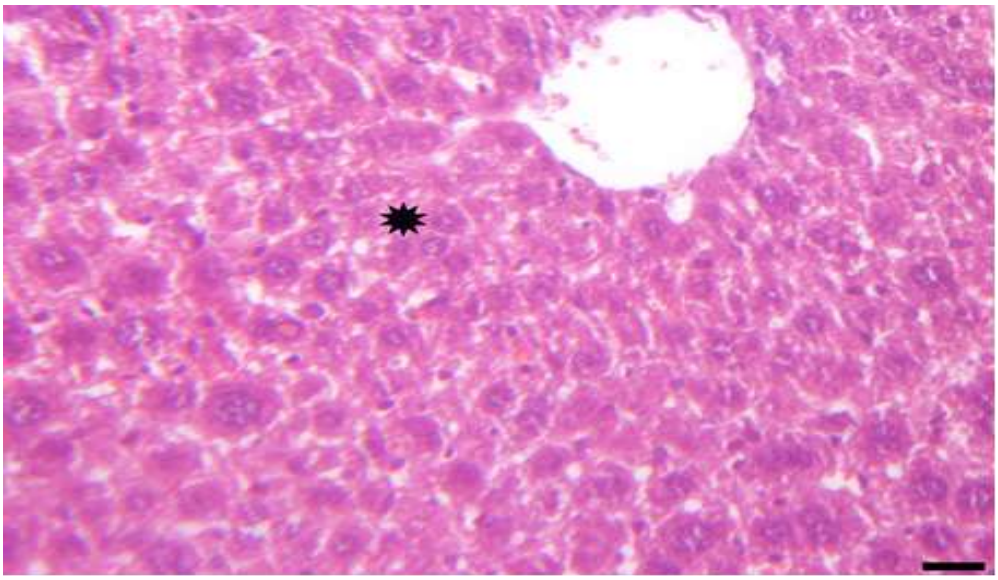


Figure 4.12. Representative photomicrograph of H&E-stained liver from the HCC+ Indian Costus group: The trabecular growth pattern (asterisk), low N/C ratio, minimal nuclear atypia (are highly indicative of Hepatocellular Carcinoma, likely of a well-differentiated grade (Grade I). Scale bar = 50 μm. H= Hepatocyes, CV= Central vein.

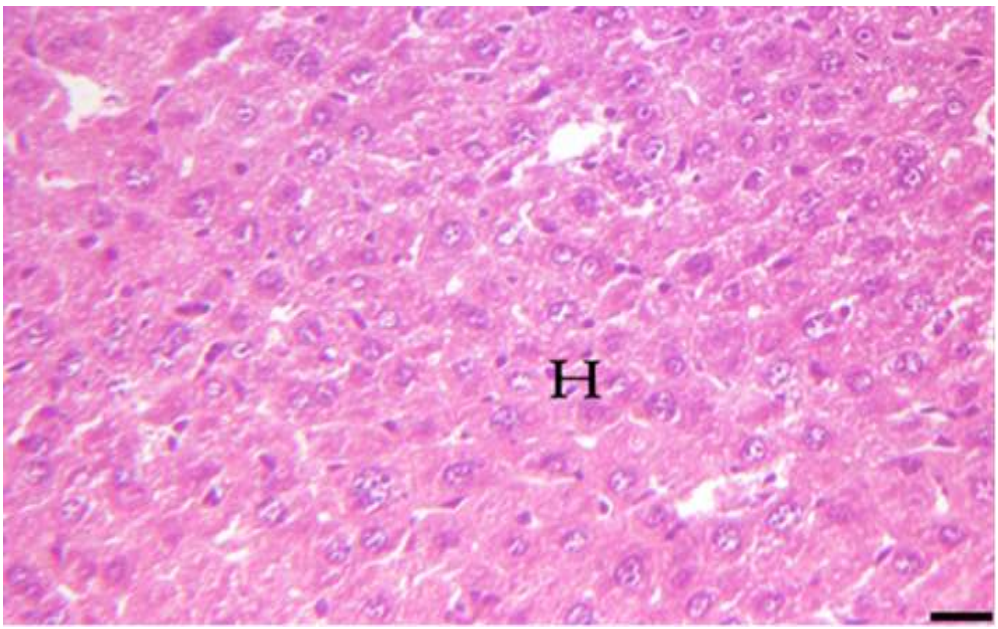


Figure 4.13 Representative photomicrograph of H&E-stained liver from HCC+ Indian Costus (Nanoparticles) group: The preserved lobular architecture, thin hepatic cords (1-2 cells thick), and uniform hepatocytes with a low N/C ratio are characteristic of normal liver parenchyma. Scale bar = 50 μm. H= Hepatocyes.

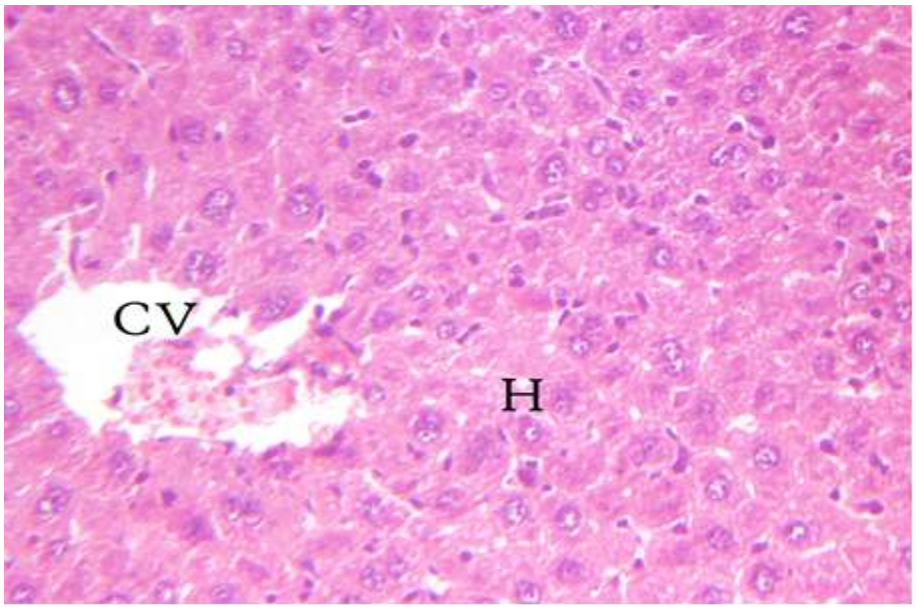


Figure 4.14 Representative photomicrograph of H&E-stained liver from HCC+ Indian Costus (Nanoparticles) group: The preserved lobular architecture, thin hepatic cords (1-2 cells thick), and uniform hepatocytes with a low N/C ratio are characteristic of normal liver parenchyma. Scale bar = 50 µm. H= Hepatocyes.

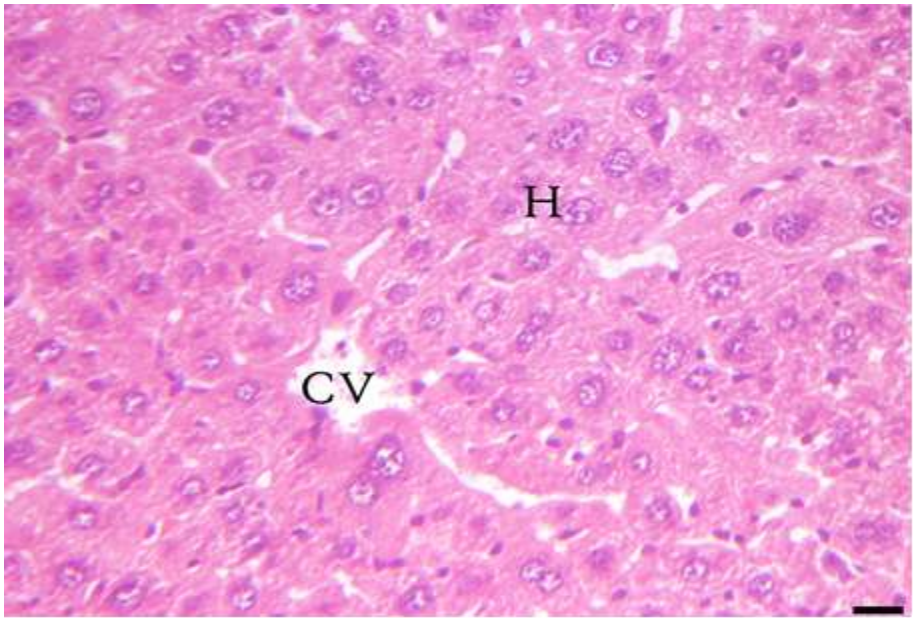


Figure 4.15 Representative photomicrograph of H&E-stained liver from HCC+ Indian Costus (Nanoparticles) group: The preserved lobular architecture, thin hepatic cords (1-2 cells thick), and uniform hepatocytes with a low N/C ratio are characteristic of normal liver parenchyma. Scale bar = 50 µm. H= Hepatocyes**.**

4.3. In Vivo Efficacy

Summary of the results obtained from the DAB/PB-induced hepatocellular carcinoma (HCC) model in Rats using crude methanolic extract (CME) and its nano-form (SC-CNPs). The primary focus is on the histological features, gross liver findings, and the total amount of hepatic protection offered by each treatment.

Table (4.1) Pathological Lesions Grading and Incidence:

	G 1 (control) (n=3)	G 2 (HCC) (n=3)	G 3 (HCC+ Indian Costus) (n=3)	G 4 (HCC+ Indian Costus (Nano) (n=3)
- The trabecular growth pattern	- (3/3)	+++ (3/3)	++ (3/3)	- (3/3)
- High N/C ratio	- (3/3)	+++ (3/3)	+ (3/3)	- (3/3)
- Cellular pleomorphism	- (3/3)	+++ (3/3)	+ (3/3)	- (3/3)
- prominent eosinophilic nucleoli	- (3/3)	+++ (3/3)	- (3/3)	- (3/3)
- Hepatocellular Carcinoma grade	- (3/3)	a moderately differentiated HCC (Grade II) (3/3)	a well-differentiated HCC (Grade I) (2/3)	- (3/3)

4.3.1. Gross Liver Findings

Experiment results demonstrated statistically significant differences between the four groups based on in-depth analysis of liver samples. The organs from the G1 control group displayed characteristic features of healthy livers, including a uniform reddish-brown color, a smooth exterior, and well-defined margins. The livers of the group that received both DAB and PB (G2), on the other hand, appeared significantly different. Their surfaces exhibited an uneven texture, with several spots resembling tiny growths or tumors. Liver tissue also seemed enlarged, yellowish, and lumpy. Exposure to toxic substances over an extended

period of time causes these major alterations, which include persistent inflammation, scarring of the liver tissue, and the growth of malignant cells.

