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RESEARCH ARTICLE

# THE ANTIOXIDANT AND HEPATOPROTECTIVE ACTIVITIES OF THE ETHANOLIC EXTRACT OF *TINOSPORA CORDIFOLIA* LEAVES: *IN VITRO* AND *IN VIVO* STUDIES

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## ABSTRACT

*Tinospora cordifolia* has been used in Ayurvedic medicine to treat a number of diseases for a long time. The antioxidant and hepatoprotective activities of the ethanolic extract of *T. cordifolia* leaves (TC extract) were tested in the present study on the cultured rat hepatocytes and thioacetamide (TAA)-treated rats. For *in vivo* experiments, forty male Wistar rats were allotted randomly into eight groups (n=5): group "1" acted as the control group, group "2" represented the TAA-treated rats, groups "3-5" were treated with different doses of TC extract (50, 100, and 200 mg/kg body weight, respectively, orally/daily for 4 weeks); while groups "6-8" were TAA-treated rats that received 50, 100, and 200 mg, respectively, of TC extract/kg body weight (orally/daily for 4 weeks). The TC extract exhibited no marked cytotoxic activity against the cultured hepatocytes at $IC_{50} \leq 500$ µg/mL (after 48 hours) in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The *in vivo* experiments revealed that the TC extract (at doses ≥100 mg/kg body weight) modulated significantly ($P<0.05$) the increase in the plasma aminotransferases and C-reactive protein levels, and lactate dehydrogenase activity, in the TAA-treated rats. In addition, the TC extract (at doses ≥50 mg/kg body weight) boosted significantly ($P<0.05$) the superoxide dismutase and catalase activities, and the glutathione content, in the hepatic tissues of the TAA-treated rats. Moreover, the histological results confirmed the hepatoprotective effects of the TC extract in the TAA-treated rats. In conclusion, the TC extract showed a hepatoprotective activity in the TAA-treated rats by its powerful antioxidant potential.

## INTRODUCTION

Plants have been employed as therapeutic aids; many people across the world have relied on natural remedies for thousands of years. The most well-known herbal methods, such as Ayurveda, Siddha, Unani, and Homeopathy, are practiced around the world, with an increased demand at present,

in various medical systems, such as the Indian and Chinese medicine systems[1]. More recently, the public's understanding of plant-based remedies has expanded significantly[2]. Recently, Sreenivasulu and Fernie[3] stated that around 20000 plant species provide herbal medicines used by about 80% of the world's population, who rely on them for their essential medicinal and health care needs. Medical plants are still being used in drug discovery, which is yielding new and promising results in the treatment of a wide range health problems[4].

Apart from eliminating xenobiotics *via* enzymatic modification, the liver plays a critical role in avoiding their build-up[5]. Alex Merrick[6] stated that the liver is susceptible to injury during detoxification of different environmental toxins and pharmaceuticals. The global mortality and morbidity rate from liver disease is a serious public health concern[7]. One of these causative mechanisms in drug-induced liver injury is regarded as a major public health concern and is of great interest worldwide[8]. Hepatotoxins are extensively used to induce acute liver injury in animal models[9]. Thioacetamide (TAA) is one of the popular centrilobular hepatotoxicants used in animal models[10]. TAA toxicity has been attributed to two-step bio-activation. The first step produces thioacetamide sulphoxide, then thioacetamide-S and S-dioxide cause initiation of cellular necrosis[11].

The use of synthetic compounds in treating some diseases has many severe, unwanted side effects, primarily on hepatic tissue. Therefore, the usage of traditional herbal products claiming to protect the liver should be evaluated using scientific study methodology[12,13]. *Tinospora cordifolia* is a herbaceous plant that is native to the Indian subcontinent and China. It is a member of the Menispermaceae family and is extensively dispersed throughout the region. The plant possesses a wide range of therapeutic qualities; it is used in traditional medicine to treat a variety of diseases due to its anti-inflammatory and antioxidant activities[14,15]. Therefore, the purpose of the current study was to analyse the hepatoprotective ability of the ethanolic extract of *T. cordifolia* leaves (TC extract) in TAA-treated rats in order to understand its benefits through attenuation of oxidative stress.

## MATERIAL AND METHODS

### The TC extract

*T. Cordifolia* leaves were brought from Nisarg Life Sciences India Pvt. Ltd; Indore (Madhya Pradesh, India). The selected plant was identified and authenticated by Dr. Ahmed Banhawey (Botany Department, Faculty of Science, Suez Canal University) on the basis of taxonomic characters with a voucher number (ZT#012018). Plant leaves were ground into a fine powder. For extraction, 100 g of the powdered leaves were soaked in 500 mL of 75% ethanol for 72 hours, then filtered, and evaporated (using a rotary evaporator, model: IKA RV 8 Basic V-C, Asynt Ltd, Ely, UK). The yield was 12% of raw material. The sticky residue (TC extract) was kept at –20°C in a sealed container.

### Gas chromatography-mass spectrometry (GC-MS) analysis for TC extract

According to Tayade *et al.*[16], GC-MS analysis of TC extract was performed using Thermo Finnigan POLARIS Q - Mass Spectrometer (Hertfordshire, UK). Electron impact with an ionisation energy of 70eV, Helium (99.99%), was employed as the carrier gas, with a constant flow rate of 1.21 mL/min. The injector was preheated to a temperature of 200°C, whereas the temperature of the mass transfer line was set at 240°C. The temperature within the oven was adjusted from 70-220°C at a rate of 10°C per minute, held at an isothermal state for two minutes, and then raised to 300°C for ten minutes. In the splitless mode, diluted samples with a volume of 2 μL were manually injected. The split ratio was set at 1:40, and the mass scan ranged from 50 to 650 atomic mass units. The GC-MS has a running time of around 50 minutes. The data was analysed using Finnigan Xcalibur

2.0, data collection and processing software (ThermoQuest LC/MS Division, Waltham, MA, USA). The normalised peak area percentage was used to estimate the TC extract's relative concentration of various ingredients. Wiley and Tandem Mass (NITS) Libraries, which create a comprehensive Kovat index, were used for the identification of compounds by matching mass spectra to their Kovat index, in order to transform retention periods into system-independent constants[17].

### *In vitro* experiments

#### Rat hepatocytes isolation

A two-step collagenase perfusion method was used to separate intact hepatocytes from rat as previously described[18]. The isolated cells were cultured in William's E medium supplemented with 50 µg/mL gentamicin (Invitrogen; Breda, Netherlands). During the attachment phase (4 hours of incubation), the medium was supplemented with 50 nmol/L dexamethasone (Sigma, St. Louis, MO, USA) and 5% foetal calf serum. Then, the hepatocytes were washed and grown in a serum-free medium without dexamethasone in a humidified incubator at 37°C with 5% $CO_2$. According to the trypan blue exclusion assay, the viability of hepatocytes was consistently higher than 90%, and their purity was higher than 95% as quantified by light microscopy.

