DOI: https://doi.org/10.31351/vol32issSuppl.pp145-155

Possible Protective Anticancer Effect of Ethanol Fraction of Iraqi *Hibiscus tiliaceus L.* Leaves Extract on Diethylnitrosamine-induced Hepatocarcinogenesis in Male Rats [#]

Zahraa Maan Abdul-Azeez[*,1] and Shihab Hattab Mutlag[2]

[#] 2nd Scientific Conference for Postgraduate Students Researches

[1] Ministry of Health and Environment, Medicolegal Directorate, Baghdad, Iraq.

[2]Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad, Baghdad, Iraq.

Abstract

Liver cancer with hepatocellular carcinoma is a serious clinical illness that progresses quickly and has a bad prognosis because of increased malignancy. Fibrosis is the precursor of liver cancer, which progresses to cirrhosis and carcinoma Diethylnitrosamine (DEN) is a chemical molecule that has been used as a carcinogenic agent to promote cancer in test animals because of its strong carcinogenic potential. Herbal plants have long been used as inexpensive, effective alternatives to pharmaceuticals in various liver-associated complications since they contain many bioactive compounds useful in liver disorders. *Hibiscus tiliaceus* L. (Malvaceae) contains various phytochemicals in the plant extracts such as flavonoids, phenols, alkaloids, tannins, saponins, quinones, coumarins, triterpenoids, glycosides, and steroids. Thus, has various health benefits, and medicinal and therapeutic values, It could be a source of natural antioxidants, antimicrobials, hepatoprotective compounds, and antimutagenic effects.

The study was designed to study the possible protective role of Iraqi *Hibiscus tiliaceus* L. ethanol leaves extract administered in two different doses on diethylnitrosamine-induced liver carcinoma in male Wistar Albino rats. Rats utilized in this study were randomized into 4 groups (six rats per each group); Group I: (Control) Rats were administered an oral daily dose of 1ml /kg/day of distilled water for 20 weeks; Group II: rats intraperitoneally injected with 70 mg/kg diethyl nitrosamine once per week for 10 continuous weeks; Group III: Rats were administered 250mg/kg of Iraqi *Hibiscus tiliaceus* L. ethanol leaves extract as chronic oral administration with food for 5 days per week for 20 weeks and subsequently intraperitoneal dose of diethylnitrosamine (70 mg/kg) once per week for 10 continuous weeks; Groups IV: Rats were administered 500mg/kg of *Hibiscus tiliaceus L.* ethanol leaves extract as chronic oral administration with food for 5 days per week for 20 weeks with subsequently intraperitoneal dose of diethylnitrosamine (70 mg/kg) once per week for 10 continuous weeks. The finding revealed that Iraqi *Hibiscus tiliaceus L.* ethanol leaves extract showed significant reduction ($P<0.05$) in malondialdehyde, alpha-fetoprotein (AFP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST), total bilirubin (TSB), pro-inflammatory cytokines include transforming growth factor beta (TGFβ), tumor necrosis factor-alpha (TNFα) and significant elevation ($P<0.05$) of anti-oxidant enzyme superoxide dismutase (SOD) in a dose-dependent manner. In conclusion, this study demonstrated that the chemoprotective role of *Hibiscus tiliaceus L.* ethanol leaves extract might be explained by lowered levels of hepatic enzymes, pro-inflammatory cytokines, and antioxidant levels, as well as decreased levels of alpha-fetoprotein (AFP), gold standard for detecting hepatocellular carcinoma.

Keywords: Hepatocellular carcinoma, Diethylnitrosamine, *Hibiscus tiliaceus* L. (Malvaceae), Alpha-fetoprotein.

التأثير الوقائي المحتمل المضاد للسرطان لجزء الإيثانول من خلاصة أوراق الكركديه العراقية على السرطان الكبدي الناجم عن ثنائي إيثيل نيتروسامين في ذكور الجرذان[#]

زهراء معن عبدالعزيز[*,١] و شهاب حطاب مطلك[٢]

#المؤتمر العلمي الثاني لطلبة الدراسات العليا

[١] وزارة الصحة والبيئة العراقية ، دائرة الطب العدلي ، بغداد، العراق

[٢]فرع الأدوية والسموم، كلية الصيدلة، جامعة بغداد، بغداد، العراق

الخلاصة

يُعد سرطان الكبد المصاب بسرطان الخلايا الكبدية حالة سريرية حرجة مصحوبة بسرطان خبيث متزايد وتطور سريع وضعف التشخيص. يبدأ سرطان الكبد على شكل تليف ، ويتطور على شكل تليف الكبد ، وينتج عنه السرطان. ثنائي إيثيل نيتروسامين هو مركب كيميائي يعرض نشاطًا مسرطنًا قويًا يستخدم كعامل مسرطن لتحفيز السرطانات في حيوانات التجارب. تم استخدام النباتات العشبية على نطاق واسع كدواء بديل بتكلفة منخفضة وآثار جانبية أقل في العديد من المضاعفات المرتبطة بالكبد ، لأنها تحتوي على العديد من المركبات النشطة بيولوجيًا المفيدة في اضطرابات الكبد.نبات الكركديه تيليسيوس العراقي تحتوي على مواد كيميائية نباتية مختلفة في المستخلصات النباتية مثل الفلافونويد والفينولات

[1]Corresponding author E-mail: zahraa89atos@gmail.com
Received:17 /4 /2023
Accepted: 8/6 /2023

والقلويدات والعفص والصابونين والكينون والكومارين والتربتربينويد والجليكوزيدات والمنشطات. وبالتالي ، فإن لها فوائد صحية مختلفة ، ولها قيم طبية وعلاجية ، ويمكن أن تكون مصدرًا محتملاً للعوامل الطبيعية المضادة للميكروبات ومضادات الأكسدة ويمكن أن تمتلك خصائص كبدية ومضادة للطفرات.