The G3 group, which got the crude methanolic extract, had slightly better liver function. Despite a few minor abnormalities and light-colored patches, these livers seemed more typical in size and color when contrasted with the carcinogen-exposed group. It appears that the extract may have had some protective effects on the liver.

Significant improvements were observed in the group that was treated with the nano-formulation (G4). Their livers were nearly colorless, smoother, and more uniformly shaped than those of the other groups. These findings suggest that the use of SC-CNPs, as opposed to the non-nano extract, provides a better level of protection for the liver and may also help in the regeneration of liver tissue.

4.3.2. Microscopic Findings

Histopathological analysis confirmed the initial macroscopic observations and gave a detailed look at how much tissue damage occurred and how well it healed in each group:

- In the control group (G1), the liver samples showed a normal structure with clear lobular organization. The hepatocytes had a uniform cytoplasm and a low ratio of nucleus to cytoplasm. They also had typical central veins and thin hepatic cords made up of just one or two cells. There was no sign of any abnormal cells, inflammation, or cell death.
- In the group that was exposed to the HCC model (G2), the liver tissue showed a big change in structure. The growth pattern was trabecular, meaning the cells grew in sheets. The cells varied in size and shape (pleomorphism), and their

nuclei were larger in comparison to the cytoplasm, with noticeable nucleoli. The hepatic cords were thicker, consisting of four to five cells. There were scattered signs of cell death, the sinuses were widened, and the normal lobular pattern was disturbed. These features are consistent with Grade II moderately differentiated hepatocellular carcinoma, and they support the idea that the DAB/PB method is effective in causing HCC.

- When the group received the crude methanolic extract of *S. costus* (G3), the liver tissue showed clear improvement compared to G2. The hepatic cords were thinner, the changes in the nuclei were less severe, and the areas of dead cells were smaller, even though some cancerous growth patterns were still present. Overall, the tissue looked more like a well-differentiated HCC (Grade I), with some recovery of the normal lobular structure. This suggests that the crude extract provides some level of protection to the liver cells and may help control cancer development.
- The group that was treated with both the nano-extract of *S. costus* (G4). There was also very little inflammation. These findings suggest that the nano-extract has strong liver-protecting properties and could also have anti-cancer effects, along with a significant reduction in the harmful changes caused by the cancer model.

4.3.3. Histopathology Grading

Consistent with the qualitative histology findings, the quantitative evaluation of the primary pathological alterations in the tissue samples revealed a high nucleus-to-cytoplasm ratio, diverse cell morphologies, proliferation in a pattern of thick bundles, and clearly visible, brightly stained nucleoli. A negative sign (-) indicates that the control group did not have any lesions, as seen clearly in Table 4.2. Conversely, a score of +++ across all categories indicated extremely severe

abnormalities in the hepatocellular carcinoma (G2) group. The crude methanolic extract group (G3) showed some improvement, but it wasn't enough to completely undo the malignant changes; their scores ranged from mild (+) to moderate (++). Group 4 (nanoparticle recipients) scored similarly to the control group (-), indicating that they had nearly no abnormalities. The histological results, in conjunction with the biochemical and antioxidant data that will be covered later, give compelling evidence that the SC-CNPs, offer superior protection to the liver structure compared to the crude extract.

4.4. Mechanistic Considerations

Mechanistically, the enhanced therapeutic impact of SC-CNPs seems to be due to the enhanced physicochemical properties (nanometric size, positive surface charge, sustained release) and the enhancement of the inherent antioxidant and anti-inflammatory activities of S. costus phytoconstituents. Here, integrate biochemical, oxidative stress, and gene expression data to explain the seen histological protection.

4.4.1. Biochemistry (Serum Liver Function Markers)

When DAB/PB causes liver cancer, it can throw off blood tests that measure liver function. Compared to the control group, the rats in the G2 group who developed liver cancer had substantially lower levels of total protein and albumin. Also, total bilirubin, ALT, AST, ALP, and GGT levels were significantly increased. This alteration in the liver's function may be due to cell membrane abnormalities, protein deficiencies, bile duct obstruction, or cell death.

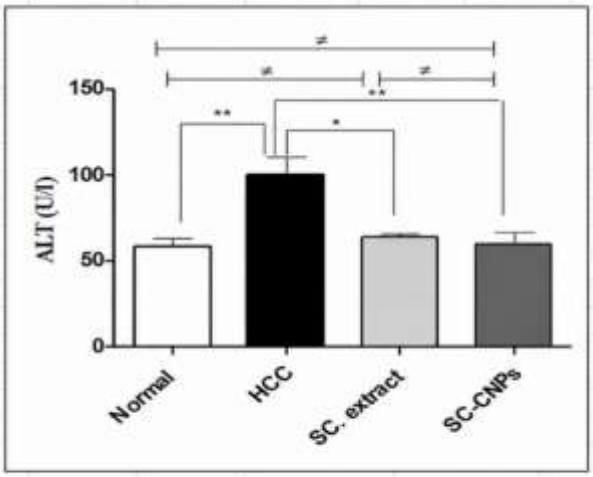
Many of these indicators began to improve following administration of the G3 crude extract. In contrast to the G2 group, this one had lower levels of ALT and

GGT and higher levels of total protein and albumin. It appears that the crude extract is insufficient to completely protect the liver, as these markers did not return to normal levels.

Rats administered Sc.CNPs (G4), in contrast, fared considerably better. Their bilirubin, ALT, AST, ALP, and GGT levels were near those of the control group, much lower than the G2 group. Both total protein and albumin levels changed for the better. This supports the idea that the active substances are better able to travel throughout the body thanks to nano-encapsulation, which in turn helps to lessen liver damage and promote improved liver function generally.

The blood tests' findings corroborate those of the tissue samples. Significant indicators of liver health were restored, proving the efficacy of the nano-formulated material. When tested with ALT blood, the various groups demonstrated significantly varied outcomes. Levels of ALT were greatest in the HCC group, an indicator of significant liver damage. A common indicator of liver injury is the presence of high ALT values, which indicate the leakage of liver cells. Lower ALT levels were seen in the group that received *Saussurea costus* extract. Subjects administered the nano-formulated extract (Sc.CNPs) exhibited significantly reduced ALT levels. Because of nanoparticles' enhanced absorption, localized targeting, and controlled release of active compounds over time, this improvement is probably attributable to them.

sample	OD		
Normal 1	0.0375	65.475	58.2291
Normal 2	0.0352	61.4592	
Normal 3	0.0363	63.3798	
Normal 4	0.0244	42.6024	
HCC1	0.0658	114.8868	100.0022
HCC2	0.0542	94.6332	
HCC3	0.0412	71.9352	
HCC4	0.0679	118.5534	
TK1	0.0384	67.0464	64.20915
TK2	0.0363	63.3798	
TK3	0.0385	67.221	
TK4	0.0339	59.1894	
TN1	0.0239	41.7294	60.01875
TN2	0.0351	61.2846	
TN3	0.0411	71.7606	
TN4	0.0374	65.3004	



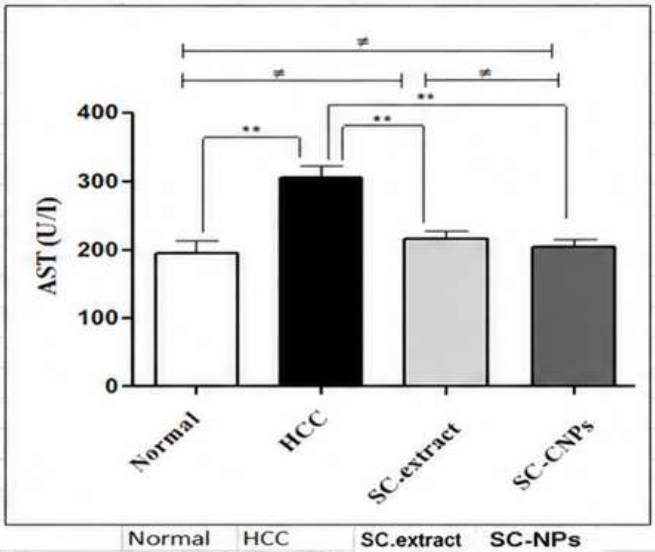
	Normal	HCC	SC.extract	SC-CNPs
Mean	58.23	100	64.21	60.02
Std. Devia	10.55	21.47	3.786	12.93
Std. Error	5.273	10.73	1.893	6.467

Figure 4.16 Serum ALT levels (U/L) in Normal, HCC, SC-extract, and SC-CNPs groups showing significant elevations in the HCC group and marked reductions following treatment.

Variations in blood aspartate aminotransferase (AST) levels across the study's several groups provided conclusive evidence that carcinogen exposure produced varying degrees of liver damage. Figure (4.17) shows that AST levels were significantly greater in the HCC group than in the control group, indicating that this was a liver cancer diagnosis. An extremely high AST level indicates that the liver is in distress, which could have an effect on the cell membranes and mitochondria—two crucial components of liver cells. On the other hand, the treatment groups' much lower AST levels suggest that the methanolic extract of *Saussurea costus* may be protecting the liver, in contrast to the HCC group. The nano-formulated extract (SC-CNPs) group fared better than the control group, as their AST levels were significantly lower. There is mounting evidence, and these results add to it, that nanoparticles may be the best method for drug delivery currently available. Reason being, nanoparticles have the ability to enhance drug

absorption, focus drug delivery to specific tissues, and slow drug release over time, all of which contribute to more efficient and tailored treatment.

sample	OD		
Normal 1	0.0854	149.1084	192.5838
Normal 2	0.1312	229.0752	
Normal 3	0.0958	167.2668	
Normal 4	0.1288	224.8848	
HCC1	0.1653	288.6138	306.2048
HHC2	0.1895	330.867	
HCC3	0.1956	341.5176	
HCC4	0.1511	263.8206	
TK1	0.1364	238.1544	215.5437
TK2	0.1101	192.2346	
TK3	0.1318	230.1228	
TK4	0.1155	201.663	
TN1	0.1208	210.9168	203.6709
TN2	0.1178	205.6788	
TN3	0.1299	226.8054	
TN4	0.0981	171.2826	



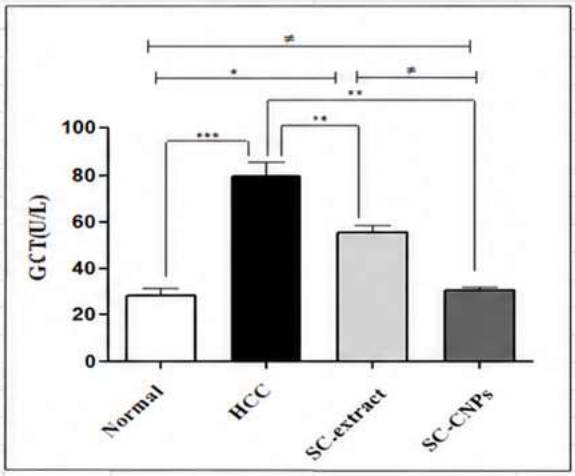
	Normal	HCC	SC.extract	SC-NPs
Mean	192.6	306.2	215.5	203.7
Std. Devia	40.44	36.34	22.06	23.39
Std. Error	20.22	18.17	11.03	11.69

Figure 4.17 Serum AST concentrations (U/L) across the Normal, HCC, SC-extract, and SC-CNPs groups, showing significant elevation in the HCC group and marked reductions following treatment, particularly with the nano-form.