#### Cytotoxicity assay

In order to assess the cytotoxicity of the TC extract on the rat hepatocytes, they were first seeded in 96-well plates and then cultivated at 37°C for 24 hours. The hepatocytes ($1\times10^6$/mL) were incubated in Dulbecco's modified Eagle medium (DMEM) with TC extract at various concentrations (0, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, and 500 µg/mL) in five duplicates for each concentration. There was no TC extract in the wells designated as control samples, even though the medium included 1% ethanol, as vehicle. It was necessary to remove the growing media from the cells after 48 hours of incubation in order to determine the vitality of the cells that had been treated with the TC extract. A 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium-bromide (MTT) colorimetric assay (Cell Applications, Inc., San Diego, CA, USA) was used to determine cytotoxicity of the TC extract. After being taken in by live cells, MTT undergoes a transformation from yellow to purple formazan by cellular dehydrogenases. The MTT cytotoxic assay was performed as described by Gerlier and Thomasset[19]. The absorbance of the resulting solution was measured using plate reader (PerkinElmer; Bengaluru, Karnataka, India) at 490 nm test wavelength using a reference wavelength of 650 nm. The cytotoxic concentration of TC extract was determined from dose-response curves. Then, the antioxidant defence system and lipid peroxidation in the cultured hepatocytes was evaluated at the extract's concentration (15.6 µg/g cells) that showed no observed adverse effect level (NOAEL). The homogenates of TC extract (15.6 µg/g cells) treated and untreated hepatocytes were prepared as follows:

Using a gentle pipetting motion, the cell suspensions were transferred from the wells to Eppendorf tubes. After centrifugation at 1800 ×*g*; the precipitated cells were rinsed with ice-cold $KH_2PO_4$ (50 mmol, pH 7.4), and the isolated cells ($2\times10^6$ cells/mL) were harvested. A glass-porcelain ultrasonic homogenizer was used to break down the cells (Jencons Scientific Co., Herts, UK). The homogenate was centrifuged for 15 minutes at 11200 ×*g*. All procedures were conducted at 4°C. The supernatant fraction was isolated and stored at −20°C until used for the quantification of the antioxidants and lipid peroxidation.

### *In vivo* experiments

#### Animals

A total of 60 healthy male Wistar albino rats were purchased from the National Research Centre (Giza, Egypt). Polystyrene cages were utilised to house the animals (five animals per cage), room temperature was maintained at 12-h light/12-h dark cycle at

23 ± 2°C. Access to food and water were allowed *ad libitum.* The animals were kept in appropriate laboratory conditions for two weeks before initiation of the experimental protocol, which is achieved according to the guidelines of the Committee of Scientific Research Ethics in Suez Canal University, Egypt.

**Oral acute toxicity of TC extract**

Acute toxicity of TC extract was analysed on rats, according to Rispin *et al*.[20]. Twenty male albino rats weighing 150-180 g were divided into control and 3 experimental groups. Each group included five animals. The experimental group received TC extract (dissolved in distilled water) by gavage with a metal gastric needle's aid at a single dose of 100, 1000, and 3000 mg/kg of the animal weight, while the control group received distilled water. All of the animal groups were monitored for changes in skin and fur, eyes, mucous membranes, musculature, and respiratory symptoms for the first four hours after the dose was administered and once daily for the next 14 days.

**Experimental groups**

Experimental animals were randomly allotted into eight major groups (n=5); group "1": control group received orally the distilled water, group "2": rats treated with TAA (350 mg/kg body weight dissolved in saline, once *via* intraperitoneal injection) for induction of acute liver injury according to Salama *et al*.[21]. Groups "3-5": rats treated with 50, 100, and 200 mg/kg body weight of TC extract dissolved in distilled water, respectively, *via* gavage daily for 4 weeks, group "6-8": TAA-treated rats that received TC extract (50, 100, and 200 mg/kg, respectively, dissolved in distilled water) *via* gavage daily for 4 weeks.

**Blood and tissue sampling**

The rats were anaesthetized with isoflurane inhalation at the end of the experimental period. Blood samples were collected by cardiac puncture as described by Parasuraman *et al*.[22] and transferred immediately into heparinized tubes for the separation of plasma by centrifugation at 3000 ×*g* for 10 min; then kept at –20°C until used to estimate the intended biochemical parameters. The liver tissue was immediately dissected and washed by phosphate buffer saline; part of it was homogenized for biochemical analysis. Approximately, 10% of the homogenate was prepared in ice-cold phosphate buffer (pH = 7.0) and protein content in tissue homogenate was measured according to the method of Lowry *et al*.[23]. For histological evaluation, the other half was fixed in formalin (10%).

**Determination of plasma biochemical parameters**

Plasma was used for the determination of enzymes' concentrations/activities, as well as the C-reactive protein (CRP) concentration, using the standard procedures of a commercial diagnostic sandwich ELISA and colorimetric kits. The alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphate (ALP) concentrations were assessed by ELISA kits specific for rats purchased from MyBioSource (catalogue number: MBS2024403, San Diego, CA, USA) and Elabscience (catalogue numbers: E-EL-R0076 and E-BC-K091-S, Houston, TX, USA), respectively, according to the manufacturer's guides. Lactate dehydrogenase (LDH) was measured colorimetrically using LDH kit (catalogue number: E-BC-K046-M) from Elabscience. CRP was determined using ELISA Kit specific for rats form Elabscience (catalogue Number: E-EL-R0506) according to the manufacturer's guide.

**Determination of antioxidant/oxidative markers in rat hepatocytes and liver tissues**

The reduced glutathione (GSH) concentration was determined spectrophotometrically at 412 nm by using the Beutler method[24]. According to McCord[25], the superoxide dismutase (SOD) activity was tested using a commercial kit (Randox Lab, Crumlin, UK) at 505 nm and 37°C. A commercial kit from Randox Lab was used to estimate catalase

(CAT) activity at 340 nm and 37°C according to method described by Goth[26]. According to Clairbone[27] the catalase activity was expressed as a µmol/min/mg protein. In order to detect lipid peroxidation, malondialdehyde (MDA) level was measured colorimetrically according to the method published by Hong *et al*.[28].

**Histopathological studies**

After being fixed in 10% formalin, the liver tissue was successively dehydrated in ethanol ranging from 50% to 99%, purified in xylene, and ultimately embedded in paraffin. Haematoxylin and eosin dyes were used to stain the hepatic tissue microsections after they were prepared.