صممت الدراسة لدراسة الدور الوقائي المحتمل لمستخلص الإيثانول لأوراق الكركديه تيليسيوس العراقي. المعطى بجرعتين مختلفتين من سرطان الكبد الناجم عن ثنائي إيثيل نيتروسامين في ذكور الجرذان. تم تقسيم الجرذان المستخدمة في هذه الدراسة بصورة عشوائية إلى ٤ مجموعات (ستة جرذان لكل مجموعة). المجموعة الأولى: الجرذان تلقت جرعة يومية فموية قدرها ١ مل / كجم / يوم من الماء المقطر لمدة ٢٠ أسبوعًا ؛ المجموعة الثانية: الجرذان المحقونة داخل الصفاق بـ ٧٠ ملغم / كغم من ثنائي إيثيل نيتروسامين مرة واحدة في الأسبوع لمدة ١٠ أسابيع متواصلة ؛ المجموعة الثالثة: تلقت الجرذان ٢٥٠ ملجم / كجم من مستخلص الإيثانول لأوراق كركديه تيليسيوس العراقي كإعطاء عن طريق الفم مع الطعام لمدة ٥ أيام في الأسبوع لمدة ٢٠ أسبوعًا وبعد ذلك جرعة داخل الصفاق من ثنائي إيثيل نيتروسامين (٧٠ مجم / كجم) مرة واحدة في الأسبوع لمدة ١٠ أسابيع متواصلة ؛ المجموعات الرابعة: تلقت الفئران ٥٠٠ مجم / كجم من خلاصة الإيثانول لأوراق كركديه تيليسيوس العراقي كإعطاء عن طريق الفم مع الطعام لمدة ٥ أيام في الأسبوع لمدة ٢٠ أسبوعًا مع جرعة داخل الصفاق من ثنائي إيثيل نيتروسامين (٧٠ مجم / كجم) مرة واحدة في الأسبوع لمدة ١٠ أسابيع متواصلة. أظهرت النتائج أن مستخلص الإيثانول لأوراق كركديه تيليسيوس العراقي. أظهر انخفاضًا معنويًا في انزيمات الكبد، إجمالي البيليروبين، انخفاض مستوى ألفا فيتوبروتين (علامة ذهبية لسرطان الكبد) وتقليل المالوندايلدهايد و السيتوكينات الالتهابية بطريقة تعتمد على الجرعة. في الختام أوضحت هذه الدراسة أن الدور الوقائي الكيميائي لمستخلص الإيثانول لأوراق كركديه تيليسيوس العراقي. يمكن أن يعزى إلى: انخفاض مستوى ألفا فيتوبروتين (علامة ذهبية لسرطان الكبد) إلى جانب تقليل الإنزيمات الكبدية ، وخفض مستوى السيتوكينات المؤيدة للالتهابات ، وتحسين مستوى مضادات الأكسدة.

الكلمات المفتاحية: سرطان الخلايا الكبدية ، ديثيل نيتروسامين ، كركديه تيليسيوس ، بروتين فيتوبروتين ألفا.

Introduction

Hepatocellular carcinoma (HCC), a kind of liver cancer, is the third-leading cause of cancer-related mortality globally [(1)]. Nearly all HCC cases are accompanied by cirrhosis or hepatic fibrosis brought on by long-term liver damage. Fibrosis and cirrhosis are recognized as crucial variables in the carcinogenesis of liver tissue and the severity of the necrotic-inflammatory tissue damage, the creation of fibrosis, and the disease's progression to HCC. Each state may include various carcinogenic pathways [(2)]. Fibrotic animal models are closely associated with testing HCC medicines for their efficacy against tumor start and/or progression [(3)]. The fibrotic liver is characterized by changed hepatic vascularization, extracellular matrix composition, and drug metabolism [(4)]. Hepatocarcinogenesis, which mimics the pathological process of developing HCC in humans, includes liver damage, hepatocyte proliferation, liver fibrosis/cirrhosis, disorganized vasculature, chronic inflammation, and modulations of the immune microenvironment in the diethyl nitrosamine (DEN) chronically induced HCC rat model [(5)]. Nitrosamines including DEN have toxic and mutagenic process Alkylating guanine's N-7 results in DNA instability and enhanced DNA breaking [(6)(7)], and enhance oxidative stress, DNA damage, lipid peroxidation, and protein adduct formation by producing reactive oxygen species such superoxide (O2) and hydrogen peroxide (H2O2) [(8,9)].

Phytochemicals, which are bioactive substances found in medicinal plants that have therapeutic benefits, come in a wide variety. Plants have analgesic, anti-inflammatory, antiviral, antimalarial, and anticancer properties. [(10)], Plants also produce antioxidative phenolic chemicals like flavonoids, which are known antioxidants, to protect themselves from oxidative damage brought on by ultraviolet (UV) radiation. [(11)].

Sea hibiscus, sometimes known as a cotton tree, is a tropical and subtropical mangrove-associated coastal plant. *H. tiliaceus* trees grow quickly, reaching a height of 10 m and generating low, spreading branches. Heart-shaped and grouped in spirals, the leaves. Flowers are bell-shaped, orange-red in the morning, becoming orange-red in the evening, and mauve the next morning. Their heart and stigma are maroon in color [(12)]. An increase in flavonols and anthocyanins may be the cause of the yellow-to-red color shift in flowers. [(13)].

Hibiscus tiliaceus L. (Malvaceae) was used as a traditional medication to cure typhoid, diarrhea, dysentery, ear infections, coughs, chest congestion, and dry throat [(14)], According to the phytochemistry test, the *H. tiliaceus* leaves included phenols, flavonoids, tannins, glycosides, terpenoids, and steroid chemicals in addition to carbohydrates and proteins. [(15)].

and the extract of *H. tiliaceus* was considered to have a variety of interesting pharmacological properties, including anti-inflammatory, anthelmintic, antibacterial, and antioxidant activity, and was used as a therapeutic and chemopreventive drug [(16)].

Materials and Methods

Chemicals and drugs

Diethyl nitrosamine (DEN) obtained from Sigma Aldrich

Plant material

H. tiliaceus L. leaves were collected in Master of Regents City, Karbala-Baghdad Rd, Karbala, in April 2022, and authenticated By Dr. Prof. Khazal Wadi Al-Jubori at the University of Diyala/ Collage of Science.

Extraction of the plant

The leaves of the plant were dried in the shade at room temperature, then rendered into a fine powder by using an electrical mill and weighed.

The pulverized plant material (roughly 150 g) was defatted separately with enough hexane solvent for 24 hours to remove chlorophyll and the hexane soluble compounds like waxy material, followed by placing it in the thimble chamber of the Soxhlet apparatus and putting it through the soxhlet extractor using solvent ethanol (80 %; v/v) To create semi-solid material, the extract was filtered and concentrated at 30–40°C in a rotary evaporator [17,18].

Phytochemical Screening

The extract was subjected to a conventional procedure for phytochemical screening for flavonoids, coumarins, tannins, saponin, and terpenoids [19,20].

Experimental animals and treatment

Twenty -four male albino rats, aged 1-2 months and weighing an average of (100-150gm), were obtained from the animal house of the College of Pharmacy/University of Baghdad. Before treatment, the animals had a 2-week acclimatization period in a typical laboratory setting. Water and a regular diet were freely available to them. They were kept under normal conditions of temperature (30ºC), cycles of light and dark, and humidity. All of the experimental experiments were carried out in violation of our college's ethical protocol guidelines for the care and use of experimental animals. The animals were used in this study divided equally into four groups, each group with 6 rats they were treated as follows:

Group I: (negative control) received an oral daily dose of 1ml /kg/day of distilled water (DW) for 20 weeks. Group II: (positive control), in which rats intraperitoneally (I.P.) were injected with only 70 mg/kg diethyl nitrosamine (DEN) once per week for 10 continuous weeks [21].