The serum gamma-glutamyl transferase (GGT) assay revealed notable differences between the various experimental groups, which clearly point to a decline in liver function. As shown in Figure 4.18, the HCC group exhibited significantly higher GGT levels when compared to the normal control group. This increase in GGT levels is linked to heightened oxidative stress, a reduction in glutathione metabolism, and impaired detoxification processes in the liver, all of which are indicative of liver cell damage and the progression of liver cancer. The methanolic extract of *Saussurea costus* was found to lower GGT levels in the HCC group, suggesting that it may help protect the liver against oxidative stress and support its detoxification mechanisms. Remarkably, the nano-formulated version of the extract, known as SC-CNPs, led to a significant reduction in GGT activity,

bringing it down to levels comparable to those of the normal group. This enhanced effect is most likely due to improved cellular uptake, greater stability of the active components, and more effective delivery of the compounds to the liver, thanks to the advanced nanoparticle technology.

sample	g/dl		
Normal 1	0.0203	29.435	
Normal 2	0.0146	21.17	
Normal 3	0.0218	31.61	
Normal 4	0.0137	19.865	
HCC1	0.0658	95.41	74.27625
HHC2	0.0513	74.385	
HCC3	0.0492	71.34	
HCC4	0.0386	55.97	
TK1	0.0395	57.275	50.9675
TK2	0.032	46.4	
TK3	0.0297	43.065	
TK4	0.0394	57.13	
TN1	0.0165	23.925	28.52875
TN2	0.0223	32.335	
TN3	0.0193	27.985	
TN4	0.0206	29.87	



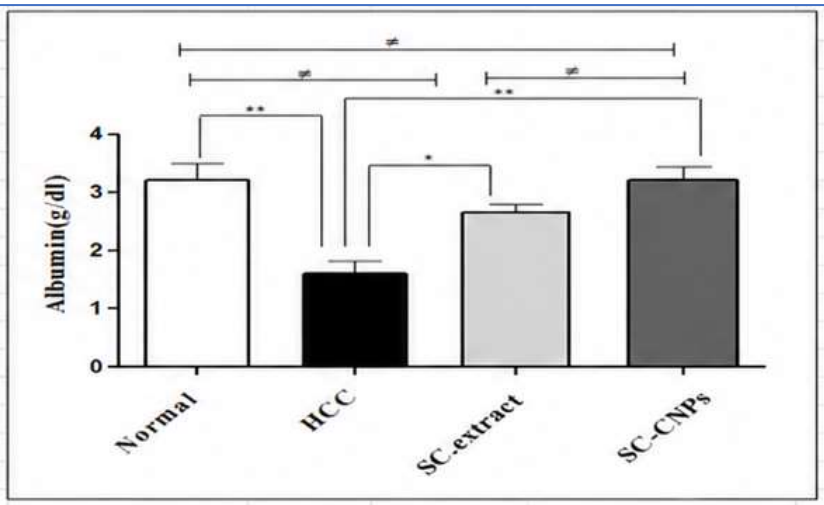
	Normal	HCC	SC.extract	SC.NPs
Mean	25.52	74.28	50.97	28.53
Std. Devia	5.868	16.23	7.327	3.549
Std. Error	2.934	8.116	3.664	1.774

Figure 4.18 Serum GGT concentrations (U/L) across the Normal, HCC, SC-extract, and SC-CNPs groups, showing significant elevation in the HCC group and marked reductions following treatment, particularly with the nano form.

Significant variations in serum albumin levels were found among the different experimental groups, shedding light on the liver's response to cancer-related stress. Figure 4.19 shows that the albumin levels were significantly lower in the group with hepatocellular carcinoma (HCC) as compared to the normal control group. The liver's capacity to manufacture proteins has drastically decreased, as shown by the drastic drop in albumin content; this is a hallmark of severe liver dysfunction. This malfunction is associated with tumor growth and harm to hepatocytes, the cells that make up the liver.

It appears that the liver's protein-producing abilities were partially recovered after therapy with the methanolic extract of *Saussurea costus*, since the serum albumin levels in the HCC group demonstrated a substantial improvement. This means that the liver's synthetic functions have improved to some extent. The results with the nano-formulated extract (SC-CNPs) were even more encouraging; albumin levels were greater than in the HCC group and were getting close to normal group values. Because of the special characteristics of the nanoparticle system, this improved effect is possible. The drug's continuous presence in the body is helped by the nanoparticles' ability to release the active components more slowly and consistently. Nanoparticles not only increase the drug's efficacy, but they also boost the drug's bioavailability and absorption into the bloodstream. All of these things work together to help the liver's metabolism and cell regeneration.

sample	g/dl
Normal 1	4.12
Normal 2	3.05
Normal 3	2.85
Normal 4	2.96
HCC1	2.09
HHC2	1.57
HCC3	1.9
HCC4	0.95
TK1	2.33
TK2	2.72
TK3	2.86
TK4	2.98
TN1	3.07
TN2	3.94
TN3	2.77
TN4	3.17



	Normal	HCC	SC.extract	SC-NPs
Mean	3.245	1.628	2.723	3.238
Std. Devia	0.589	0.5002	0.2824	0.4982
Std. Error	0.2945	0.2501	0.1412	0.2491

Figure 4.19 Serum albumin (g/dl) levels across the Normal, HCC, SC-extract, and SC-CNPs groups, showing severe reduction in the HCC group and marked improvement following treatment, particularly with the nano-form.

Looking at the overall amount of serum proteins in the blood is an important technique to determine the liver's protein-making ability. Total protein levels varied significantly among the study's various groups. Figure (4.20) shows that total protein levels were significantly lower in the hepatocellular carcinoma (HCC) group than in the normal control group. A normal symptom of liver cancer is a decrease in protein levels, which indicates that the liver is not producing these vital proteins as it should. Because of alterations in nutrition processing and injury to liver tissue, the body's capacity to manufacture vital proteins is significantly diminished.

The overall serum protein levels in the HCC group improved after treatment with *Saussurea costus* extract, indicating that the liver was able to restore some of its protein-making abilities. This means that the liver's function has partially returned. Nevertheless, protein levels were nearly identical to those in the control group when the extract was transformed into SC-CNPs, or nanoparticles. This provides more evidence that the extract's nanoparticle form is superior for reestablishing liver function. The improved outcome is most likely attributable to the fact that the nanoparticles are more biocompatible, more stable, and capable of delivering the medication straight to the cells in the liver. The liver is able to function better and mend itself more efficiently because of these benefits.

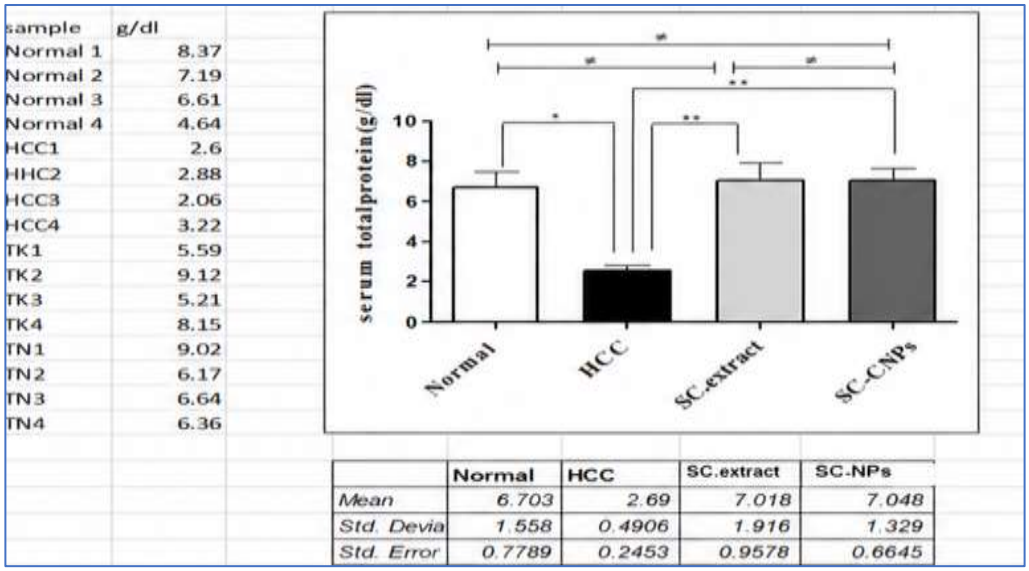


Figure 4.20 Total serum protein (g/dl) across Normal, HCC, SC-extract, and SC-CNPs groups, illustrating the significant reduction in the HCC group and the marked recovery following treatment, particularly with the nano-form.

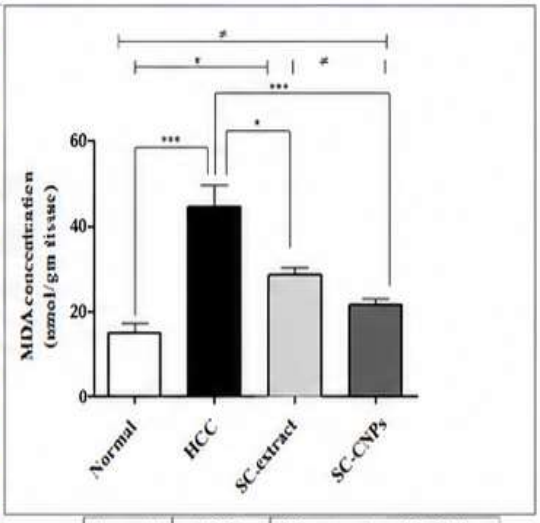
4.4.2. Antioxidant and Oxidative Stress Markers