**Statistical analysis**

Statistical analysis was performed by one-way ANOVA followed by a Duncan's post-hoc test using the SPSS for Windows, version 20.0. Differences were considered significant at $P<0.05$[29].

## RESULTS

### The phytochemical components of TC extract

Figure "1" showed the GC-MS chromatogram of the major constituents of TC extract. The heptadecanoic acid was the major component (31.53%) followed by trimethoxy (12.30%), dihydroxypropyl ester (8.62%), linoleic acid ethyl ester (7.53%), quercetin (6.36%), cis-13-octadecenoic acid (4.53%), and lucenin 2 (2.89%) as shown in Table "1". Other chemical compounds represent less than 2%. Table "1" summarises the major constituents of TC extract, their retention durations, molecular weight, and chemical formula.

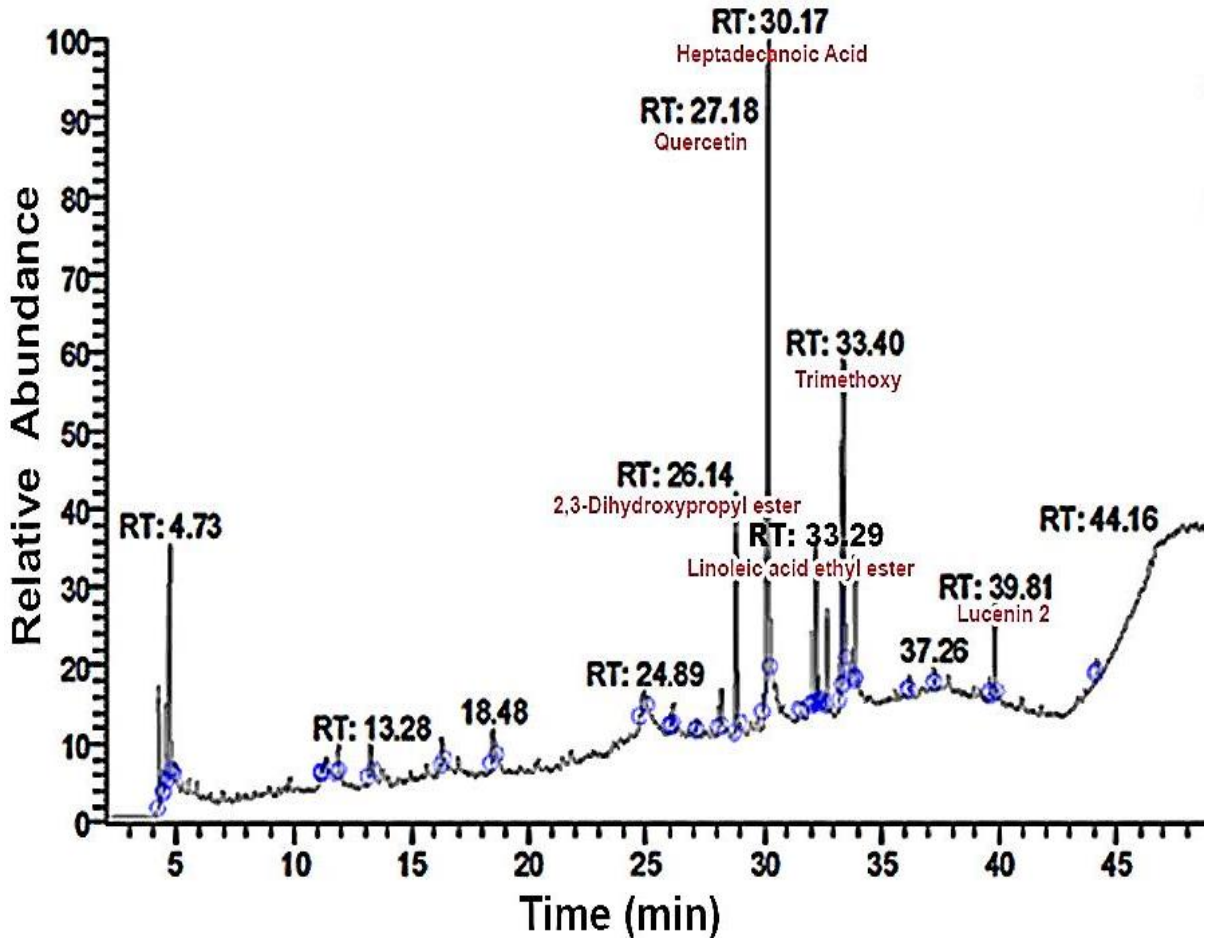


**Figure 1:** GC-MS chromatogram of the major constituents of the ethanolic extracts of *Tinospora cordifolia* leaves.

**Table 1:** GC-MS analysis of chemical components of the ethanolic extracts of *Tinospora cordifolia* leaves.

| Retention Time (min) | Compound name | Molecular formula | Molecular weight | Area (%) |
|---|---|---|---|---|
| 26.14 | 2,3-Dihydroxypropyl ester | $C_{21}H_{42}O_4$ | 358 | 8.62 |
| 27.18 | Quercetin | $C_{18}H_{16}O_7$ | 344 | 6.36 |
| 30.17 | Heptadecanoic Acid | $C_{18}H_{36}O_2$ | 284 | 31.53 |
| 32.20 | Cis-13-octadecenoic acid, methyl ester | $C_{19}H_{36}O_2$ | 296 | 4.53 |
| 33.29 | Linoleic acid ethyl ester | $C_{20}H_{36}O_2$ | 308 | 7.53 |
| 33.40 | Trimethoxy | $C_{20}H_{38}O_2$ | 310 | 12.30 |
| 39.81 | Lucenin 2 | $C_{27}H_{30}O_{16}$ | 610 | 2.89 |

GC-MS: Gas chromatography-mass spectrometry
Area (%) represents the concentration of each constituent in the TC extract.

**Cytotoxicity of TC extract and its effect on antioxidant/oxidant markers of cultured rat hepatocytes**

According to the MTT cytotoxic assay, the maximum dose of TC extract (15.6 µg/g cells) showed no observed NOAEL, as calculated from the dose-response curve (Figure 2). The inhibitory activity of TC extract under these experimental conditions did not exceed 25% up to $IC_{50}$ = 500 µg/mL (Figure 2). The TC extract (15.6 µg/g cells) induced a significantly decrease ($P$<0.05) in the MDA level and a significant increase ($P$<0.05) in the GSH content and the activities of the antioxidant enzymes (CAT and SOD) in the cultured hepatocyte, as compared with the control (untreated) hepatocytes (Table 2).