Group III: received 250mg/kg [22] of Iraqi *H. tiliaceus* L. ethanol leaves extract as chronic oral administration of 1:2000 with food [23] for 5 days per week for 20 weeks with subsequently intraperitoneal dose of DEN (70 mg/kg) once per week [21] for 10 continuous weeks.

Group IV: received 500mg/kg [22] of *H. tiliaceus* L. ethanol leaves extract as chronic oral administration of 1:4000 with food [23] for 5 days per week for 20 weeks with subsequent intraperitoneal dose of DEN (70 mg/kg) once per week [21] for 10 continuous weeks.

After 24 h of the end of the experimental period (20 weeks), all the animals were sacrificed by euthanizing rats by diethyl ether anesthesia and then by cervical dislocation and in clean test tubes the blood samples were collected and allowed to clot and centrifuged at room temperature to obtain serum and liver tissue obtained for tissue homogenization.

Biochemical assessment

To determine the levels of the enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), total serum bilirubin (TSB), alpha-fetoprotein (AFP), interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), the serum was separated by centrifugation for 20 minutes at 3600 revolutions per minute (rpm).

Preparation of liver tissue homogenate

After the rat was euthanized by anesthetic ether, the liver was quickly excised, rinsed in ice-cold buffer phosphate saline pH 7.4 to remove excess thorough blood and weighed before homogenization, and add phosphate buffer saline (PBS) to tissue in a ratio 1:9 (1gm of tissue and 9 ml of PBS) than the tissue homogenized by homogenizer after putting the tube in a beaker containing ice. After that the homogenate was then centrifuged for 20 min at 10000 rpm using a cold centrifuge and the supernatant was utilized for the estimation of glutathione peroxidase (GPX), superoxide dismutase (SOD), and malondialdehyde (MDA) levels.

Enzyme-Linked Immunosorbent Assay

Using ELISA kits, the quantities of serum tumor necrosis factor (TNF-α) and interleukin-1 (IL-1β), as well as tissue homogenate supernatant, were determined. Briefly, the assay plate was prepared and filled with the serum, standards, and blank for 1 hour at 37°C. Following incubation, stop solutions, chromogenic substrate, enzyme conjugate, biotinylated-specific antibody, and were added in that sequence. The optical density (OD) at 450 nm was determined using a microplate reader.

Statistical analysis

Utilizing graph prism version 9, data were examined. The mean and standard error of the mean were used to express the numerical data. Post hoc analysis was used to establish the statistical significance of each group's comparison to the induction group, and the one-way ANOVA test was used to compare each group to all others. All of the results given in this study were considered significant if the p-value was less than 0.05 (p0.05).

Result

Preliminary Phytochemical Screening of Hibiscus tiliaceus ethanol leaves extract

Testing for flavonoids: involved adding conc. H2SO4 and 5ml of diluted ammonia solution to a quantity of crude extract. Each extract had a yellow coloration that suggested the presence of flavonoids.

Testing for coumarin: involved adding 10% NaOH to the extract and adding chloroform to look for a yellow color, which indicates the presence of coumarin.

Test for tannins (Ferric chloride) test: One or two drops of 10% ferric chloride were given to 2 ml

of the sample in a test tube bluish-black color developed so tannins were present.

Test for saponins: Crude extract was mixed with 5 ml of distilled water in a test tube and it was shaken vigorously leading to a 1 cm foam formation that indicates the presence of saponin

the Salkowski test for terpenoids: 5 ml of extract was combined with 2 ml of chloroform, and 3 ml of concentrated H2SO4 to make a layer. The contact developed a reddish-brown coloration as proof that terpenoids were present.

Phytochemicals	***Hibiscus tiliaceus* ethanol leaves extract**
Flavonoids	+
Tannins	+
Saponins	+
Terpenoids	+
coumarin	+

Effect on Alpha-fetoprotein (AFP)

Group II (induction by DEN) rats showed significant elevation ($p<0.05$) in the level of AFP Mean ±SEM (13.53±0.7872) compared to the corresponding level in the control (group I) rats Mean ±SEM (1.610±0.05049).

Group III in which rats have induction by DEN and received 250mg/kg of Iraqi *H. tiliaceus* L. ethanol leaves extract revealed a considerable decline ($p<0.05$) in AFP level mean ±SEM (7.05±0.3547) compared to the corresponding level in group II HCC rats where mean ±SEM (13.53±0.7872).

Group IV in which rats have induction by DEN and received 500mg/kg of Iraqi *H. tiliaceus* L. ethanol leaves extract revealed a considerable decline ($p<0.05$) in AFP level mean ±SEM (3.09±0.264) compared to the corresponding level in group II HCC rats where mean ±SEM (13.53±0.7872) as shown in table 1 below and Figure 1.

Effects on alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and total serum bilirubin (TSB)

Group II (induction by DEN) rats demonstrated a clear rise ($p<0.05$) in the level of ALT, AST, and TSB compared to the corresponding level in the control (group I) rats, Mean ±SEM of ALT, AST, and TSB were respectively (60.16±3.266), (51.03±2.35) and (1.59±0.08449) compared to (27.32±0.557), (22.62±0.5702) and (0.3982±0.0106).

Group III in which rats have induction by DEN and received 250mg/kg of Iraqi *H. tiliaceus* L. ethanol leaves extract showed significant decrease ($p<0.05$) in ALT, AST, and TSB compared to the corresponding level in group II rats where mean ±SEM of ALT, AST, and TSB were respectively (30.83±0.1087), (24.08±0.2614), (0.4567±0.009387) compared to (60.16±3.266), (51.03±2.35) and (1.59±0.08449).

Group IV in which rats have induction by DEN and received 500mg/kg of Iraqi *H. tiliaceus* L. ethanol leaves extract showed a significant decrease ($p<0.05$) in ALT, AST, and TSB compared to corresponding level in group II rats where mean ±SEM of ALT, AST, and TSB were respectively (17.73±0.5083), (16.64±0.3619) and (0.3307 ± 0.005959) compared to (60.16±3.266), (51.03±2.35) and (1.59±0.08449) as shown in table 1 below and Figure 1.

Effects on interleukin 1 β (IL1β) and tumor necrosis factor α (TNFα)

Group II (induction by DEN) rats showed significant elevation ($p<0.05$) in the level of (IL1β) and (TNFα) compared to the corresponding level in the control (group I) rats Mean ±SEM of IL1-β and TNFα were respectively (7.363±0.8231) and (2276±149) compared to (0.798±0.1018) and (367±64.27).

Group III in which rats have induction by DEN and received 250mg/kg of Iraqi *H. tiliaceus* L. ethanol leaves extract showed a significant decrease ($p<0.05$) in the level of (TNF-α) where mean ±SEM (1737±33.49) compared to corresponding level in group II rats where mean ±SEM (2276±149) and the decrease in the level of IL1β that is non-significant Mean ±SEM (5.534±0.6136) compared to corresponding level in group II rats where mean ±SEM (7.363±0.8231).