Dangerous compounds, such as DAB and PB, can cause oxidative stress, which in turn increases the risk of cancer and liver damage. Reactive oxygen species (ROS) are dangerous molecules that can damage cells and tissues; an excess of their synthesis causes oxidative stress. When these harmful substances are present and the body's defense systems are overwhelmed, this disease develops. Producing glutathione (GSH) from the amino acid glutathione, the glutathione peroxidase (GPx) enzyme aids in the body's defenses against oxidative stress. All three of these molecules work together as antioxidants, boosting the immune system and assisting the body in its fight against dangerous toxins. Malondialdehyde (MDA), on the other hand, is an important marker for determining the extent to which oxidative stress has compromised cell membranes. The elevated levels of malondialdehyde (MDA) demonstrated that the G2 group of rats with liver cancer had an elevated amount of reactive oxygen species (ROS) and cell membrane

damage. Reduced glutathione and glutathione peroxidase (GPx) levels were lower in the G2 group compared to the healthy group, indicating that the body's internal defenses against ROS damage had diminished. G3, the unprocessed extract of S. costus, provided some resistance to oxidative stress. In addition to lowering MDA levels, this extract helped bring back some of the lost GSH and GPx. The recovery was incomplete, though, because oxidative stress markers were still significantly high. Among the groups treated with SC-CNPs, the G4 group showed the most antioxidant benefits. When contrasted with the G2 and G3 groups, this one had very low MDA levels, nearly identical to the control group. Also, in this group, the levels of GPx and GSH went back to normal. In light of these results, it is reasonable to conclude that nanoparticle encapsulation of plant active compounds enhances their bioavailability and stability. The group that was treated with nanoparticles may have had better results because the compounds were more regulated and had higher bioavailability. Because of their healing and antioxidant capabilities, SC-CNPs can help restore balance and maintain homeostasis, two factors that are critical in halting the spread of liver cancer.Different groups had significantly different levels of malondialdehyde (MDA), notwithstanding MDA's reliability as a marker for lipid breakdown and tissue injury. Figure 4.21 demonstrates that in comparison to the normal control group, the HCC group had a substantially higher MDA concentration. This upsurge is consistent with the hallmarks of liver cancer progression, which include oxidative stress and membrane damage. Those who took the *Saussurea costus* extract showed a decrease in malondialdehyde levels, which may indicate a protective effect against oxidative stress. The nano-formulated extract (SC-CNPs) works wonders since it restores normal levels of MDA by a substantial margin. This improved result is probably due to the enhanced characteristics of the nanoparticle system. Active phytochemicals are delivered to cells more efficiently, have a longer release time,

and are more bioavailable when encased in nanoparticles. *Saussurea costus* extract significantly reduced oxidative damage, as indicated by significantly lower MDA levels in the group that received it compared to the HCC group. In addition to lowering MDA levels, the nano-formulated extract (SC-CNPs) brought them closer to normal. Better penetration of active phytochemicals into the body, enhanced bioavailability, and prolonged release are the three primary ways in which nanoparticle technology improves performance.

MDA		(nmol/gm tissue)			
	OD	OD standard		standard conc	nmol/gm tissue
Normal 1	0.2966	0.1623	1.82748	10	88.27479975
Normal 2	0.1236		3.761553	10	7.615526802
Normal 3	0.227		1.398644	10	13.98644487
Normal 4	0.3158		1.915779	10	19.45779421
HCC1	0.0767		5.511399	10	55.11398644
HCC2	0.7937		4.890327	10	48.90326555
HCC3	0.7295		4.494763	10	44.94762785
HCC4	0.51192		3.154159	10	31.54158965
TK1	0.3988		2.45533	10	24.55229636
TK2	0.4763		2.934689	10	29.34668848
TK3	0.5075		3.126925	10	31.26925447
TK4	0.4935		3.040665	10	30.40605434
TN1	0.3611		2.224892	10	22.24892175
TN2	0.3118		1.921134	10	19.21133703
TN3	0.4118		2.537277	10	25.37276846
TN4	0.3429		2.312754	10	21.12754159



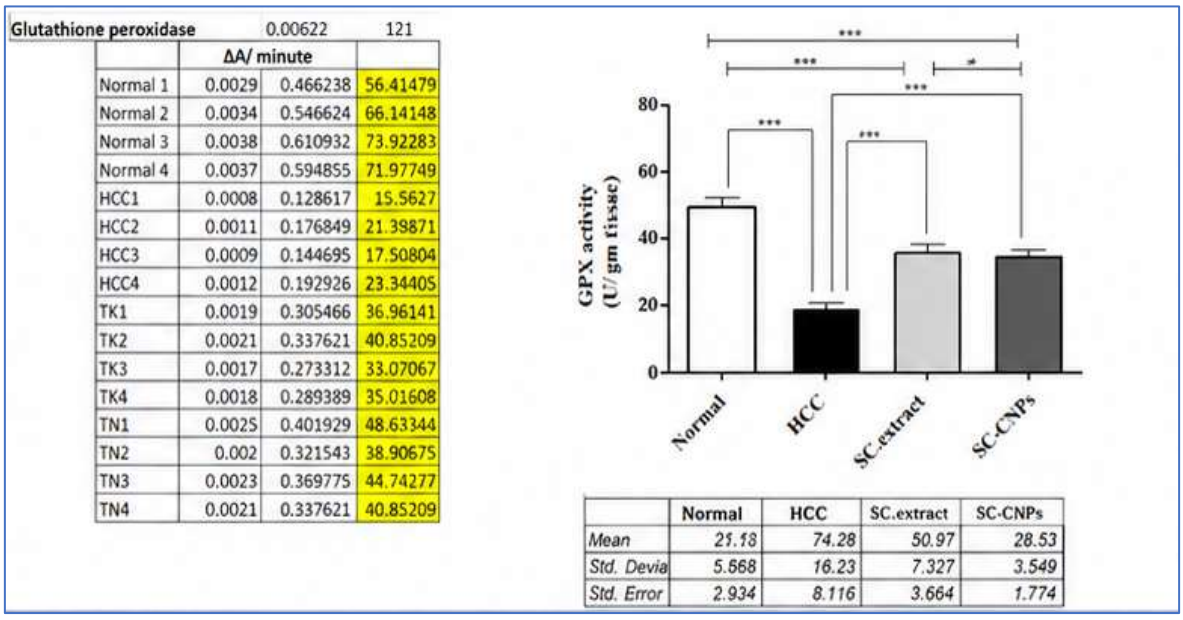
	Normal	HCC	SC.extract	SC-CNPs
Mean	14.63	45.13	28.89	21.99
Std. Devia	5.355	9.977	2.999	2.58
Std. Error	2.678	4.988	1.499	1.29

Figure 4.21 MDA concentration (nmol/g tissue) in Normal, HCC, SC-extract, and SC-CNPs groups, illustrating severe oxidative damage in the HCC group and substantial improvement following treatment, especially with the nano-formulation.

A key enzyme involved in the body's natural defense against oxidative damage, glutathione peroxidase (GPx), showed significant changes across all the groups studied. In the HCC group, GPx activity dropped considerably when compared to the normal control group, as clearly shown in Figure (4.22). This decrease is linked to a buildup of harmful substances known as reactive oxygen species (ROS) and a

weakened ability to combat them, which leads to increased oxidative stress and supports the growth of tumors.

When compared to the HCC group, treatment with *Saussurea costus* extract helped improve GPx activity, indicating some recovery of the enzyme's ability to protect cells from oxidative damage. However, the nano-formulation made from chitosan nanoparticles (SC-CNPs) had an even more pronounced effect, boosting GPx activity to levels that closely resembled those found in the normal group. This superior performance is likely due to the unique qualities of the nanoparticle system, such as its better ability to reach deeper into tissues, stay in the body longer, and release its active components more effectively. These properties work together to strengthen the body's natural antioxidant defenses and help repair the damage caused by oxidative stress.



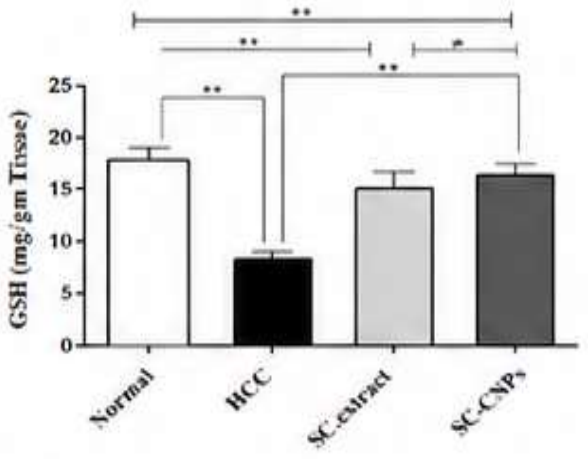
Glutathione peroxidase	0.00622		121
	ΔA/ minute		
Normal 1	0.0029	0.466238	56.41479
Normal 2	0.0034	0.546624	66.14148
Normal 3	0.0038	0.610932	73.92283
Normal 4	0.0037	0.594855	71.97749
HCC1	0.0008	0.128617	15.5627
HCC2	0.0011	0.176849	21.39871
HCC3	0.0009	0.144695	17.50804
HCC4	0.0012	0.192926	23.34405
TK1	0.0019	0.305466	36.96141
TK2	0.0021	0.337621	40.85209
TK3	0.0017	0.273312	33.07067
TK4	0.0018	0.289389	35.01608
TN1	0.0025	0.401929	48.63344
TN2	0.002	0.321543	38.90675
TN3	0.0023	0.369775	44.74277
TN4	0.0021	0.337621	40.85209

	Normal	HCC	SC.extract	SC-CNPs
Mean	21.18	74.28	50.97	28.53
Std. Devia	5.668	16.23	7.327	3.549
Std. Error	2.934	8.116	3.664	1.774

Figure 4.22 GPx activity (U/g tissue) in Normal, HCC, SC-extract, and SC-CNPs groups, showing severe depletion in HCC and substantial recovery following treatment, particularly in the nano-form-treated group.

The research looked into how levels of reduced glutathione, which is an essential antioxidant, differ among various groups of Rats. This antioxidant plays a key role in helping the body manage the balance between oxidation and reduction reactions that occur inside cells. From Figure 4.23, it's clear that individuals with hepatocellular carcinoma, a type of liver cancer, had much lower levels of this important antioxidant compared to those in the normal control group. This big decrease in glutathione suggests that the disease might be causing major changes in the body, such as using up more antioxidant resources, increasing the bodys exposure to harmful oxidative stress, and reducing its ability to break down and get rid of harmful substances. On the other hand, rats who received *Saussurea costus* extract had much higher glutathione levels than those with liver cancer. This hints that this treatment could be helping the body to recover some of its antioxidant functions. However, the group that took the nano-formulated version of the extract, known as SC-CNPs, had the most significant improvement. Their glutathione levels were almost the same as those in the standard control group. This is likely because the nanoparticle form of the extract makes it easier for the body to take in the active compounds from the plant. These nanoparticles may help the body absorb the ingredients more effectively, spread them better to the cells, and overall have a stronger effect in supporting the bodys natural ability to fight against harmful free radicals.

sample	OD	mg/gm tissue
Normal 1	0.307	20.46462
Normal 2	0.292	19.46472
Normal 3	0.275	18.3315
Normal 4	0.197	13.13202
HCC1	0.1339	8.925774
HCC2	0.0948	6.319368
HCC3	0.1195	7.96587
HCC4	0.0900	6.000600
HCC4	0.0904	6.026064
TK1	0.1686	11.238876
TK2	0.2645	18.96477
TK3	0.2578	17.184948
TK4	0.1958	13.052028
TN1	0.2522	16.811652
TN2	0.3191	21.271206
TN3	0.1966	13.109356
TN4	0.2145	14.29857