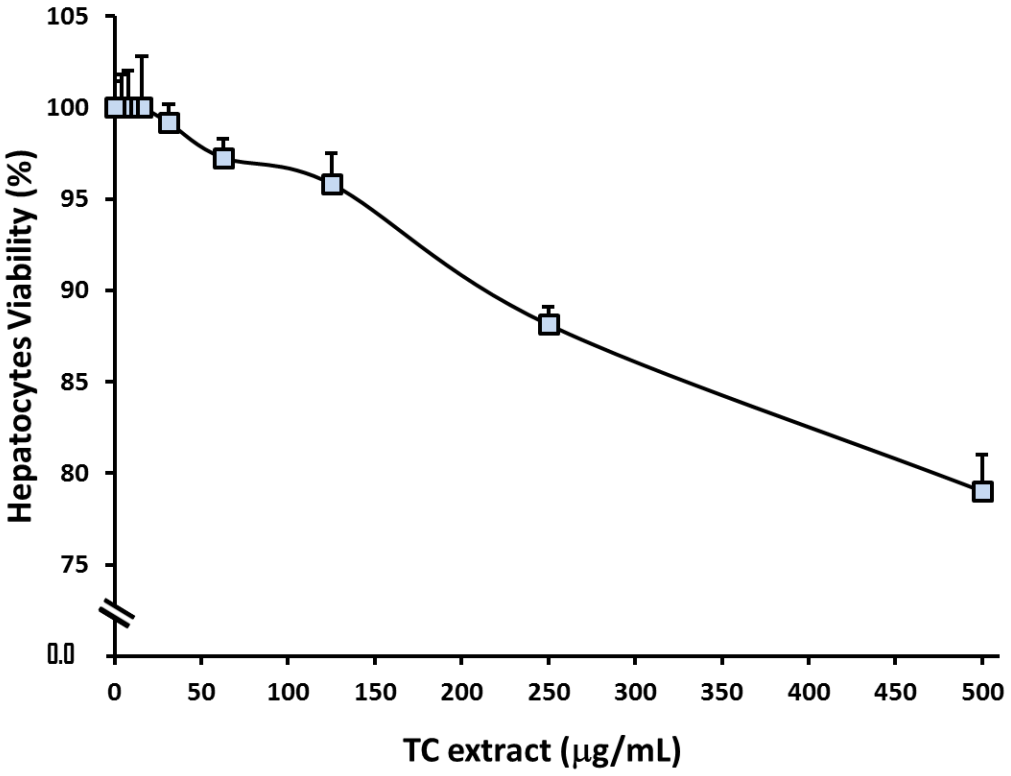


**Figure 2:** Toxicity effects of the ethanolic extracts of *Tinospora cordifolia* leaves (TC extract) on cultured rat hepatocytes after 48 hours of incubation.

**Table 2:** Effect of the ethanolic extracts of *Tinospora cordifolia* leaves (TC extract) at dose of 15.6 µg/g cells on antioxidant/oxidative markers of cultured rat hepatocytes.

| | Control | TC extract |
|---|---|---|
| MDA (µmol/$10^6$ cells) | 762.50±25.61 | 498.50±24.47* |
| GSH (nmol/$10^6$ cells) | 791.75±18.19 | 957.75±21.97* |
| SOD (U/mg protein) | 98.82±4.77 | 139.00±2.72* |
| CAT (µmol/min/mg protein) | 6.87±0.27 | 12.55±0.14* |

Values were expressed as means ± their standard errors, *: significantly different ($P$<0.05) from the control group, MDA: malondialdehyde, GSH: reduced glutathione, SOD: superoxide dismutase, CAT: catalase.

***In vivo* oral acute toxicity and the hepatoprotective activity of TC extract**

The TC extract (at dosages of 100, 1000, and 3000 mg/kg of the animal weight) did not induce any harmful impact on the skin and fur, eyes, mucous membranes, musculature, and respiratory symptoms for the first four hours after the dose was administered and throughout the next 14 days, thus the plant materials were found to be harmless. Table "3" summarized the effect of TC extract on the plasma markers of inflammation and tissue injury in TAA-induced hepatotoxicity in rats. The ALT, AST, and ALP concentrations, as well as LDH activity, and CRP level were increased significantly ($P$<0.05) by TAA compared with the control group. However, TC extract at doses ≥100 mg/kg body weight significantly decreased the ALT, ALT, AST, and CRP levels, as well as LDH activity, at a dose dependent manner (Table 3).

According to the current findings, the TAA-treated rats showed higher levels of hepatic MDA compared with the control group (Table 4). In a dose-dependent way, the treatment with TC extract considerably inhibited ($P$<0.05) the elevation in the hepatic lipid peroxidation. However, there was a considerable decrease in the activities of hepatic SOD and CAT, as hepatic GSH content, in the TAA-treated group compared with the control group (Table 4). The treatment with TC extract displayed a protective effect against the TAA intoxication, which detected by the elevation induced in SOD and CAT activities, as well as GSH content, when compared with the TAA-treated group that not received TC extract (Table 4).

**Modulatory effects of TC extract on the histopathological alterations in hepatic tissue of TAA-treated rats**

The liver of control and TC extract treated groups showed normal hepatic tissue. The section of normal rat liver showed large number of hepatic lobules (Figures 3a and 4a). The hepatic lobule contains numerous cords of hepatocytes radiating from the central vein to the periphery of the lobule. The hepatocytes are separated by narrow sinusoids as seen in the liver sections. Hepatic cells are arranged concentrically around the central vein. The large hepatocytes have more or less centrally situated one large round nucleus and homogenous cytoplasm (Figures 3a and 4a). The sections of the liver of the TAA group showed histopathological abnormalities such as: loss of normal architecture with focal necrosis associated with cell infiltration, hydropic degenerated cells with karyolysed nuclei, and dilatation of blood sinusoids (Figures 3b,c and 4b,c). In comparison with the TAA group, the histopathological changes in TAA+TC extract-treated groups were less sever and this was evident by absence of necrotic hepatocytes in the hepatic tissue, the hepatic vacuolation was mostly seen as variable

**Table 3:** Effect of the ethanolic extracts of *Tinospora cordifolia* leaves (TC extract) on the plasma markers of inflammation and tissue injury in the thioacetamide (TAA)-treated rats.