Group IV, in which rats have induction by DEN and received 500mg/kg of Iraqi *H. tiliaceus* L. ethanol leaves extract, showed a significant decrease ($p<0.05$) in the level of (IL1-β) and (TNF-α) where mean ±SEM (1.339±0.1382) and (563.9±64.11) compared to the corresponding level in group II rats Mean ±SEM were respectively (7.363±0.8231) and (2276±149) as shown in table 1 below and Figure 2.

Effects on transforming growth factor beta (TGFβ)

Group II (induction by DEN) rats showed significant elevation ($p<0.05$) in the level of (TGF-β) Mean ±SEM (342.1±33.75) compared to the corresponding level in control (group I) rats Mean ±SEM (59.57±6.615)

Group III in which rats have induction by DEN and received 250mg/kg of Iraqi *H. tiliaceus* L. ethanol leaves extract showed a significant decrease ($p<0.05$) in the level of (TGFβ) Mean ±SEM (177.1±17.76) compared to the corresponding level in group II rats where mean ±SEM (342.1±33.75).

Group IV in which rats have induction by DEN and received 500mg/kg of Iraqi *H. tiliaceus* L. ethanol leaves extract showed a significant decrease ($p<0.05$) in the level of (TGF-β) Mean ±SEM (59.86±2.366) compared to the corresponding level in group II rats where mean ±SEM (342.1±33.75) as shown in table 1 below and Figure 2.

Effects on malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPX)

Group II (induction by DEN) rats showed significant elevation ($p<0.05$) in the level of MDA Mean ±SEM (5.259±0.4098) compared to the corresponding level in the control (group I) rats Mean ±SEM (0.7113±0.665).

While rats have induction by DEN for HCC in group II showed a significant decrease ($p<0.05$) in the level of SOD and GPX Mean ±SEM respectively (30.13±3.928), (5.179±0.2825) compared to the corresponding level in control (group I) rats Mean ±SEM (106.2±6.693) and (12.56±1.113).

Group III in which rats have induction by DEN and received 250mg/kg of Iraqi *H. tiliaceus* L. ethanol leaves extract showed a significant decrease ($p<0.05$) in the level of MDA Mean ±SEM were (3.603±0.2178) compared to the corresponding level in group II HCC rats where mean ±SEM (5.259±0.4098) while showed non-significant increase ($p<0.05$) in the level of SOD and GPX Mean ±SEM were respectively (37.03±2.674) and (5.531±0.1307) compared to corresponding level in group II HCC rats mean ±SEM were respectively (30.13±3.928) and (5.179±0.2825).

Group IV in which rats have induction by DEN and received 500mg/kg of Iraqi *H. tiliaceus* L. ethanol leaves extract, showed a significant decrease ($p<0.05$) in the level of MDA Mean ±SEM (0.5145±0.01037) compared to the corresponding level in group II HCC rats where mean ±SEM (5.259±0.4098) and showed a significant increase ($p<0.05$) in the level of SOD Mean ±SEM were (78.81±5.004) compared to the corresponding level in group II HCC rats mean ±SEM were (30.13±3.928) while showed a non-significant increase ($p<0.05$) in the level of GPX Mean ±SEM (7.255±0.2820) compared to the corresponding level in group II HCC rats mean ±SEM (5.179±0.2825) as shown in table 1 below and Figure 2.

Table 1. Effect of low and high doses of *Hibiscus tiliaceus L*. leaves ethanol extract on serum level of ALT, AST, total bilirubin, AFP, IL1β, TNFα and tissue homogenate of MDA, SOD, GPX

	Control group (Group I)	(DEN)Induction (group II)	Group III	Group IV
AFP	1.610±0.05049	13.53±0.7872*	7.05±0.3547#	3.09±0.264#
ALT	27.32±0.557	60.16±3.266*	30.83±0.1087#	17.73±0.5083#
AST	22.62±0.5702	51.03±2.35*	24.08±0.2614#	16.64±0.3619#
TSB	0.3982±0.0106	1.59±0.08449*	0.4567±0.009387#	0.3307±0.005959#
MDA	0.7113±0.665	5.259±0.4098*	3.603±0.2178#	0.5145±0.01037#
GPX	12.56±1.113	5.179±0.2825*	5.531±0.1307	7.255±0.2820
SOD	106.2±6.693	30.13±3.928*	37.03±2.674	78.81±5.004#
TNFα	367±64.27	2276±149*	1737±33.49#	563.9±64.11#
IL1β	0.798±0.1018	7.363±0.8231*	5.534±0.6136	1.339±0.1382#
TGFβ	59.57±6.615	342.1±33.75*	177.1±17.76#	59.86±2.366#

Value are expressed as mean ±SEM; n=6 rats in each group;(*) refer to significant difference in groups($p<0.05$) compared to group I; (#) refer to significant difference in groups($p<0.05$) compared to group II