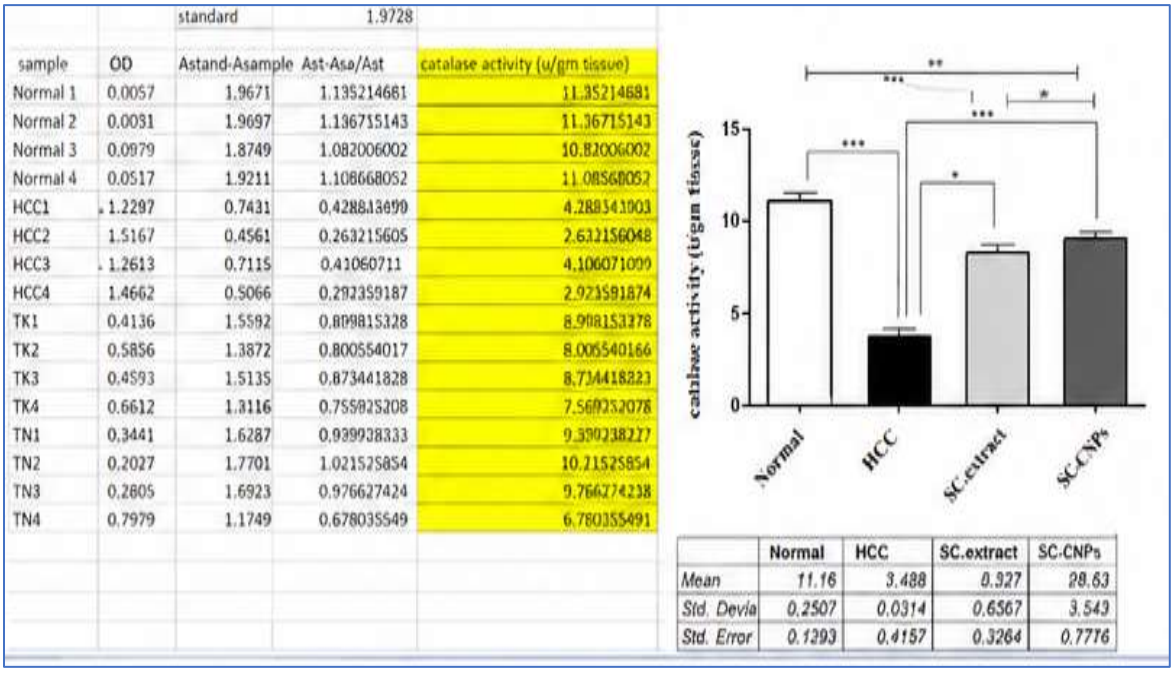


	Normal	HCC	SC.extract	SC-CNPs
Mean	17.85	7.309	15.11	16.37
Std. Devia	3.263	1.375	3.577	3.613
Std. Error	1.631	0.6874	1.789	1.807

Figure 4.23 GSH concentration (mg/g tissue) in Normal, HCC, SC-extract, and SC-CNPs groups, showing severe depletion in the HCC group and marked recovery following treatment, particularly with the nano-formulation.

Catalase is an important antioxidant enzyme in the body that breaks down hydrogen peroxide into water and oxygen, which are not harmful. This mechanism is very important for stopping harmful reactive oxygen species (ROS) from building up in the body, since they can hurt cells and tissues. Figure 4.24 shows that the group with hepatocellular carcinoma (HCC) had considerably lower catalase activity than the normal control group. This substantial decline in catalase activity demonstrates that rats with HCC have more oxidative stress and that the liver's natural antioxidant defenses are quite poor. Treatment with *Saussurea costus* extract caused a substantial increase in catalase activity compared to the HCC group. This suggests that parts of the antioxidant capabilities were partially restored. When the extract was made into nanoparticles, which are called SC-CNPs, the catalase activity went up a lot more, almost back to the level seen in the normal group. This huge change is probably because the nanoparticle form is better

at getting into cells, staying in the body longer, and getting the active chemicals to the proper tissues more effectively.



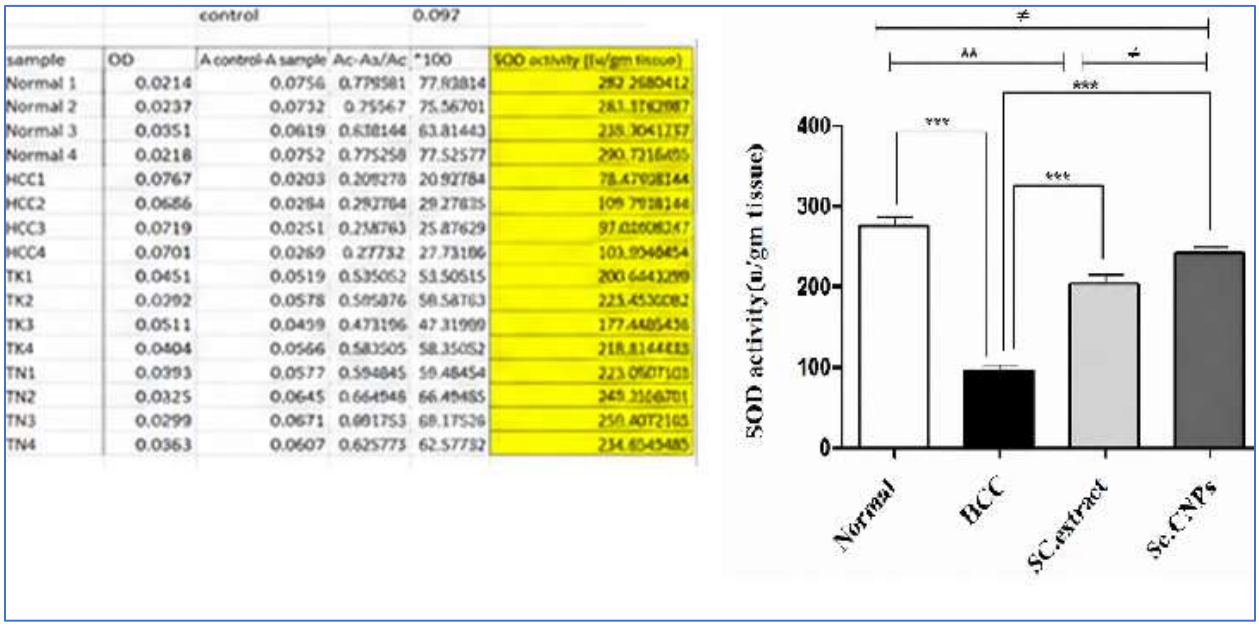
		standard	1.9728	
sample	OD	Astand-Asample	Ast-Asa/Ast	catalase activity (u/gm tissue)
Normal 1	0.0057	1.9671	1.135214681	11.35214681
Normal 2	0.0031	1.9697	1.136715143	11.36715143
Normal 3	0.0979	1.8749	1.082006002	10.82006002
Normal 4	0.0517	1.9211	1.108668052	11.08568052
HCC1	1.2297	0.7431	0.428813693	4.288341903
HCC2	1.5167	0.4561	0.263215605	2.632156048
HCC3	1.2613	0.7115	0.41060711	4.106071099
HCC4	1.4662	0.5066	0.292359187	2.923591874
TK1	0.4136	1.5592	0.899815328	8.998153278
TK2	0.5856	1.3872	0.800554017	8.005540166
TK3	0.4593	1.5135	0.873441828	8.734418283
TK4	0.6612	1.3116	0.755825208	7.558252078
TN1	0.3441	1.6287	0.939938333	9.399238277
TN2	0.2027	1.7701	1.021525854	10.21525854
TN3	0.2805	1.6923	0.976627424	9.766274238
TN4	0.7979	1.1749	0.678035549	6.780355491

	Normal	HCC	SC.extract	SC-CNPs
Mean	11.16	3.488	8.327	28.63
Std. Devia	0.2507	0.0314	0.6567	3.543
Std. Error	0.1293	0.4157	0.3264	0.7776

Figure 4.24 Catalase concentrations (U/L) acrss the Normal, HCC, SC-extract, and SC-CNPs groups, showing significant elevation in the HCC group and marked reductions following treatment, particularly with the nano-form.

Superoxide dismutase, or SOD, is an enzyme that is the body's first line of defense against reactive oxygen species (ROS). SOD is one of the most important antioxidant enzymes in the body. Its main job is to change toxic superoxide radicals into hydrogen peroxide, which is less hazardous. Figure (4.25) shows that the SOD activity levels were much lower in the group of rats with hepatocellular carcinoma (HCC) than in the healthy control group. This drop-in SOD activity is a clear sign that antioxidant protection is not as strong. When this reduced defense system is paired with excessive amounts of oxidative stress, it can harm liver cells and eventually cause tumors to grow.

Using *Saussurea costus* extract dramatically increased SOD activity compared to the HCC group. This suggests that the body's ability to get rid of damaging free radicals was largely recovered. But the outcomes were even better when the extract was turned into nanoparticles, which are called SC-CNPs. In this instance, the SOD activity not only elevated but also approached levels comparable to those observed in the healthy control group. The nanoparticle system's unique features are what made this amazing progress possible. These nanoparticles help the extract get into the cells more quickly, keep the active substances more stable, and help the body absorb and use them better.



	control		0.092		
sample	OD	A control-A sample	Ac-As/Ac	*100	SOD activity (u/gm tissue)
Normal 1	0.0214	0.0756	0.778581	77.83814	282.2680412
Normal 2	0.0237	0.0752	0.75567	75.56701	261.3762887
Normal 3	0.0351	0.0619	0.638144	63.81443	235.3041237
Normal 4	0.0218	0.0752	0.775258	77.52577	290.7216495
HCC1	0.0767	0.0203	0.209278	20.92784	78.47938144
HCC2	0.0686	0.0264	0.272784	29.27835	109.7938144
HCC3	0.0719	0.0251	0.258763	25.87629	97.03608247
HCC4	0.0701	0.0269	0.27732	27.73196	103.9948454
TK1	0.0451	0.0519	0.535052	53.50515	200.6443299
TK2	0.0292	0.0578	0.595876	59.58763	223.4536082
TK3	0.0511	0.0459	0.473196	47.31959	177.4484536
TK4	0.0404	0.0566	0.583505	58.35052	218.8144433
TN1	0.0393	0.0577	0.594845	59.48454	223.0671103
TN2	0.0325	0.0645	0.664948	66.49485	249.3106701
TN3	0.0299	0.0671	0.691753	69.17526	259.4072165
TN4	0.0363	0.0607	0.625773	62.57732	234.6546485

Figure 4.25 SOD activity (U/gm tissue) in Normal, HCC, SC-extract, and SC-CNPs groups, illustrating severe reduction in HCC and marked recovery following treatment, particularly with the nano-formulation.