| | AST (pg/mL) | ALT (pg/mL) | ALP (pg/mL) | LDH Activity (U/L) | CRP (ng/mL) |
|---|---|---|---|---|---|
| Control | 80.6±2.2[1] | 31.2±3.6 | 343.0±2.1 | 342.0±33.4 | 199.3±9.5 |
| TAA (350 mg/kg body weight) | 122.9±15.2[a] | 53.1±3.1[a] | 371.0±24.2[a] | 1303.0±27.7[a] | 1232.0±31.7[a] |
| | 52.4%[2] | 69.9% | 7.4% | 281.0% | 517.1% |
| TC extract (50 mg/kg body weight) | 84.4±3.6 | 31.3±2.9 | 295.3±45.3 | 364.7±36.4 | 258.3±36.0 |
| | 4.7%[2] | 0.1% | –13.9% | –22.6% | 29.6% |
| TC extract (100 mg/kg body weight) | 81.2±5.6 | 30.5±2.6 | 274.3±38.0 | 331.7±52.1 | 233.0±30.1 |
| | 0.8%[2] | –2.4% | –20.0% | –3.2% | 16.9% |
| TC extract (200 mg/kg body weight) | 74.1±4.6 | 24.4±2.2 | 243.7±69.7 | 342.3±47.2 | 191.0±18.7 |
| | –8.1%[2] | –21.8% | –29.0% | –0.1% | –4.2% |
| TAA + TC extract (50 mg/kg body weight) | 117.0±6.0 | 52.0±3.8 | 370.7±34.7 | 544.7±34.7[b] | 769.7±25.6[b] |
| | –4.8%[3] | –2.1% | –0.1% | –58.2% | –37.5% |
| TAA + TC extract (100 mg/kg body weight) | 97.7±1.4[b] | 33.7± 2.3[b] | 311.3±74.6 | 523.7±33.1[b] | 498.3±77.2[b] |
| | –20.5%[3] | –36.4% | –16.1% | –59.8% | –59.6% |
| TAA + TC extract (200 mg/kg body weight) | 95.2±5.5[b] | 31.9±2.2[b] | 263.7±51.1 | 392.7±17.2[b] | 324.7±27.6[b] |
| | –22.5%[3] | –39.8% | –28.9% | –69.9% | –73.4% |

[1]means ± their standard errors, [2]% of changes of relative to the control group, [3]% of changes of relative to the TAA group, [a]Significant different ($P$<0.05) compared with the control group, [b]Significant different ($P$<0.05) compared with the TAA group, AST: aspartate aminotransferase, ALT*:* alanine aminotransferase, ALP: alkaline phosphatase, LDH: lactate dehydrogenase, CRP: C-reactive protein.

sized cytoplasmic vacuoles around centrally located nucleus, and the sever type of the vacuolar degeneration was very minimal. Most of areas of hepatic lobules especially around the central vein maintained normal hepatic architecture (hepatic cords) in groups treated with TAA and treated with TC extract in a dose dependent manner (Figures 3d-f and 4d-f). Also, no irregularly shaped neoplastic hepatocytes were observed in pervious groups.

## DISCUSSION

Hepatocytes are primarily responsible for the vital function that the liver plays in the

**Table 4:** Effect of the ethanolic extracts of *Tinospora cordifolia* leaves (TC extract) on the antioxidant/oxidative markers in liver tissues of the thioacetamide (TAA)-treated rats.

| | MDA (nmol/g tissue) | GSH (mmol/g tissue) | SOD (U/g protein) | CAT (µmol/min/mg protein) |
|---|---|---|---|---|
| Control | 23.6±1.6[1] | 1.8±0.04 | 283.3±11.4 | 2.2±0.2 |
| TAA (350 mg/kg body weight) | 49.8±3.6[a] | 0.6±0.1[a] | 187.3±13.3[a] | 0.9±0.1[a] |
| | 111.4%[2] | –86.3% | –33.9% | –56.3% |
| TC extract (50 mg/kg body weight) | 23.4±2.2 | 1.6±0.1 | 296.7±44.4 | 2.0±0.02 |
| | –0.6%[2] | –13.9% | 4.7% | –9.1% |
| TC extract (100 mg/kg body weight) | 24.8±2.5 | 1.6±0.2 | 315.7±38.2 | 2.1±0.2 |
| | 5.1%[2] | –9.4% | 11.4% | –3.3% |
| TC extract (200 mg/kg body weight) | 15.8±1.1[a] | 1.7±0.1 | 323.3±22.1 | 2.1±0.2 |
| | –33.2%[2] | –8.3% | 14.1% | –4.4% |
| TAA + TC extract (50 mg/kg body weight) | 37.9±3.8[b] | 1.0±0.2[b] | 193.3±12.7[b] | 1.4±0.1[b] |
| | –24.0%[3] | 79.0% | 3.2% | 42.4% |
| TAA + TC extract (100 mg/kg body weight) | 31.2±2.6[b] | 1.0±0.1[b] | 286.3±24.8[b] | 1.6±0.3[b] |
| | –73.4%[3] | 82.5% | 52.9% | 70.3% |
| TAA + TC extract (200 mg/kg body weight) | 23.1±2.6[b] | 1.2±0.2[b] | 292.7±13.8[b] | 2.1±0.3[b] |
| | –53.6%[3] | 110.5% | 56.2% | 124.0% |

[1]means ± their standard errors, [2]% of changes of relative to the control group, [3]% of changes of relative to the TAA group, [a]Significant different ($P$<0.05) compared with the control group, [b]Significant different ($P$<0.05) compared with the TAA group, MDA: malondialdehyde, GSH: reduced glutathione, SOD: superoxide dismutase, CAT: catalase.

regulation of the body's internal environment, known as homeostasis. When investigating xenobiotic metabolism, as well as a variety of liver diseases, the use of isolated primary hepatocytes has emerged as an indispensable instrument. Part of the present study concerned with the investigation of the effect of TC extract on endogenous antioxidant of the isolated rat hepatocytes. In this study, the isolated rat hepatocytes were exposed to TC extract at concentrations of 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, and 500 µg/mL for 48 hours. The extract exhibited no significant cytotoxic activity against the cultured rat hepatocytes achieving a weak inhibitory activity under these experimental conditions with IC50≤500 µg/mL.