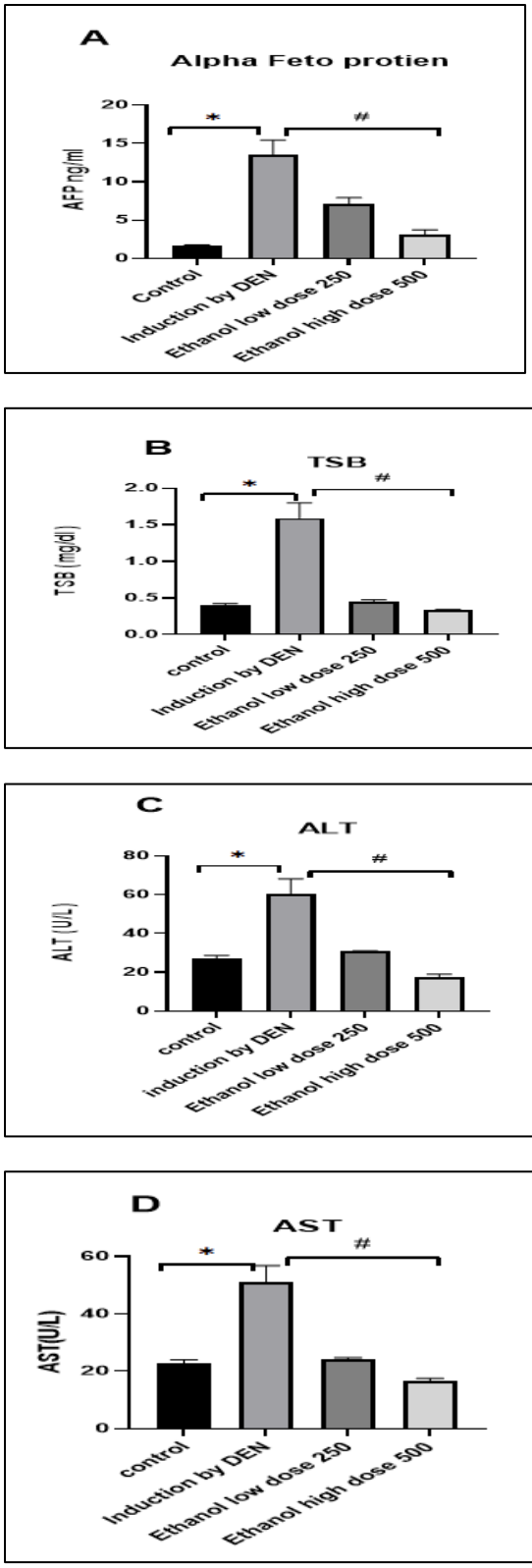

Figure 1. Hibiscus tiliaceus L. ethanol leaves extract effect in two doses on (A) alpha-fetoprotein (AFP) liver cancer marker and on (B) total serum bilirubin (TSB) and liver enzyme (C)ALT and (D)AST

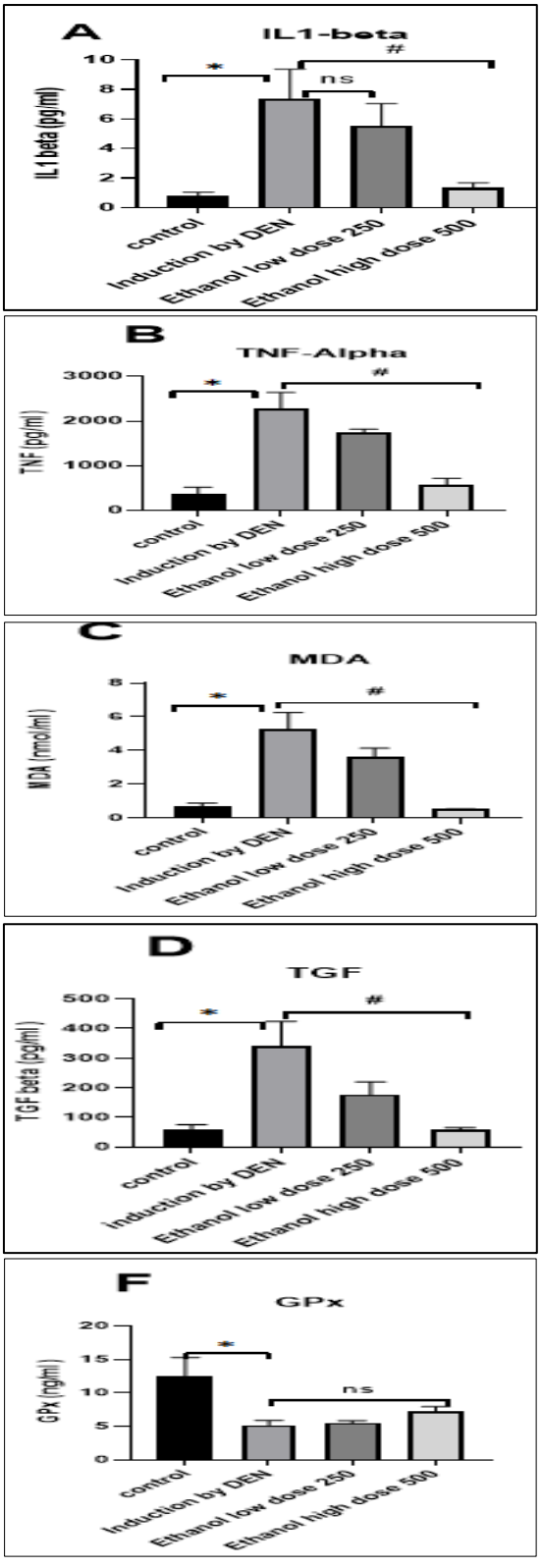

Figure 1. Hibiscus tiliaceus L. ethanol leaves extract in two doses affect inflammatory mediators (A)interleukin 1 beta (IL1 beta), (B)tumor necrosis factor alfa (TNF alfa), and (D)transforming growth factor beta (TGF beta) and on (C) Malondialdehyde (MDA) antioxidant enzymes (E) superoxide dismutase (SOD) and (F) glutathione peroxidase (GPX)

Histopathologic examination:

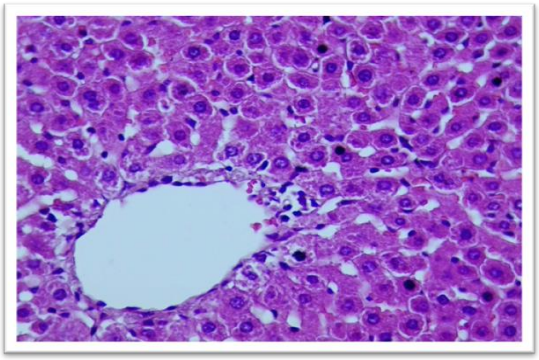

Figure 3. The normal liver architecture of Group I: (negative control) received distilled water only. H.&E (x100)

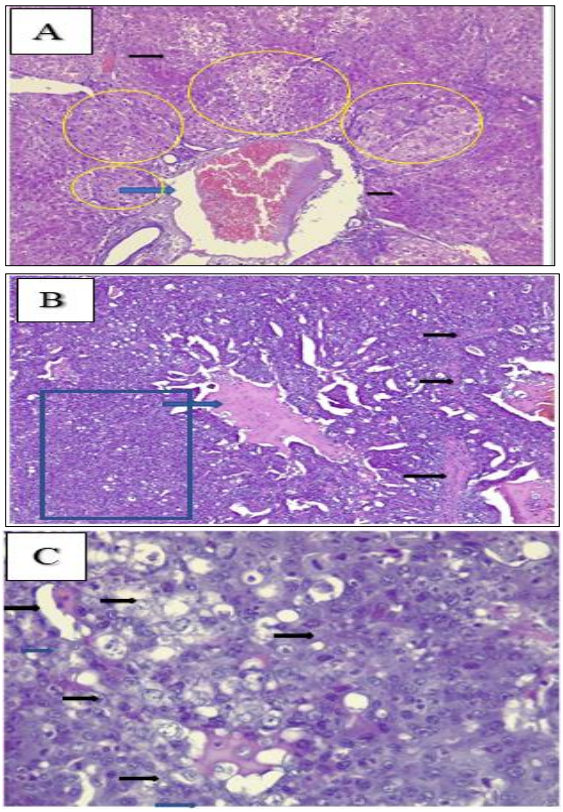

Figure 4. Group II, positive control in which rats intraperitoneally were injected with (DEN) developed HCC, **(A)** A dilated portal triad with extensive dilatation to the branch of the portal vein (⟹) surrounded by multiple focal necrotic nodules (circled), making it lose the normal architecture of the liver, with diffused areas of eosinophilic cell alteration (➡), **(B)** Complete loss for the normal liver architecture. The central vein (⟹) is ruptured and disrupted, with the absence of normal hepatic cords arrangement (▭). Fibrous tissue is seen (➡), **(C)** Ballooning degeneration of multiple hepatocytes (⟹), which is a manifestation of apoptosis (single-cell necrosis). Complete loss for the original liver architecture with the colloid formation in more than one area (⟹). H.&E (x100)