4.4.3. PCR Analysis and Molecular Markers

We examined the molecular foundations of the observed protective advantages using quantitative real-time PCR, a technique that quantifies specific gene levels in the liver. The findings indicated that some genes associated with inflammation and

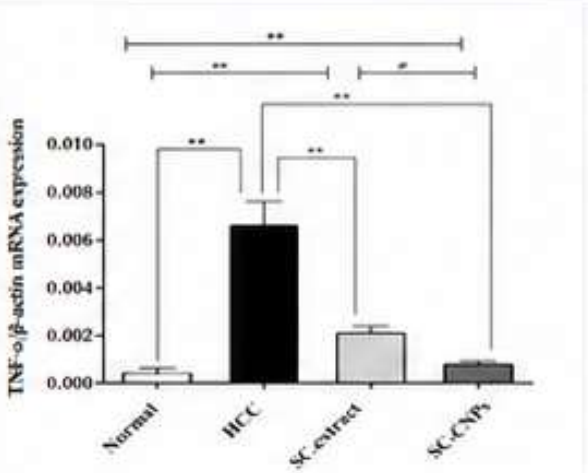
apoptosis, such as Bax, TNF-α, IL-1β, and TGF-β, exhibited heightened activity group (Group G2) compared to their typical expression levels. On the other hand, genes that stop cells from dying, such Bcl-2, were less active. These patterns of gene activity reveal that there was ongoing inflammation, scarring of tissues (fibrosis), and abnormal cell growth, all of which are connected to the development of liver cancer (HCC). When the group had CME treatment (Group G3), some nice things transpired. The activity of genes that cause scarring and inflammation was lowered, and the balance between Bax and Bcl-2, which are genes that control cell death, was partially restored. The group who took the nano-formulated extract (Group G4) showed the largest alterations. This group had genes that are linked to inflammation and scarring that were considerably less active, and genes that help protect cells that were much more active. This suggests that the gene activity pattern in this group was similar to what is seen in typical, healthy situations.

These results support the idea that SC-CNPs (nanoparticles made from Sc) can change important signaling pathways related to inflammation, apoptosis, and tissue fibrosis, while also reducing harmful oxidative damage. The improved repair of liver tissue and greater liver function in animals receiving the nano-formulated extract are likely due to simultaneous alterations in numerous biological pathways induced by the treatment.

TNF-α, a pro-inflammatory cytokine, is intimately associated with hepatic damage, oxidative stress, and tumor development. Figure (4.26) demonstrates that the levels of TNF-α mRNA were significantly elevated in the HCC group compared to the normal control group. This increase indicates an overactive inflammatory response, which is common in hepatocellular carcinoma, where chronic inflammation significantly contributes to tumor growth and dissemination. A large decline was found in TNF-α levels when looked at the HCC group and the group that got

Saussurea costus extract. This indicates that signals that were linked to inflammation were substantially weaker. The group that had the nano-formulated extract (SC-CNPs) experienced the highest drop in TNF-α levels, though. The TNF-α levels in this group were almost the same as those in the normal group. The stronger anti-inflammatory effect is probably because nanoparticles are better at staying stable, having more biological activity, and being easier for cells to take in.

sample	TNF-α	β-actin	ΔCT	
Normal 1	20.339	31.1282	10.7872	0.000565886
Normal 2	20.0995	32.344	12.2445	0.000206081
Normal 3	20.633	31.424	10.791	0.000564198
Normal 4	19.404	32.4102	12.9162	0.000129371
HCC1	24.3556	32.3722	8.0166	0.003861561
HCC2	25.1266	32.976	7.8494	0.004336059
HCC3	26.0313	32.9289	6.8976	0.008387171
HCC4	23.8964	30.6694	6.7775	0.009131072
TK1	21.7631	29.3164	7.5533	0.005323803
TK2	16.8425	28.1103	11.2678	0.000405559
TK3	20.5805	31.4689	10.8884	0.000527552
TK4	20.1941	29.7455	9.5494	0.001334579
TN1	19.7261	29.2668	9.5407	0.001342651
TN2	18.9168	29.2095	10.4807	0.000699834
TN3	20.9197	31.149	10.2293	0.000833055
TN4	21.2259	31.891	10.6651	0.000615864



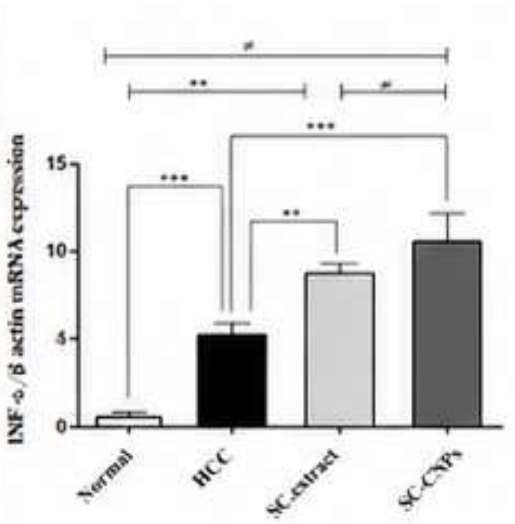
	Normal	HCC	SC.extract	SC-CNPs
Mean	0.0003664	0.006429	0.001895	0.0008773
Std. Deviation	0.0002316	0.002715	0.002321	0.0001126
Std. Error	0.0001158	0.001357	0.00116	0.000160

Figure 4.26 TNF-α / β-actin mRNA expression levels across Normal, HCC, SC-extract, and SC-NP groups, showing pronounced elevation in HCC and significant reductions following treatment, particularly with the nanoparticle formulation.

IFN-α, or interferon-alpha, is an essential protein that aids the immune system in combating viruses and reducing the proliferation of cancer cells. Rats without cancer have higher amounts of IFN-α mRNA, in contrast to those with hepatocellular carcinoma (HCC), a kind of liver cancer, as seen in Figure 4.27. This mRNA instructs cells on the production of IFN-α. The immune system may be performing worsening duties as liver cancer progresses, as indicated by the decrease in IFN-α levels. Because of this, the body may find it more difficult to

fight against diseases and cancer may progress more easily. The administration of an extract from the *Saussurea costus* plant to HCC resulted in a small rise in IFN-α levels, suggesting a potential restoration of immunological functioning. Furthermore, it is worth noting that the IFN-α levels increased to about the same level as in the normal control group when the identical extract was transformed into a unique form utilizing SC-CNPs, nanoparticles. The nanoparticles probably boost the extract's performance, which is why there is such a marked improvement. Maybe the nanoparticles will make the extract more bioavailable, increase its absorption by cells, and keep it where it needs to be. The immune system's function is likely to be restored, and the body's ability to fight diseases is enhanced, by all of these benefits working together.

sample	INF alpha	B-actin		
Normal 1	34.785	31.1262	-3.6588	12.63015
Normal 2	35.9474	32.344	-3.6034	12.15434
Normal 3	34.8843	31.424	-3.4603	11.00662
Normal 4	35.79	32.4102	-3.3798	10.40929
HCC1	33.0591	32.3722	-0.6869	1.609821
HCC2	34.7002	32.976	-1.7242	3.303969
HCC3	33.6927	32.9289	-0.7638	1.697957
HCC4	31.2543	30.6694	-0.5849	1.499935
TK1	32.2543	29.3164	-2.9379	7.662951
TK2	31.6815	28.1103	-3.5712	11.88607
TK3	34.6824	31.4689	-3.2135	9.275982
TK4	32.5592	29.7435	-2.8157	7.040608
TN1	33.1548	29.2668	-3.888	14.80467
TN2	33.1966	29.3995	-3.7971	13.90084
TN3	34.3617	31.149	-3.2127	9.27084
TN4	34.5861	31.891	-2.6951	6.475987



	Normal	HCC	SC.extract	SC-CNPs
Mean	11.55	2.038	6.066	11.11
Std. Devia	1.021	0.6545	2.162	3.978
Std. Error	0.5106	0.4273	1.081	1.964

Figure 4.27 IFN-α / β-actin mRNA expression levels across Normal, HCC, SC-extract, and SC-NP groups, showing severe downregulation in HCC and substantial restoration following treatment, particularly with the nano-formulation.

Chapter Five

Conclusion and Recommendations

5.1 Conclusion

The mortality rate is significantly higher when hepatocellular carcinoma (HCC) is in its later stages when compared to earlier stages. At the present time, once the disease has advanced, there is currently no known cure. There are several factors that can contribute to the development of HCC, such as oxidative stress, exposure to harmful chemicals, chronic inflammation, and visible signs of liver damage. A combination of eco-friendly chitosan nanoparticles with a methanolic extract from *Saussurea costus* has been considered as a potential tool for fighting cancer, reducing oxidative stress, and serving as a preventive measure. To test this theory, scientists used Rats that were specially bred to develop HCC, a disease that has distinct biochemical, genetic, and histological features. The Rats were genetically engineered to acquire liver cancer by exposing them to carcinogens such as phenol and p-dimethylaminoazobenzene. Tests revealed that the serum of these animals had elevated levels of several enzymes, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and lactate dehydrogenase (LDH). These substances appeared to have a severe impact on the liver's ability to process nutrients and eliminate waste. A rise in systemic malondialdehyde (MDA) levels also indicated increased oxidative stress. The body has certain enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase, which it uses to protect itself from damage caused by free radicals. The harmful substances altered gene expression by interfering with biological systems that regulate responses to stress, inflammation, and cell death. Genes that are associated with cell death and inflammation, such as TNF-α, IL-1, and Bax, showed overexpression, while genes that promote cell protection, like Bcl-2 and Beclin-1, had lower levels of expression. The liver tissue was severely damaged and showed abnormal cells,

inflammation, necrosis, and tumor-like abnormalities. Research using a methanolic *Saussurea costus* extract in vivo showed promising results in preventing these harmful changes. The extract improved liver function indicators, increased the activity of antioxidant enzymes, and reduced oxidative stress. One additional benefit was that it helped restore the structure of the liver. The active components in S. costus, such as phenolic compounds, sesquiterpene lactones, and flavonoids, are responsible for its medicinal benefits. The anti-inflammatory and antioxidant properties of these compounds are particularly noteworthy. When chitosan nanoparticles were added to the extract, the results were much better than when the extract was used alone. These nanoparticles demonstrated a significant reduction in oxidative stress markers, an improvement in gene regulation, a restoration of antioxidant enzyme activity, and an enhancement in liver tissue repair. These nanoparticles' unique chemical and physical properties are likely behind their improved performance. The advantages of this method include a slow and steady release of the extract, better stability and bioavailability, and a more favorable environment for the nanoparticles to accumulate in the liver. These findings support the idea that combining traditional herbal therapy with environmentally friendly nanotechnology could be a new and effective way to protect the liver and prevent cancer. Nanoparticles can increase the effectiveness of natural plant extracts, reduce their toxicity, and minimize their environmental impact. This approach offers numerous health benefits, including reducing oxidative stress, enhancing the activity of antioxidant enzymes, repairing liver tissue, and improving overall health. The improved bioavailability, delayed release, and increased accumulation in the liver are likely responsible for the nanoparticles' greater effectiveness. The study's authors suggest a novel approach to the treatment of cancer and liver disease by combining herbal medicine with non-toxic

nanotechnology. This strategy has the potential to improve public safety and protect the environment.