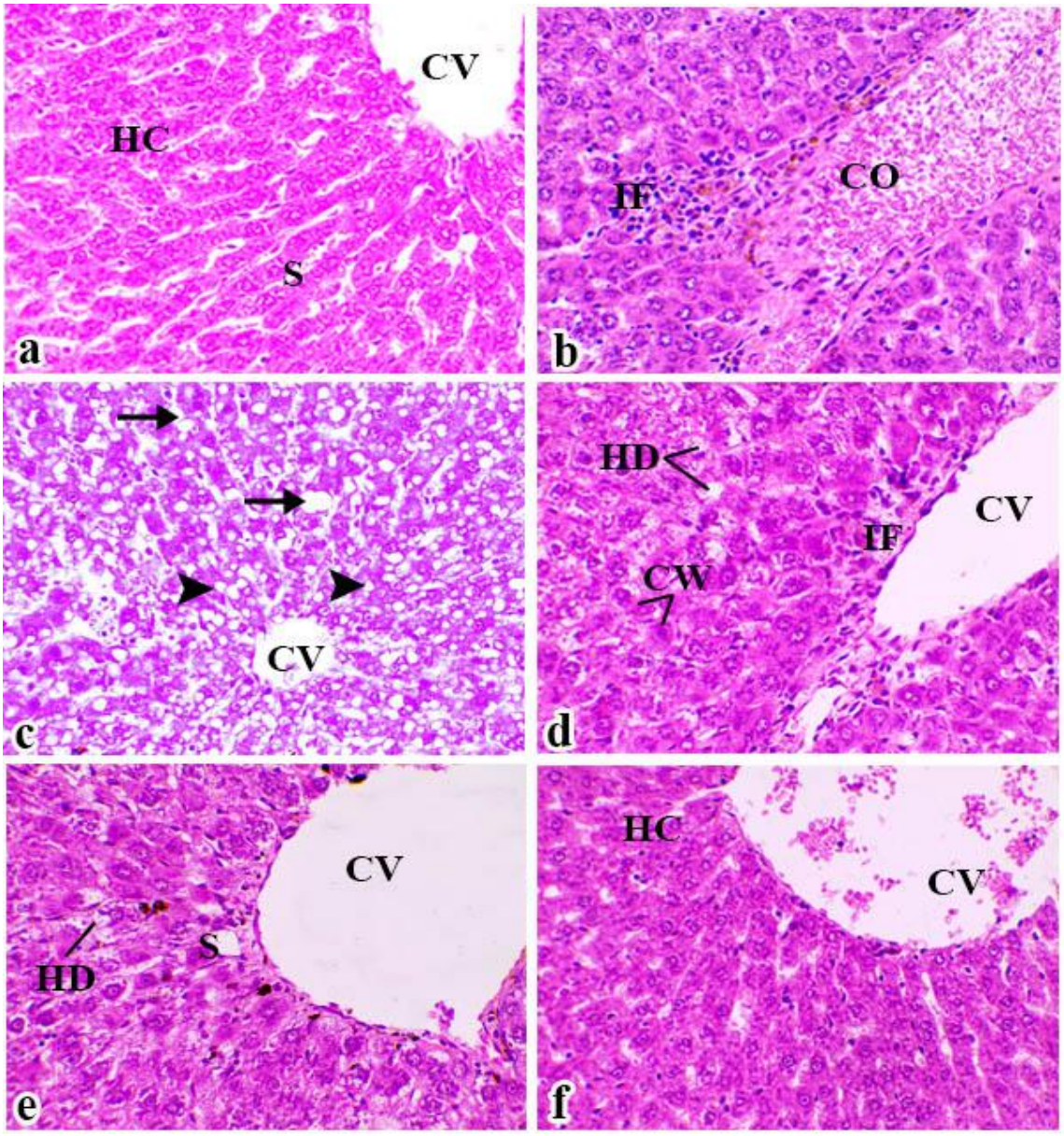


**Figure 3:** Light microphotographs of sections from rat's liver (central area; haematoxylin and eosin stain; magnification: 200×). (**a**): control group exhibited normal hepatic strands (HC) around the central vein (CV) with sinusoids (S) between the hepatocyte strands. (**b** and **c**) Thioacetamide (TAA)-treated group showed focal necrosis associated with severe mononuclear cellular infiltration (IF) at the central area, besides congestion (CO) of central vein, microvesicular steatosis (arrow head) and macrovesicular steatosis (arrows) were also observed. (**d**) TAA-treated rats received the ethanolic extracts of *Tinospora cordifolia* leaves (TC extract, 50 mg/kg body weight) showing the central area of hepatic lobule with absence of fatty changes, mild hydropic degeneration (HD), and cloudy swelling (CW) of some hepatocytes, besides mild lymphatic infiltration (IF). (**e**) TAA-treated rats received the TC extract (100 mg/kg body weight) showing the central area of hepatic lobule with normal hepatocytes architecture around central vein (CV), some hepatocytes exhibited hydropic degeneration (HD), and some sinusoids exhibited mild dilatation (S). (**f**) TAA-treated rats received the TC extract (200 mg/kg body weight) exhibited normal hepatocytes within the cell cord, which were extending from the central vein. Hepatocytes are distinguished by their central euchromatic nucleus and their ability to maintain a consistent and uniform cytoplasm (HC).