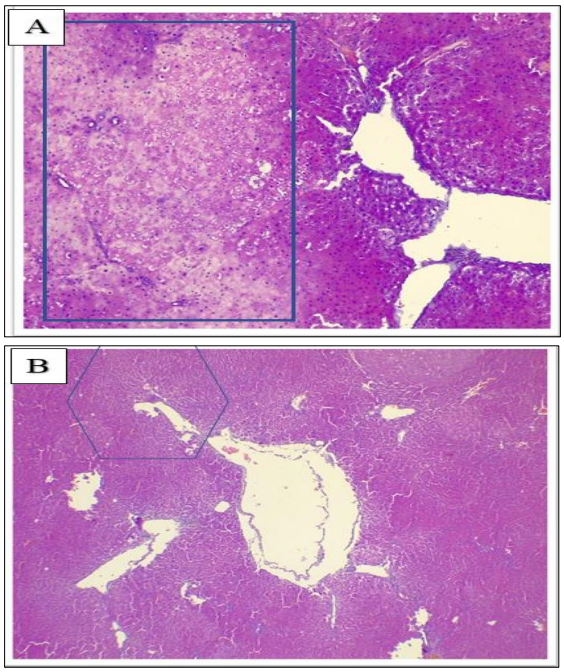

Figure 5. (A) The liver of a rat induced for HCC with DEN and treated with 250mg/kg of Iraqi *Hibiscus tiliaceus* L. ethanol leaves extract demonstrated complete disruption for the hepatic architecture with diffused areas of hepatocellular vacuolar degeneration involving a large area of fatty change and cytoplasmic collagen is well-noticed, **(B)** The liver of a rat induced for HCC with DEN and treated with 500mg/kg of Iraqi *Hibiscus tiliaceus* L. ethanol leaves extract demonstrating zones of normal liver architecture are noticed (encircled), with no evidence of necrosis or hepatic cell degeneration. H.&E (x100)

Discussion

The most prevalent kind of primary liver cancer, hepatocellular carcinoma, is distinguished by a growing prevalence and a high fatality rate. Hepatocellular carcinoma animal models are frequently used to research the biology of cancer and evaluate new treatments. The pathogenesis of human hepatocellular carcinoma, including liver damage, chronic inflammation, hepatocyte proliferation, liver fibrosis and cirrhosis, disorganized vasculature, and modulations of the immune microenvironment, is mimicked in the rat model of DEN-induced hepatocellular carcinoma, preclinical drug testing using the DEN-induced rat liver cancer model [(2)].

Hepatocarcinogenesis is caused by the frequently used hepatocarcinogen DEN, which impacts cancer start by causing DNA strand breaks and carcinogen adducts by alkylation [(24)]. Hepatocellular carcinogenesis caused by DEN is influenced by reactive oxygen species (ROS) [(25)].

H. tiliaceus possess hepatoprotective and antimutagenic characteristics, is a source of natural antibacterial agents, and antioxidants, and has medical and therapeutic benefits; as a result, it has a

wealth of phytochemicals such as terpenoids, alkaloids, phenols, and flavonoids [26][27].

Therefore, the purpose of the current investigation was to examine any potential protective effects that an ethanolic leaf extract of *H.tiliaceus* could have against HCC.

For predicting HCC, alpha-fetoprotein (AFP, or α-fetoprotein) is recognized as the gold standard. It is a big serum glycoprotein that is a member of the once-development family [28]. It has a half-life of 5-7 days and is largely produced in the first trimester of pregnancy by the fetal digestive tract, vital line sac cells, and embryonic liver. After birth, the blood content of AFP rapidly decreases, and in adulthood, its expression is suppressed. In HCC, AFP is increased pathologically [29].

Our findings demonstrate that the group II model of DEN-induced HCC rats, where rats were intraperitoneally (I.P.) administered with 70 mg/kg (DEN) once per week for 10 continuous weeks showed significantly increased levels of AFP as compared with the normal control group inconsistent with other studies [30][31]. And the administration of *H.tiliaceus* leaves ethanol extract caused a significant decrease in AFP level as compared with HCC-induced rats in group II in a dose-dependent manner.

The liver function tests include Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin. (TBS), These tests can locate liver damage, and the pattern of increase can help with a differential diagnosis [32,33]. While AST is present as cytosolic and mitochondrial isoenzymes and is present in the liver, cardiac muscle, skeletal muscle, kidneys, brain, pancreas, lungs, leucocytes, and red cells, ALT is a cytosolic enzyme that is found in high amounts in the liver. In most cases of liver illness, when both enzymes are primarily active in the cytosol of hepatocytes, ALT is often greater than AST [34]. These enzymes are released into the bloodstream in response to cell death and hepatocellular damage. Normal males have greater AST and ALT readings than normal females. [35][36]. They are important indicators that are frequently employed in animal research to identify and track the development of hepatocarcinogenesis [31,37].

According to our findings in the rat model of DEN-induced HCC, group II showed significantly increased levels of ALT, AST, and TSB as compared to the normal control group consistent with other studies [21][30][31], and the administration of *H.tiliaceus* leaves ethanol extract cause a significant decrease in the levels of ALT, AST, and TSB in a dose-dependent manner as compared with HCC induced rats in group II.

Under a variety of pathological circumstances, oxidative stress is a key component in the development of HCC, ROS-induced oxidative stress damages hepatocytes and increases inflammation [38].

A typical indicator of oxidative stress and the antioxidant state in cancer patients is the quantity of malondialdehyde [39,40]. Our study shows that the DEN-induced HCC rat model in group II showed significant elevation in the levels of MDA as compared with the normal control group consistent with other studies [21][30]. the administration of *H.tiliaceus* leaves ethanol extract caused a significant decrease in the level of MDA in a dose-dependent manner as compared with HCC-induced rats in group II due to its high contents of polyphenols and flavonoids [41,42].

The most significant antioxidant enzyme in aerobic cells, superoxide dismutase (SOD), eliminates superoxide radicals. SOD catalyzes the dismutation of molecular oxygen and hydrogen peroxide. The primary enzyme that breaks down hydrogen peroxide into water in cells is glutathione peroxidase (GPX). SOD and GPX can directly counteract the oxidant onslaught and shield cells from DNA damage [43,44].

The DEN-induced HCC rat model in group II showed a significant decrease in the levels of SOD and GPX as compared with the normal control group in consistent with other studies [30][31].

