



## Liver histopathological of purification cinnamic acid activity against endoxan in mice

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### Abstract

The study was carried out to purified the cinnamic acid from cinnamon bark by high performance liquid chromatography (HPLC) to obtain 96% cinnamic acid from cinnamon bark and study the hepatic cells damage. Examined biological effect in bioassay system (88 albino mice) by two purification concentration to cinnamic acid (56 and 28 mg/Kg) was comparison with vitamin C (180 mg/Kg) against the mutagenic influence of endoxan (50 mg/Kg), which is a chemical compound that damage hepatic cells and has mutagenic effects. Two concentration of purified cinnamic acid (56 and 28 mg/kg) were evaluated to choose the suitable concentration which remembered the negative control. In order to use in the interaction experiments, included two types to treatments pre- endoxan and post- endoxan. Comparing between perfect concentration of purification cinnamic acid (28mg/kg), pre-endoxan and post- endoxan treatment found that results after endoxan treatment was the best and with low damage in liver cells enzymes, while results before endoxan treatment were showed necrosis and high enzymes leak, comparison with both vitamin C before and after endoxan treatment, therefore, cinnamic acid can be considered as cure hepatocytes from acute liver damage at first degree and prevention from necrosis hepatocells.

**Keywords:** Liver, Histopathological, Cinnamic acid, Endoxan.

### Introduction

The liver is the key organ regulating homeostasis in the body, it's involve with almost all the biochemical pathways related to growth, fight against disease, nutrient supply, enemy provision and reproduction (Ward *et al.*, 1999). The liver is expected not only to perform physiological functions but also to protect against the hazards of harmful drugs and chemicals. In spite of tremendous scientific advancement in the field of hepatology in recent years, liver problems are on the rise jaundice and hepatitis are two major hepatic disorders, that account for a high death rate (Pang *et al.*, 1992) and heaptocellular carcinoma is one of the ten most common tumors in the world with over 2,500,000 new case each year. Liver protective herbal drugs contain a variety of chemical constituents like phenols, coumarins, lignaus, essential oil, monoterpenes, earotingids, glycosides, flavanoids, organic acids, lipids, alkaloids and xanthenes, Plant extracts of many crude drug are also used for the treatment plants of liver disorders .

Extracts of different, plants of about 25 plants have been reported to care liver disorders (Sharma *et al.*, 2002). *Cinnamon aromaticum* (Laurales) is popular spice consumed in many parts of the world

and grows, naturally in India, Vietnam, China and Indonesia, the bark is peel off, dried and allows to form a roll-the cinnamon stick. It's most popular know Chinese cinnamon and containing cinnamaldehyde, cinnamic acid, phenol compounds essential oil, monotrepenoid and others (Evan,1996). Cinnamic acid is a white crystalline hydroycinnamic acid, the most sonynames (phenylacrylic acid, 3-phenyl-proponoic acid, 3-phenylacrylic acid, cinnama). The molecular formula  $C_9H_8O_2$  (Camarat *et al.*, 2006). Cinnamic acid is slightly soluble in water (Cinnamon, 2011). It's obtained from oil of cinnamon or from balsams such as storax. It's also found in Shea butter and is the best indication of its environmental history and post-extraction conditions can also be made synthetically. Cinnamic acid is used in flavors synthetic indigo and certain pharmaceutical, though its primary used in the manufacturing of methyl and benzyl esters for perfume industry (Camarat *et al.*, 2006). It has a honey floral odor and cinnamic acid and its move volatile ethyl ester (ethyl cinnamte) are flavor components in the essential oil of cinnamon in which related cinnamaldehyde is the major constituent. Cinnamic

acid is also part of the biosynthetic shikimate and phenylpropanoid pathways. Its biosynthesis performed by action of enzyme phenylalanine ammonia-lyase (PAL) on phenylalanine. It is soluble in diethyl ether, insoluble in hexane (Maria *et al.*, 2006). Among the two isomers of the cinnamic acid, the isomer *trans* is the most encountered and exhibits the highest biological activity, *trans*-cinnamic acid is a competitive inhibitor for all isomers of phenylalanine ammonia-lyase, the enzyme that induces the conversion of phenylalanine to cinnamic acid, unlike *cis*-cinnamic acid which only one isomer of this enzyme (Camarat *et al.*, 2006).

A study made to test cinnamic acid and 15 derivatives in CCl<sub>4</sub> induced acute liver damage models which is dependent on oxidative stress mechanisms and as a result the 3,4 methylenedioxy moiety in the caffeic acid derivatives seem to be the main features required for the hepatoprotection in it (Yeon *et al.*, 2008).