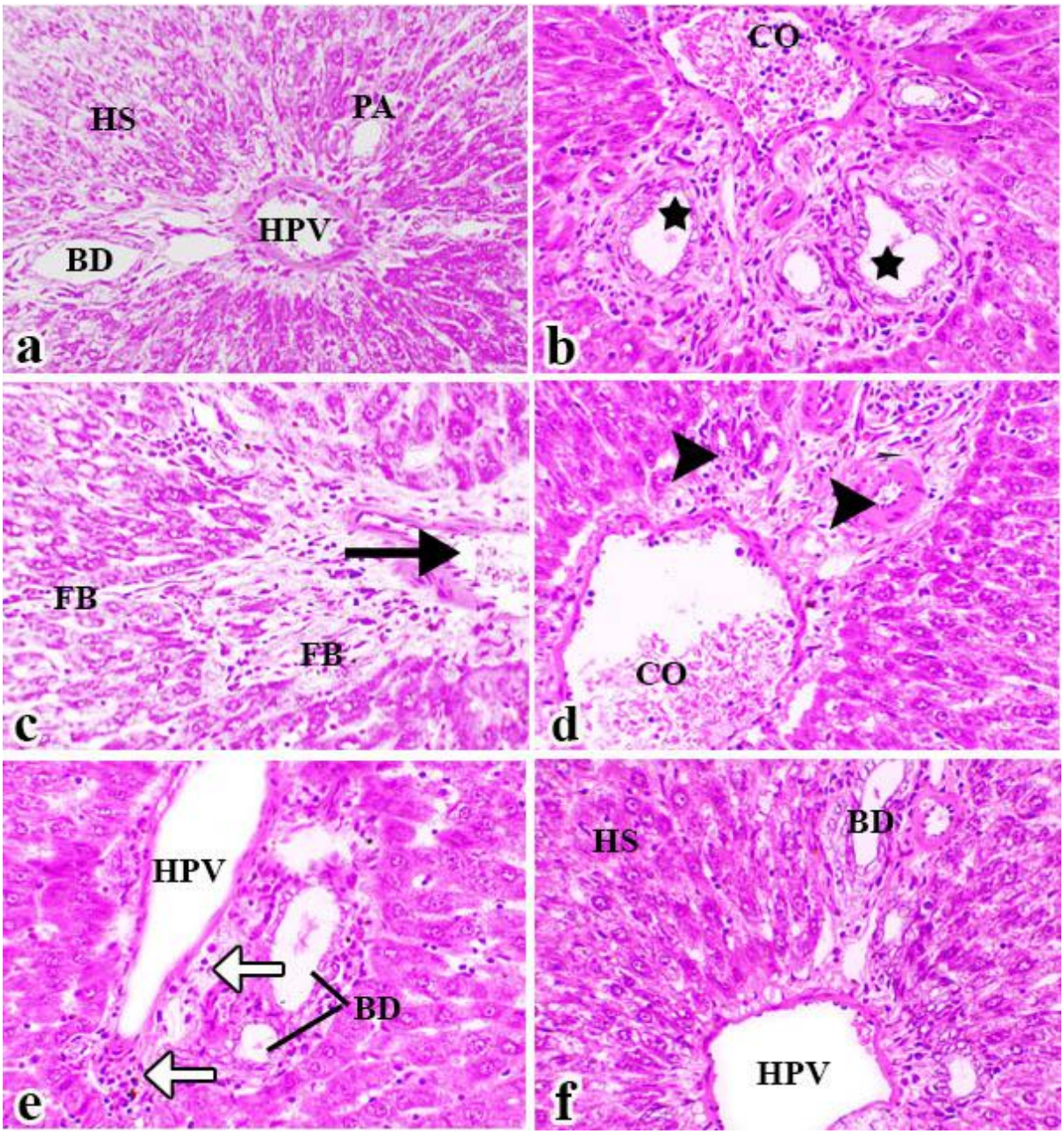


**Figure 4:** Light microphotographs of sections from rat's liver (portal area; haematoxylin and eosin; magnification: 200×). (**a**) Control group exhibited normal hepatocyte strands (HS) around hepatic portal vein (HPV), and branches of portal vein (PA) besides bile duct (BD) in connective tissue area. (**b** and **c**) Thioacetamide (TAA)-treated group showed dilatation of bile duct (stars), which surrounded by mononuclear inflammatory cells and congested hepatic vein (CO). Portal vein (black arrow) surrounded by (oedematous) fibrotic tissue (FB) with a lymphocytic infiltration. (**d**) TAA-treated rats received the ethanolic extracts of *Tinospora cordifolia* leaves (TC extract, 50 mg/kg) displayed mild congestion (CO) in portal vein, besides bile duct (head arrow) lined by regenerating epithelial cells associated with moderate infiltration of mononuclear inflammatory cells. (e) TAA-treated animals received the *T. cordifolia* extract (100 mg/kg body weight) showing portal area of hepatic lobule with normal hepatocytes architecture around hepatic portal vein (HPV), mild lymphatic infiltration (white arrows) appears around regenerated bile ducts (BD). (**f**) TAA-treated rats received the TC extract (200 mg/kg body weight) revealed normal hepatocyte strands (HS) around hepatic portal vein (HPV), besides normal appearance of bile duct (BD).

The maximum dose at 15.6 µg/g cells had NOAEL on cultured rat hepatocytes. Pre-incubation of hepatocytes with TC extract (15.6 µg/g cells) increased significantly ($P$<0.05) the intracellular GSH content, and CAT and SOD activities, as well decreased significantly ($P$<0.05) the MDA level, as compared with the untreated hepatocytes. These results declared the role of TC extract in decreasing oxidative stress by increasing the endogenous hepatocytes antioxidant defence system. According to the results of GC-MS of TC extract, heptadecanoic acid, was the major compound followed by trimethoxy, dihydroxypropyl ester, linoleic acid ethyl ester, quercetin, cis-13-octadecenoic acid, and lucenin 2. Al-Douri and Shakya[30] reported the antioxidant properties of heptadecanoic acid, which is the most abundant constituent of the extract under investigation. In addition, other studies reported the antioxidant and anticancer activities of the trimethoxy and quercetin[31-33]. Moreover, Huang *et al*.[34] found that the octadecenoic acid extracted from endophytic fungal of the plant "*Nerium oleander*" have antioxidant and antimicrobial activities. These finding support our results, which revealed the role of the TC extract continents as potent antioxidants.

The liver is a common target for medication-induced harm due to its role in concentrating and metabolizing the majority of medicines[35-37]. Acetaminophen (paracetamol) is the most researched hepato-toxic medication. Anticancer treatments, antibiotics, and heart medication are just a few of the many medications that might cause liver damage[36]. Acute hepatic failure mortality and morbidity have decreased dramatically in the last 40 years because of advances in medical knowledge and therapy. Progress has been gained through studying animal models that mimic the human syndrome's clinical, biochemical, and histological pattern. TAA has been used extensively in the development of animal models of acute liver injury[37,38]. Rather than causing direct harm to other organs, earlier studies have shown that the large doses of TAA can cause oxidative stress, lipid peroxidation, and a decrease in the antioxidant status that limit its pathogenic effects to acute liver injury[39] and hepatic cirrhosis[40].

Despite the fact that medical plant drug development remains an important source of novel therapeutic leads, a number of obstacles exist, including obtaining plant materials, selecting and implementing appropriate high-throughput screening bio-assays, and scaling up active molecules[41]. Due to their well-known lack of side effects compared with pharmaceuticals, natural compounds with therapeutic value are progressively gaining prominence in clinical research. *T. cordifolia*, known as "Guduchi" in old Ayurvedic literature, is widely used in the treatment of a wide range of ailments[42].

The TAA treatment resulted in a significant decrease in the hepatic GSH content, as well as SOD and CAT activities, in the current study. TAA was hazardous to the liver since it changed several of the organ's parameters. In addition, the rise in CRP in TAA-treated rats indicated an enhancement of inflammation. The histo-pathological lesions ranged from mild to severe in TAA-treated rats. TAA, a sulphur containing compound, is a carcinogenic agent[43]. It is commonly used for inducing fulminant hepatic failure[44]. Biotrans-formation of TAA to form bothflavin-containing monooxygenase and cytochrome P450[45] precedes oxidative damage, which cause liver injury.

The concentration of blood ALT, AST, ALP, and LDH, can be used to estimate tissue injury including liver[46,47]. In our study, significant increases in ALT, AST, and ALP, and the activity of LDH, were seen in the TAA group. A possible explanation for the modulatory effects of the TC extract against leakage of plasma markers for tissue damage in the TAA-induced liver toxicity rat model is the membrane stabilising effect of the TC extract.