The administration of *H.tiliaceus* leaves ethanol extract caused a significant increase in the levels of SOD at a dose of 500mg/kg [42].

The production of proinflammatory TNF and IL1 in response to tissue damage is linked to an acceleration of the cell cycle, oxidative stress-induced DNA damage, and regulation of tumor development in HCC [45]. Our findings are similar to prior research in that group II in the DEN-induced HCC rat model displayed significantly higher levels of proinflammatory TNF and IL1 than the healthy control group [30][31]. The administration of *H.tiliaceus* leaves ethanol extract caused a significant decrease in the levels of TNFα in a dose-dependent manner as compared with HCC-induced rats in group II, and cause a significant decrease in the levels of IL1β when administered only in dose 500mg/kg as compared with HCC induced rats in group II [22].

Malignant cells may frequently decrease their suppressive TGF-β signaling by changing the expression of its receptors. TGF-β controls various inflammatory processes, which typically result in the suppression of cellular activities, including proliferation, differentiation, and surviving carcinogenesis [(46)]. Recently, it was claimed that TGF-β enhanced the potential for improved chemoresistance by inducing the expression of drug-efflux transporters via activation of the PXR nuclear receptor for xenobiotics [(47)]. Our findings are similar to prior research in that group II in the DEN-induced HCC rat model had significantly higher levels of the cytokine TGF-β relative to the normal control group [(48)], administration of *H.tiliaceus* leaves ethanol extract caused a significant decrease in the level of TGF-β in a dose-dependent manner as compared with HCC induced rats in group II.

Funding

No financial assistance was received

Ethics Statements

This research was approved by the Ethics Committee of the College of Pharmacy, Baghdad University.

Conflict of interest

The authors declare no conflict of interest

Author contributions

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved the submission.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians. 2021 May;71(3):209-49.
2. Matter MS, Marquardt JU, Andersen JB, Quintavalle C, Korokhov N, Stauffer JK, Kaji K, Decaens T, Quagliata L, Elloumi F, Hoang T. Oncogenic driver genes and the inflammatory microenvironment dictate liver tumor phenotype. Hepatology. 2016 Jun;63(6):1888-99.
3. Macek Jilkova Z, Kurma K, Decaens T. Animal models of hepatocellular carcinoma: the role of immune system and tumor microenvironment. Cancers. 2019 Oct 2;11(10):1487.
4. Shankaraiah RC, Gramantieri L, Fornari F, Sabbioni S, Callegari E, Negrini M. Animal models of hepatocellular carcinoma prevention. Cancers. 2019 Nov 14;11(11):1792.
5. Kurma K, Manches O, Chuffart F, Sturm N, Gharzeddine K, Zhang J, Mercey-Ressejac M, Rousseaux S, Millet A, Lerat H, Marche PN. DEN-induced rat model reproduces key features of human hepatocellular carcinoma. Cancers. 2021 Oct 4;13(19):4981.
6. Swann PF, Magee PN. Nitrosamine-induced carcinogenesis. The alkylation of nucleic acids of the rat by N-methyl-N-nitrosourea, dimethylnitrosamine, dimethyl sulphate and methyl methanesulphonate. Biochemical Journal. 1968 Nov;110(1):39-47.
7. Otteneder M, Lutz WK. Correlation of DNA adduct levels with tumor incidence: carcinogenic potency of DNA adducts. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 1999 Mar 8;424(1-2):237-47.
8. Chuang CH, Hu ML. Synergistic DNA damage and lipid peroxidation in cultured human white blood cells exposed to 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone and ultraviolet A. Environmental and molecular mutagenesis. 2006 Mar;47(2):73-81.
9. Lim IK. Spectrum of molecular changes during hepatocarcinogenesis induced by DEN and other chemicals in Fischer 344 male rats. Mechanisms of ageing and development. 2002 Nov 1;123(12):1665-80.
10. Raina H, Soni G, Jauhari N, Sharma N, Bharadvaja N. Phytochemical importance of medicinal plants as potential sources of anticancer agents. Turkish Journal of Botany. 2014;38(6):1027-35.
11. Larson RA. The antioxidants of higher plants. Phytochemistry. 1988 Jan 1;27(4):969-78.
12. Chan EW, Wong SK, Chan HT. A review on the phytochemistry and pharmacology of two Hibiscus species with spectacular flower colour change: H. tiliaceus and H. mutabilis. International Journal of Pharmacognosy and Phytochemical Research. 2016;8(7):1200-8.
13. Shimokawa S, Iwashina T, Murakami N. Flower color changes in three Japanese Hibiscus species: further quantitative variation of anthocyanin and flavonols. Natural Product Communications. 2015 Mar;10(3):1934578X1501000319.
14. Ramproshad S, Afroz T, Mondal B, Haque A, Ara S, Khan R, Ahmed S. Antioxidant and antimicrobial activities of leaves of medicinal plants Hibiscus tiliaceus L. Pharmacologyonline. 2012 Dec 30;3:82-7.
15. Samsudin MS, Andriani Y, Sarjono PR, Syamsumir DF. Study on Hibiscus tiliaceus leaves as antibacterial and antioxidant agents. Alotrop. 2019 Dec 19;3(2).
16. Vijay T, Rajendra B. Phytochemical screening and anthelmintic activity of wood and leaves of Hibiscus tiliaceus Linn. World Journal of Pharmacy and Pharmaceutical Sciences (WJPPS). 2014;3(10):880-9.
17. Mahire SP, Patel SN. Extraction of phytochemicals and study of its antimicrobial

and antioxidant activity of Helicteres isora L. Clinical Phytoscience. 2020 Dec;6:1-6.

18. Mutlag SH, Hamad MN, Abbas IS, Ismael SH. The evaluation of ethyl acetate fraction of Cressa cretica effect on mitotic index and micronucleous Frequency in Mice. Int J Pharm Sci Rev Res. 2017;45(28):147-50.

19. Mutlag SH, Ismael SH, Hassan AF, Abbas RF. Genotoxic effect of ethyl acetate fraction of cressa cretica on chromosomal aberration on bone marrow cells and spleen cells in mice. Int. J. Pharm. Sci. Rev. Res. 2017 Mar;43(2):220-3.

20. Khamees AH, Kadhim EJ, Sahib HB, Mutlag SH. In vitro analysis of antioxidant and antimicrobial activity of Iraqi Bryonia dioica. Int J Pharm Sci Rev Res. 2017;43(1):248-52.

21. Song J, Ding W, Liu B, Liu D, Xia Z, Zhang L, Cui L, Luo Y, Jia X, Feng L. Anticancer effect of caudatin in diethylnitrosamine-induced hepatocarcinogenesis in rats. Molecular Medicine Reports. 2020 Aug 1;22(2):697-706.