In the other side cinnamic acid exhibited depigmenting activity on the UV-B induced hyperpigmentation of brown guinea pig skin and as a skin whitening agent via inhibition of tyrosinase activity and expression within melanocytes. The displacement experiment shows that cinnamic acid can bind to the subdomain IIA (corresponding to sulfhydryl drug binding site I), the distance between the tryptophan residues in BSA and cinnamic acid bound to site he was estimated to be 1.63nm using Förster's equation on the basis of fluorescence energy transfer. The decreased binding constant in the presence of common ions indicates that common ions have effect on drug-BSA system (Hedong *et al.*, 2007).

The antioxidant activity of four derivatives of benzoic acid was systematically compared with the activity of the four homologous derivatives of cinnamic acid. The couples of compounds differed for the kind of aromatic substitution (*p*-hydroxy, *p*-hydroxymethoxy, *p*-hydroxydimethoxy, dihydroxy). The antioxidant activity was measured using a competition kinetic test, to measure the relative capacity to quench peroxy radical and the in vitro oxidative modification of human low-density lipoprotein (LDL), initiated by 2,2'-azobis (amidinopropane) dihydrochloride or catalyzed by Cu (II). In both models, cinnamic acids were more efficient than their benzoic counterparts (Fausta *et al.*, 1999). Some other derivatives of cinnamic acid show ability to cure from some diseases (Dominique *et al.*;1998) maintain that used some ascorbic and cinnamic acid derivatives show antioxidant activity *in vivo* and also protective against reperfusion injury

chlorogenic acid (CGA) is the ester of cinnamic acid and caffeic acid are antioxidants in vitro and prevention of type 2 diabetes mellitus and cardiovascular disease and the green coffee is the major source of (CGA). It has used for production of nutraceuticals, also used as weight loss (Shearea *et al.*, 2003).

## Materials and Methods

### Extraction and Purification of Cinnamic acid

**Plant material:** The bark of *cinnamom aromaticum* were collected from local market and classified by the college of Science, Baghdad University, then kept in a cool dark places until used.

**Preparation of extracts:** Mixed 150 gm of cinnamon bark powder with 2.5 L methanol for 72 hrs. in cool dark place, then filtered and dried at (30-40°C) by rotary evaporator to obtain 1/10 from the original volume and stored at 20°C till the next steps. All these steps were done away from direct light and extensive stress that led to oxidation of the plant extract (Harbone, 1984).

**Detection of polyphenol groups:** Phenolic group (C<sub>9</sub>H<sub>8</sub>OH) was detected after dissolving in NaOH 5%, by change the color from colorless to any color due to oxidation. Phenolic group in molecules can be determined by the following tests:

- 1. Ferric chloride test:** Phenolic group was detected by adding 1% aqueous or alcohol ferric chloride and change the color to green, purple, blue or black (Harbone, 1984).
- 2. Libermann reaction:** Phenolic group with a free para position can be detected by libermann reaction, which included addition 1ml conc. H<sub>2</sub>SO<sub>4</sub> and a few crystal of NaNO<sub>2</sub>, production of green or blue-violet color immediately indicates the presence of phenolic group (Sharma,1992).
- 3. Phthalein test:** Phthalein test was done by adding 2 drops of conc. H<sub>2</sub>SO<sub>4</sub> to phenolic compound sample, then heated and poured to NaOH 10%, production of a red, blue, high green and colourless indicates the presence of phenol, catechol and hydroquinone,  $\alpha$  and  $\beta$  Naphthol and *p*-cresol, respectively (Harbone, 1984).

**Isolation and purification of cinnamic acid:** The following steps were:

1. Acid hydrolysis: (Harbone, 1984).
2. Liquid-liquid portion: (Harbone, 1984).
3. High performance layer chromatography (Huiping and Huang, 2002).
4. Specific reaction of benzene (Aromatic ring) aluminium chloride (AlCl<sub>3</sub>) test (Sharma, 1992).
5. Specific test for double bond (Sharma, 1992).

**Bioassay of cinnamic acid:**

1. Phosphate buffer solution (PBS),  $K_2HPO_4$  0.87 gm was added to distilled water, then adjust the pH to 5.5 by using 1N HCl hydrochloride and completed the volume to 100 ml.
2. The endoxan solution: The proper dosage given to mice according to weight was 50 mg/kg.
3. Colchicine solution: Colchicine 1 mg (one tablet) and sterile distilled water 1 ml. The solution was used immediately after preparing 2.5 to 3 hours.
4. Vitamin C (180 mg/kg): 180 mg from it and 100 ml sterile distilled water.