The body has a comprehensive anti-oxidant defence system that works against free radicals to protect important bio-molecules. In the present work, TC leaves extract boosted significantly hepatic SOD and CAT activities in TAA-treated rats indicating that TC extract can decrease reactive free radicals and minimizing oxidative damage to tissues through enhancing the hepatic antioxidant enzyme activity. The dismutation of superoxide anion into oxygen and hydrogen peroxide is catalysed by superoxide dismutase[48,49]. GSH is a non-enzymatic tripeptide anti-oxidant found in high concentrations in the liver. It helps in eliminating free radical species such as hydrogen peroxide, super-oxide radicals, and alkoxy radicals[50]. In the present study, reduced level of GSH is implicated in the enhancement of lipid peroxidation in TAA-treated rats. This is in accordance with Bruck *et al*.[44] who demonstrated that TAA exhibited hepatic toxicity through free radical-mediated oxidative stress. In the current study, treatment with TC extract boosted significantly ($P$<0.05) the amount of GSH in a dose-dependent way, which is indicative of its capacity to scavenge these free radicals. The activity of the *T. cordifolia* as a potent antioxidant were reported in a number of previous studies[51,52].

Regarding liver histopathology in the current study, TAA treated animals revealed evident of hepatotoxicity in the form of necrosis, sometimes associated with lymphatic infiltration. Hepatocytes in some areas looked ballooned, with karylozed nuclei. Mononuclear cells were seen infiltrating the blood sinusoids. Hepatic toxicity of TAA was explained by Akhtar and Sheikh[53] who stated that TAA is a model hepatotoxicant consumed to induce acute and chronic liver injury due to its effects on protein synthesis, RNA, DNA, and γ-glutamyl transpeptidase activity. TAA undergoes a two-step of bio-activation to sulfine, and afterward to sulfene, a reactive metabolite. Sulfine is accountable for the enlargement of nucleoli, the increase in nuclear volume and intracellular con-centration of $Ca^{++}$, the change in cell permeability, and the inhibition in mitochondrial activity. At the same time sulfene is responsible for the release of nitric oxide synthase and nuclear factor-κB directing to centrilobular necrosis, protein denaturation, and lipid peroxidation[53]. In the current study, administration of TC extract in different doses displayed different extent in restoring the architecture of hepatocyte in a dose dependent manner. The histopathological analysis revealed normal hepatocytes cell cord radiating from the central vein, the hepatocytes had central euchromatic nuclei well-preserved in homogeneous cytoplasm, and the blood sinusoids were found to possess normal lining cells. These results come parallel to the results of oxidative markers in the present study.

In conclusion, this study strongly recommended the use of *T. cordifolia* as a source of natural antioxidants and a suitable food supplement for a healthy liver, as this plant contains hepatoprotective compounds. Due to the fact that plant components contribute significantly to overall bioactivity, more phytochemical studies of for *T. cordifolia* compounds are absolutely necessary.

## COMPLIANCE WITH ETHICAL STANDARDS

The *in vivo* protocol in this study was approved by the Committee of the Ethics of Animal Experiments of Suez Canal University, Egypt (approval number: 2021003); and was in compliance with the Guide for the Care and Use of Laboratory Animals mentioned in the ARRIVE guidelines 2.0.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHORS' CONTRIBUTIONS

ZIN: outset of the presented idea, critical revision of the article beside final approval of the version to be published; HMT: outset of the presented idea, data analysis and interpretation; EA: critical revision of the article; HTH: carried out the experiment in addition to data analysis and interpretation; MSN: data analysis and interpretation of biochemical parameters besides interpretation of chemical characterization analysis (GC-MS) of the tested extract. All authors have discussed the results and contributed to the final manuscript.

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# النشاطات المضادة للأكسدة والواقية للكبد للمستخلص الإيثانولي لأوراق نبات "*Tinospora cordifolia*": دراسات معملية وفي الجسم الحي

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يُستَخدم نبات "*Tinospora cordifolia*" في الطب الهندي لعلاج عدد من الأمراض منذ فترة طويلة. وفي هذه الدراسة تم اختبار النشاطات المضادة للأكسدة والواقية للكبد للمستخلص الإيثانولي لنبات "*T. cordifolia*" على خلايا كبد الجِرذان المستزرعة معمليًا وعلى الجِرذان المعاملة بالثيواسيتاميد. بالنسبة للتجارب في الجسم الحي، تم توزيع أربعين من ذكور الجِرذان المهقاء من سلالة "Wistar" بشكل عشوائي إلى ثماني مجموعات (ن=5): المجموعة "1" عوملت كمجموعة ضابطة، والمجموعة "2" تمثل الجِرذان المعاملة بالثيواسيتاميد، والمجموعات "3-5" عوملت بجرعات مختلفة من مستخلص النبات (50 و 100 و 200 مجم/كجم من وزن الجسم، على التوالي، عن طريق الفم/يوميًا لمدة 4 أسابيع)، بينما تضمنت المجموعات "6-8" الجِرذان المعاملة بالثيواسيتاميد والتي تلقت 50 و 100 و 200 مجم، على التوالي، من مستخلص النبات/كجم من وزن الجسم (عن طريق الفم/يوميًا لمدة 4 أسابيع). لم يُظهر مستخلص النبات أي نشاط سام ملحوظ ضد خلايا الكبد المستزرعة معمليًا عند $IC_{50}$≥500 ميكروجم/مل (بعد 48 ساعة) في اختبار "MTT". وأظهرت التجارب التي أجريت في جسم الحيوان الحي أن مستخلص النبات (بجرعات ≤100 مجم/كجم من وزن الجسم) قد عدل بشكل ملحوظ إحصائيا ($P<0.05$) من الزيادة في مستويات ناقلات الأمين والبروتين التفاعلي سي، ونشاط إنزيم اللاكتات ديهيدروجينيز، في مصل الدم للجِرذان المعاملة بالثيواسيتاميد. كما عزز مستخلص النبات (بجرعات ≤50 مجم/كجم من وزن الجسم) بشكل ملحوظ إحصائيا ($P<0.05$) من نشاطات الإنزيمين الديسموتاز والكاتلاز، ومحتوى الجلوتاثيون، في أنسجة الكبد للجِرذان المعاملة بالثيواسيتاميد. وأكدت النتائج النسيجية التأثيرات الواقية للكبد لمستخلص النبات في الجِرذان المعاملة بالثيواسيتاميد. الخلاصة، أظهر المستخلص الإيثانولي لنبات "*Tinospora cordifolia*" نشاطًا واقيًا للكبد في الجِرذان المعاملة بالثيواسيتاميد من خلال إمكاناته القوية المضادة للأكسدة.