5.2 Medical Implications and Practical Applications

The outcomes of this research have several important medical and practical implications:

1. **Hepatoprotective Therapy**

   For patients with long-term liver conditions such cirrhosis, hepatitis, and chemically induced liver damage, the methanolic extract of *Saussurea costus* and its nanoparticle formulation may be useful hepatoprotective medicines.

2. **Cancer Prevention and Adjunct Therapy**

   These formulations may be utilized as preventive or supplementary therapy in people at high risk of developing hepatocellular carcinoma because of their potent anti-inflammatory and antioxidant properties, especially in those who have been exposed to environmental or drug-related hepatotoxins.

3. **Development of Nano-Herbal Therapeutics**

   The findings provide credence to the creation of nano-herbal formulations as innovative therapeutic platforms that integrate cutting-edge drug delivery technologies with conventional medical expertise to produce better clinical results.

4. **Reduction of Chemotherapy-Associated Toxicity**

   Herbal antioxidants that have been nano formulated may be utilized in conjunction with traditional chemotherapeutic drugs to improve patient tolerance and lessen oxidative damage brought on by treatment.

5.3 Recommendations and Future Research Directions

Based on the findings of this study, the following recommendations are proposed:

1. **Clinical Translation**

   It is advised that more research be done, including carefully planned clinical studies, to assess the pharmacokinetic behavior, safety, and effectiveness of formulations based on *Saussurea costus* in human beings.

2. **Long-Term Toxicological Studies**

   To evaluate the long-term safety of repeatedly administering the nanoparticle formulation, longer toxicity and biodistribution studies ought to be carried out.

3. **Molecular Mechanism Elucidation**

   Future research should examine other molecular pathways, such as Nrf2/ARE, NF-κB, MAPK, and PI3K/Akt signaling pathways, that are implicated in hepatoprotection and cancer suppression.

4. **Optimization of Nanocarrier Systems**

   Comparative research employing various biodegradable nanocarriers—such as liposomes, polymeric nanoparticles, or solid lipid nanoparticles—may improve therapeutic efficacy and delivery efficiency.

5. **Expansion to Other Disease Models**

   Other oxidative stress-related conditions and other cancer models should be used to assess the therapeutic potential of *Saussurea costus* nanoformulations.

5.4 Overall Conclusion

One kind of liver cancer that can develop from chemical exposure is hepatocellular carcinoma. According to the results of this research, the methanolic *Saussurea costus* extract is highly effective against this particular malignancy. Three primary pathways are involved in the activity of this extract: anti-inflammatory actions, improvements to liver function, and increased antioxidant defenses. When combined with green-synthesized chitosan nanoparticles, this extract becomes much more effective. Green nanotechnology has the potential to enhance the performance of nanoparticle formulations, which could lead to a greater utilization of natural resources for medical purposes. The results of this study provide promising groundwork for future research into the treatment of liver cancer. Further, this work may aid in the development of safe, sustainable, and effective nano-herbal approaches for the prevention and treatment of liver cancer in the future.

References

[1] A. J. Czaja, "Hepatocellular carcinoma," *New England Journal of Medicine*, vol. 365, no. 12, pp. 1118–1127, 2011.

[2] J. Bruix and M. Sherman, "Management of hepatocellular carcinoma: An update," *Hepatology*, vol. 53, no. 3, pp. 1020–1022, 2011.

[3] R. L. Meyer, *The Liver: Biology and Pathobiology*, 5th ed. New York, NY, USA: Wiley-Blackwell, 2009.

[4] F. Bray et al., "Global cancer statistics 2018," *CA: A Cancer Journal for Clinicians*, vol. 68, no. 6, pp. 394–424, 2018.

[5] J. D. Yang and L. R. Roberts, "Hepatocellular carcinoma: A global view," *Nature Reviews Gastroenterology & Hepatology*, vol. 7, no. 8, pp. 448–458, 2010.

[6] World Health Organization, *Global Cancer Observatory (GLOBOCAN)*, WHO Press, Geneva, Switzerland, 2019.

[7] M. Forner, J. M. Llovet, and J. Bruix, "Hepatocellular carcinoma," *Lancet*, vol. 379, no. 9822, pp. 1245–1255, 2012.

[8] A. Villanueva, "Hepatocellular carcinoma," *New England Journal of Medicine*, vol. 380, no. 15, pp. 1450–1462, 2019.

[9] B. Halliwell and J. M. Gutteridge, *Free Radicals in Biology and Medicine*, 5th ed. Oxford, UK: Oxford University Press, 2015.

[10] J. D. Hayes, A. T. Dinkova-Kostova, and K. D. Tew, "Oxidative stress in cancer," *Cancer Cell*, vol. 38, no. 2, pp. 167–197, 2020.

[11] M. Valko et al., "Free radicals and antioxidants in normal physiological functions and human disease," *International Journal of Biochemistry & Cell Biology*, vol. 39, no. 1, pp. 44–84, 2007.

[12] L. P. Hsu and S. L. Yen, "Antioxidant enzymes and cancer prevention," *Journal of Biomedical Science*, vol. 24, no. 1, pp. 1–10, 2017.

[13] H. Esterbauer, R. J. Schaur, and H. Zollner, "Chemistry and biochemistry of 4-hydroxynonenal, malondialdehyde," *Free Radical Biology & Medicine*, vol. 11, no. 1, pp. 81–128, 1991.

[14] K. Del Rio, D. Stewart, and N. Pellegrini, "A review of malondialdehyde as a biomarker," *Nutrition, Metabolism & Cardiovascular Diseases*, vol. 15, no. 4, pp. 316–328, 2005.

[15] S. M. El-Serag, “Epidemiology of viral hepatitis and hepatocellular carcinoma,” *Gastroenterology*, vol. 142, no. 6, pp. 1264–1273, 2012.

[16] M. Sies, “Oxidative stress: Concept and applications,” *Oxidative Medicine and Cellular Longevity*, vol. 2017, Article ID 821981, 2017.

[17] J. R. Sohal and R. Weindruch, “Oxidative stress, caloric restriction, and aging,” *Science*, vol. 273, no. 5271, pp. 59–63, 1996.

[18] M. Farombi and O. Onyema, “Monosodium glutamate-induced oxidative damage,” *Human & Experimental Toxicology*, vol. 25, no. 5, pp. 251–259, 2006.

[19] R. Sharma et al., “Role of cytochrome P450 in chemical carcinogenesis,” *Toxicology*, vol. 254, no. 1–2, pp. 1–9, 2008.

[20] J. Klaunig et al., “Oxidative stress and carcinogenesis,” *Toxicologic Pathology*, vol. 38, no. 1, pp. 96–109, 2010.

[21] A. Abdel-Wahhab et al., “Protective role of antioxidants against aflatoxin toxicity,” *Food and Chemical Toxicology*, vol. 48, no. 7, pp. 1756–1763, 2010.

[22] R. L. Smith and J. Williams, “Phenobarbital toxicity and liver injury,” *Drug Safety*, vol. 25, no. 6, pp. 401–414, 2002.

[23] S. Gupta et al., “Phenobarbital-induced hepatic damage,” *Journal of Applied Toxicology*, vol. 34, no. 5, pp. 509–517, 2014.

[24] H. Pitot, *Fundamentals of Oncology*, 5th ed. New York, NY, USA: Marcel Dekker, 2002.

[25] M. J. Verna et al., “Genotoxic effects of azo dyes,” *Mutation Research*, vol. 277, no. 2, pp. 107–118, 1992.

[26] K. Choudhary and S. Sharma, “Chromosomal aberrations induced by carcinogens,” *Cytologia*, vol. 78, no. 4, pp. 423–430, 2013.

[27] A. Meyer et al., “CAR-mediated liver tumor promotion,” *Toxicological Sciences*, vol. 92, no. 2, pp. 444–453, 2006.

[28] J. Klaassen, *Casarett and Doull’s Toxicology*, 8th ed. New York, NY, USA: McGraw-Hill, 2013.

[29] R. Bancroft and M. Gamble, *Theory and Practice of Histological Techniques*, 7th ed. London, UK: Churchill Livingstone, 2013.

[30] M. Nadkarni, *Indian Materia Medica*, Mumbai, India: Popular Prakashan, 2007.

[31] S. Singh et al., "Phytochemistry and pharmacology of *Saussurea costus*," *Journal of Ethnopharmacology*, vol. 176, pp. 1–13, 2015.

[32] A. Sharma and R. Shukla, "Medicinal plants as hepatoprotective agents," *Pharmacognosy Reviews*, vol. 9, no. 18, pp. 152–170, 2015.

[33] A. El-Ghazaly et al., "Hepatoprotective activity of *Saussurea costus*," *Journal of Applied Pharmaceutical Science*, vol. 6, no. 5, pp. 67–75, 2016.

[34] R. Ansari et al., "Antioxidant and anticancer potential of Saussurea lappa," *Biomedicine & Pharmacotherapy*, vol. 128, 2020.

[35] M. Ahmed et al., "Costunolide and dehydrocostus lactone: Biological activities," *Phytochemistry Reviews*, vol. 19, pp. 1–20, 2020.

[36] Y. Li et al., "Nrf2 signaling in cancer prevention," *Molecular Cancer*, vol. 18, no. 1, 2019.

[37] R. K. Mishra et al., "Green synthesis of nanoparticles," *Journal of Cleaner Production*, vol. 231, pp. 127–140, 2019.

[38] S. Iravani, "Green synthesis of metal nanoparticles," *Green Chemistry*, vol. 13, pp. 2638–2650, 2011.

[39] K. Choi et al., "Nanotechnology in cancer therapy," *Journal of Controlled Release*, vol. 240, pp. 44–59, 2016.

[40] A. Grenha, "Chitosan nanoparticles in drug delivery," *International Journal of Pharmaceutics*, vol. 381, no. 2, pp. 155–162, 2009.

[41] P. Mohanpuria, N. Rana, and S. Yadav, "Biosynthesis of nanoparticles: Technological concepts and future applications," *Journal of Nanoparticle Research*, vol. 10, no. 3, pp. 507–517, 2008.

[42] S. Ahmed et al., "Green synthesis of nanoparticles and their applications," *Journal of Advanced Research*, vol. 7, no. 1, pp. 17–28, 2016.

[43] M. Nasrollahzadeh et al., "Plant-mediated green synthesis of nanoparticles," *RSC Advances*, vol. 4, pp. 48435–48458, 2014.

[44] A. Roy and M. Bulut, "Green synthesis of silver nanoparticles," *Colloids and Surfaces B: Biointerfaces*, vol. 135, pp. 352–361, 2015.