22. Narender KS, Kumar D, Kumar V. Antinociceptive and anti-inflammatory activity of Hibiscus tiliaceus leaves. International Journal of Pharmacognosy and Phytochemical Research. 2009;1(1):15-7.

23. Turrini F, Vallarino G, Cisani F, Donno D, Beccaro GL, Zunin P, Boggia R, Pittaluga A, Grilli M. Use of an animal model to evaluate anxiolytic effects of dietary supplementation with Tilia tomentosa moench bud extracts. Nutrients. 2020 Oct 29;12(11):3328.

24. Tolba R, Kraus T, Liedtke C, Schwarz M, Weiskirchen R. Diethylnitrosamine (DEN)-induced carcinogenic liver injury in mice. Laboratory animals. 2015 Apr;49(1_suppl):59-69.

25. Lin H, Liu XB, Yu JJ, Hua F, Hu ZW. Antioxidant N-acetylcysteine attenuates hepatocarcinogenesis by inhibiting ROS/ER stress in TLR2 deficient mouse. PLoS One. 2013 Oct 2;8(10):e74130.

26. Rajeswari G, Priyanka B, Amrutha RE, Rajaram C, Kanhere RS, Kumar SN. Hibiscus tiliaceus: a possible immunomodulatory agent. Journal of Pharmacy Research. 2013 Jul 1;6(7):742-7.

27. Prachiti RP, Berde PC. Phytochemical Screening of Hibiscus tiliaceus by FTIP Spectroscopic Analysis. International Jounral of Pharmacy and Biological Sciences–IJPBS. 2019;9(3):1308-19.

28. Gillespie JR, Uversky VN. Structure and function of α-fetoprotein: a biophysical overview. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology. 2000 Jul 14;1480(1-2):41-56.

29. Grizzi F, Colombo P, Taverna G, Chiriva-Internati M, Cobos E, Graziotti P, Muzzio PC, Dioguardi N. Geometry of human vascular system: is it an obstacle for quantifying antiangiogenic therapies?. Applied Immunohistochemistry & Molecular Morphology. 2007 Jun 1;15(2):134-9.

30. Zhang Y, Li X, Li X. Curcumae ameliorates diethylnitrosamine-induced hepatocellular carcinoma via alteration of oxidative stress, inflammation and gut microbiota. Journal of Inflammation Research. 2021;14:5551.

31. You Y, Zhu F, Li Z, Zhang L, Xie Y, Chinnathambi A, Alahmadi TA, Lu B. Phyllanthin prevents diethylnitrosamine (DEN) induced liver carcinogenesis in rats and induces apoptotic cell death in HepG2 cells. Biomedicine & Pharmacotherapy. 2021 May 1;137:111335.

32. Ribeiro AJ, Yang X, Patel V, Madabushi R, Strauss DG. Liver microphysiological systems for predicting and evaluating drug effects. Clinical Pharmacology & Therapeutics. 2019 Jul;106(1):139-47.

33. Zalzala MH, Kareem VS. The Safranal Effect against Cyclophosphamide-Induced Liver Injury. Iraqi Journal of Pharmaceutical Sciences (P-ISSN 1683-3597 E-ISSN 2521-3512). 2021 Dec 11;30(2):208-13.

34. Ahmad HA, Kathem SH. Thymoquinone Attenuates Immune Mediated Liver Injury Induced by Concanavalin A in Mice. Iraqi Journal of Pharmaceutical Sciences (P-ISSN 1683-3597 E-ISSN 2521-3512). 2021 Dec 9;30(2):50-7.

35. Prati D, Taioli E, Zanella A, Torre ED, Butelli S, Del Vecchio E, Vianello L, Zanuso F, Mozzi F, Milani S, Conte D. Updated definitions of healthy ranges for serum alanine aminotransferase levels. Annals of internal medicine. 2002 Jul 2;137(1):1-0.

36. Oh RC, Hustead TR, Ali SM, Pantsari MW. Mildly elevated liver transaminase levels: causes and evaluation. American family physician. 2017 Dec 1;96(11):709-15.

37. Aziz TA, Ahmed ZA, Juma'a KM, Abdulrazzaq MH, Hussain SA. Study of the protective effects of benfotiamine against CCl4-induced hepatotoxicity in rats. Iraqi J Pham Sci. 2009;18:47-53.

38. Fu Y, Chung FL. Oxidative stress and hepatocarcinogenesis. Hepatoma research. 2018;4

39. Gaweł S, Wardas M, Niedworok E, Wardas P. Malondialdehyde (MDA) as a lipid peroxidation marker. Wiadomosci lekarskie (Warsaw, Poland: 1960). 2004 Jan 1;57(9-10):453-5.

40. Alduboni NM, Al-Shawi NN. Possible protective effects of two different doses of cyanocobalamin against methotrexate nephrotoxicity in rats. Iraqi Journal of

Pharmaceutical Sciences (P-ISSN 1683-3597 E-ISSN 2521-3512). 2022 Dec 24;31(2):212-7.
41. Abd Al-Zahra JI, Ismael DK, Al-Shawi NN. Preventive effects of different doses of pentoxyfilline against ccl4-induced liver toxicity in rats. Iraqi J Pharm Sci. 2009;39
42. Wong SK, Lim YY, Chan EW. Antioxidant properties of Hibiscus: Species variation, altitudinal change, coastal influence and floral colour change. Journal of Tropical Forest Science. 2009 Oct 1:307-15.
43. Muttlag SH, Kasim AA, Jassim AR. The Possible Protective Effect of Simultaneous Administration of Alcoholic Ammi majus Seeds' Extract Against Gentamicininduced Nephrotoxicity in Rats. Al Mustansiriyah Journal of Pharmaceutical Sciences. 2012 Jun 1;11(1):71-8.
44. Gurudath S, Naik RM, Ganapathy KS, Guruprasad Y, Sujatha D, Pai A. Superoxide dismutase and glutathione peroxidase in oral submucous fibrosis, oral leukoplakia, and oral cancer: A comparative study. Journal of Orofacial Sciences. 2012 Jul 1;4(2):114.
45. Budhu A, Wang XW. The role of cytokines in hepatocellular carcinoma. Journal of leukocyte biology. 2006 Dec;80(6):1197-213.
46. Massagué J. TGFβ in cancer. Cell. 2008 Jul 25;134(2):215-30.
47. Bhagyaraj E, Ahuja N, Kumar S, Tiwari D, Gupta S, Nanduri R, Gupta P. TGF-β induced chemoresistance in liver cancer is modulated by xenobiotic nuclear receptor PXR. Cell Cycle. 2019 Dec 17;18(24):3589-602.
48. Shen Y, Wei Y, Wang Z, Jing Y, He H, Yuan J, Li R, Zhao Q, Wei L, Yang T, Lu J. TGF-β regulates hepatocellular carcinoma progression by inducing Treg cell polarization. Cellular Physiology and Biochemistry. 2015 ;35 (4) :1623-32.