[45] S. Iravani and R. S. Varma, "Green synthesis of metal nanoparticles," *Environmental Chemistry Letters*, vol. 18, pp. 703–727, 2020.

[46] R. Singh et al., "Biogenic nanoparticles: A review," *Journal of Nanoscience and Nanotechnology*, vol. 18, no. 4, pp. 1–17, 2018.

[47] N. M. Rao et al., "Chitosan nanoparticles as drug carriers," *International Journal of Biological Macromolecules*, vol. 64, pp. 453–466, 2014.

[48] V. R. Sinha et al., "Chitosan microspheres and nanoparticles," *International Journal of Pharmaceutics*, vol. 274, no. 1–2, pp. 1–33, 2004.

[49] M. Dash et al., "Chitosan—A versatile semi-synthetic polymer," *Progress in Polymer Science*, vol. 36, no. 8, pp. 981–1014, 2011.

[50] S. P. Vyas and R. K. Khar, *Targeted and Controlled Drug Delivery*, New Delhi, India: CBS Publishers, 2007.

[51] R. Duncan, "Polymer conjugates as anticancer nanomedicines," *Nature Reviews Cancer*, vol. 6, pp. 688–701, 2006.

[52] T. M. Allen and P. R. Cullis, "Drug delivery systems: Entering the mainstream," *Science*, vol. 303, no. 5665, pp. 1818–1822, 2004.

[53] Y. Zhang et al., "Nanoparticle-based drug delivery to the liver," *Journal of Controlled Release*, vol. 226, pp. 258–273, 2016.

[54] A. Gebrie et al., "Green synthesis of copper oxide nanoparticles using plant extract," *Materials Science in Semiconductor Processing*, vol. 121, 2021.

[55] A. Taha, "Microwave-assisted synthesis of CuO nanoparticles," *Journal of Molecular Structure*, vol. 1250, 2022.

[56] H. K. Patra et al., "Nano based drug delivery systems," *Journal of Nanobiotechnology*, vol. 16, no. 1, 2018.

[57] J. Panyam and V. Labhasetwar, "Biodegradable nanoparticles for drug delivery," *Advanced Drug Delivery Reviews*, vol. 55, pp. 329–347, 2003.

[58] R. S. Prasad, "Nanoparticles in cancer therapy," *Current Pharmaceutical Design*, vol. 18, pp. 2814–2825, 2012.

[59] S. Peer et al., "Nanocarriers as an emerging platform," *Nature Nanotechnology*, vol. 2, pp. 751–760, 2007.

[60] Muhammad ibn Isma'il al-Bukhari, *Sahih al-Bukhari*, Book 76: Medicine, hadith nos. 5692 and 5696.

[61] H. Maeda, “Tumor-selective delivery via EPR effect,” *Advanced Drug Delivery Reviews*, vol. 91, pp. 3–6, 2015.

[62] A. L. B. Seo et al., “Oxidative stress biomarkers in liver disease,” *Clinical Biochemistry*, vol. 46, no. 7–8, pp. 695–702, 2013.

[63] J. K. Andersen et al., “Oxidative stress in liver pathology,” *Free Radical Biology & Medicine*, vol. 38, pp. 160–167, 2005.

[64] H. Ohkawa, N. Ohishi, and K. Yagi, “Assay for lipid peroxides,” *Analytical Biochemistry*, vol. 95, no. 2, pp. 351–358, 1979.

[65] G. L. Ellman, “Tissue sulfhydryl groups,” *Archives of Biochemistry and Biophysics*, vol. 82, pp. 70–77, 1959.

[66] R. Ansari et al., “Antioxidant gene modulation by herbal extracts,” *Biochemistry Research International*, vol. 2019, Article ID 9453647.

[67] J. Harborne, *Phytochemical Methods*, 3rd ed. London, UK: Chapman & Hall, 1998.

[68] K. A. Al-Hassan et al., “Green-synthesized chitosan nanoparticles,” *International Journal of Biological Macromolecules*, vol. 148, pp. 1062–1072, 2020.

[69] R. Lowry et al., “Protein measurement with the Folin phenol reagent,” *Journal of Biological Chemistry*, vol. 193, pp. 265–275, 1951.

[70] M. Bradford, “A rapid and sensitive method for protein quantitation,” *Analytical Biochemistry*, vol. 72, pp. 248–254, 1976.

[71] K. Livak and T. Schmittgen, “Analysis of relative gene expression data,” *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[72] A. Bancroft and C. Layton, *Theory and Practice of Histological Techniques*, 8th ed. London, UK: Elsevier, 2019.

[73] S. Kiernan, *Histological and Histochemical Methods*, 4th ed. Oxford, UK: Butterworth-Heinemann, 2008.

[74] J. F. Gamble, “Tissue processing and staining,” *Journal of Histotechnology*, vol. 25, pp. 231–243, 2002.

[75] D. J. Shapiro, “Statistical analysis in biomedical research,” *Biostatistics*, vol. 9, no. 1, pp. 1–15, 2008.

[76] G. W. Snedecor and W. G. Cochran, *Statistical Methods*, 8th ed. Ames, IA, USA: Iowa State University Press, 1989.

[77] D. Montgomery, *Design and Analysis of Experiments*, 7th ed. Hoboken, NJ, USA: Wiley, 2009.

[78] M. B. Brown et al., "Animal models of hepatocellular carcinoma," *Laboratory Animals*, vol. 44, pp. 89–97, 2010.

[79] J. S. Lee et al., "Experimental models of liver cancer," *Hepatology Research*, vol. 41, pp. 783–795, 2011.

[80] A. Abdel-Wahhab et al., "Antioxidant protection against pesticide-induced toxicity," *Toxicology Letters*, vol. 172, pp. 1–8, 2007.

[81] J. L. Wallace, "Oxidative stress and liver disease," *Hepatology*, vol. 49, no. 1, pp. 1–8, 2009.

[82] S. Cichoż-Lach and A. Michalak, "Oxidative stress as a crucial factor in liver diseases," *World Journal of Gastroenterology*, vol. 20, no. 25, pp. 8082–8091, 2014.

[83] A. Parola and G. Robino, "Oxidative stress-related molecules and liver fibrosis," *Journal of Hepatology*, vol. 35, no. 2, pp. 297–306, 2001.

[84] H. Jaeschke, "Reactive oxygen and mechanisms of inflammatory liver injury," *Journal of Gastroenterology and Hepatology*, vol. 15, pp. 718–724, 2000.

[85] K. Muriel, "Role of free radicals in liver diseases," *Hepatology International*, vol. 3, pp. 526–536, 2009.

[86] R. S. Bhatti et al., "Herbal antioxidants in hepatoprotection," *Phytotherapy Research*, vol. 31, no. 2, pp. 182–196, 2017.

[87] M. S. Baliga et al., "Medicinal plants and hepatoprotection," *Journal of Clinical and Experimental Hepatology*, vol. 2, no. 4, pp. 356–364, 2012.

[88] A. R. Al-Asmari et al., "Protective effects of plant extracts against liver toxicity," *Toxicology Reports*, vol. 1, pp. 89–97, 2014.

[89] S. Gupta and R. Sharma, "Natural products as chemopreventive agents," *Pharmacological Research*, vol. 65, no. 5, pp. 493–506, 2012.

[90] D. Roy and S. S. Khanna, "Plant-derived anticancer compounds," *Current Pharmaceutical Biotechnology*, vol. 13, no. 1, pp. 192–205, 2012.

[91] N. Singh et al., "Nanomedicine: An emerging approach for cancer treatment," *Journal of Drug Targeting*, vol. 25, no. 1, pp. 1–15, 2017.

[92] K. K. Jain, *The Handbook of Nanomedicine*, 2nd ed. Totowa, NJ, USA: Humana Press, 2012.

[93] A. Etheridge et al., "The big picture on nanomedicine," *Nanomedicine*, vol. 9, no. 1, pp. 1–14, 2013.

[94] S. Arya et al., "Biogenic synthesis of nanoparticles and their biomedical applications," *Journal of Molecular Liquids*, vol. 281, pp. 566–576, 2019.

[95] P. Kuppusamy et al., "Biosynthesized nanoparticles for cancer therapy," *Materials Science and Engineering C*, vol. 92, pp. 574–588, 2018.

[96] A. M. Abdel-Aziz et al., "Chitosan-based nanoparticles for biomedical applications," *International Journal of Nanomedicine*, vol. 13, pp. 4925–4943, 2018.

[97] M. Rinaudo, "Chitin and chitosan: Properties and applications," *Progress in Polymer Science*, vol. 31, no. 7, pp. 603–632, 2006.

[98] H. Aranaz et al., "Functional characterization of chitosan," *Current Chemical Biology*, vol. 3, pp. 203–230, 2009.

[99] J. Park et al., "Biodegradable polymeric nanoparticles," *Advanced Functional Materials*, vol. 25, no. 28, pp. 4653–4674, 2015.

[100] S. J. C. de Jong and W. E. Hennink, "Polymeric drug delivery systems," *Pharmaceutical Research*, vol. 26, no. 2, pp. 360–370, 2009.

[101] J. M. Llovet et al., "Molecular therapies and precision medicine for hepatocellular carcinoma," *Nature Reviews Clinical Oncology*, vol. 15, no. 10, pp. 599–616, 2018.

[102] A. Villanueva and J. M. Llovet, "Targeted therapies for hepatocellular carcinoma," *Gastroenterology*, vol. 140, no. 5, pp. 1410–1426, 2011.

[103] S. M. El-Serag and K. L. Rudolph, "Hepatocellular carcinoma: Epidemiology and molecular carcinogenesis," *Gastroenterology*, vol. 132, no. 7, pp. 2557–2576, 2007.

[104] H. Bruix, J. Reig, and J. Sherman, "Evidence-based diagnosis, staging, and treatment of patients with hepatocellular carcinoma," *Gastroenterology*, vol. 150, no. 4, pp. 835–853, 2016.

[105] J. D. Yang et al., "Trends in hepatocellular carcinoma incidence and mortality," *Hepatology*, vol. 56, no. 2, pp. 460–468, 2012.

[106] M. Abdel-Aziz et al., "Protective role of natural antioxidants against liver carcinogenesis," *Environmental Toxicology and Pharmacology*, vol. 45, pp. 1–8, 2016.

[107] R. K. Sharma et al., “Role of medicinal plants in cancer prevention and therapy,” *Journal of Ethnopharmacology*, vol. 231, pp. 509–531, 2019.

[108] S. N. Ahn et al., “Experimental animal models for liver diseases,” *World Journal of Gastroenterology*, vol. 16, no. 45, pp. 5657–5666, 2010.

[109] M. R. Rezaei et al., “Histopathological evaluation of hepatoprotective agents,” *Toxicologic Pathology*, vol. 42, no. 3, pp. 474–482, 2014.