**Doses:**

1. Two doses from the purification of cinnamic acid which are (56 and 28) mg/kg.
2. Vitamin C (180 mg/kg) as comparative groups.
3. Endoxan compound in (50 mg/kg) as a positive control.
4. PBS as a negative control.

**Hepatoprotectivity effects:** To study the hepatotoxicity effect and the hepatoprotective in laboratory animals, the gavage was orally by syringe 1 ml size supplying with gavage instrument as thin plastic tube with turning shape and soft edge to avoid harm the mice and inserted to the digestive system of mouse, but endoxan was injected intraperitoneally because it is lost after (3-12) hours by urine (Al-kinani, 2005). The white mice were used in the experiments which is *Mus musculus* (Balb/C) in age 8-12 weeks that get from the National Center for Drug Control and Research. The mice put in plastic cages in groups depend on the experimental need in temperature room (25-32°C) and gave the water and integrated animal feed which manufacture locally.

**The Experiment:** Two concentrations of purified cinnamic acid (56 and 28 mg/kg), the concentration a count depended on the mouse weight. The experiment contains 40 mice divided into 5 groups of 8 mice each. 16 mice gaved with the two cinnamic acid concentrations (56 and 28 mg/kg), 8 mice gaved with PBS and depended as a negative control, 8 mice injected with endoxan compound and depended as a positive control, 8 mice gaved with vitamin C and depended as a comparative group and from the two controls and the comparative groups can gain primary idea about the suitable concentration to cinnamic acid.

**Study the interaction between the cinnamic acid and endoxan:** After treated with endoxan compound, 24 mice were used in this experiment, 8 gaved with the perfect concentration from the purification of cinnamic acid 56 mg/kg, other 8 gaved with vitamin C (180 mg/kg) and the last 8 mice gaved with the PBS.

**\_1<sup>st</sup> group (positive control):** Mice injected with endoxan compound 50 mg/kg in the intraperitoneally

in the first day with dose 0.1 ml and then gaved orally with the PBS for 7 days, mice dissected after 24 h from the last dose.

**\_2<sup>nd</sup> group:** Mice injected with endoxan compound 50 mg/kg in the intraperitoneally in the first day with dose 0.1 ml and then gaved orally with the vitamin C (180 mg/kg) for 7 days, mice dissected after 24 h from the last dose.

**\_3<sup>rd</sup> group:** Mice injected with endoxan compound 50 mg/kg in the intraperitoneally in the first day with dose 0.1 ml and then gaved orally with the perfect concentration of purification of cinnamic acid (28 mg/kg), mice dissected after 24 h from the last dose.

**The Histological study:** Attended the paraffin sections (Bancroft and Steven, 1982) as follows:

1. **Dehydration:** Samples passed upward concentrations of ethanol (70%, 80%, 90% and 100%) hours for each concentration, for drawing water inside the tissue.
2. **Clearing:** Leaching models have been developed in a solution of toluene for two hours and then placed in xylene for half hour.
3. **Leaching:** Placed the samples in a mixture of xylene and paraffin wax with a melting point at 54-56 °C by 1/1 for 15 min. in the oven temperature 59-60°C and then drank the same type of wax.
4. **Embedding:** Buried samples the same type of wax used in the leak, as the molten wax poured in a special template and then transferred the samples to the template with a hot needle to prevent bubbles.
5. **Sectioning:** Template has been installed in the device manual (rotary microtome), then attended the slides, clean and put the adhesive (Mayer's albumin). Cut samples with thickness of 4 microns and quoted passages tape (Ribbon) to the extent a water bath 56°C for the purpose of textile and furniture loaded on glass slides, then left to dry in the oven at a temperature 56°C for 24 hr.
6. **Staining:** Glass slides placed in toluene for half hour to remove the paraffin wax samples. Followed by a pass slides in a series of regressive of ethanol (80%, 90% and 100%) for 10 min at each concentration to restoration of water into the fabric (rehydration), passed in distilled water for 5 min, placed in a solution of haematoxyline stain for 10 min, immersed in distilled water four times and twice with alcohol acid, washed with running water for 5 min, placed in a solution of eosin stain (2-5 min), immersed in tap water for 5-7 times.

Finally, passed a series of slides progressive concentrations of ethanol (70, 80, 90 and 100%), and placed in the coloring of the leaching solution.

7. **Mounting:** Used the center of loading permanent Canada balsam and put the slides on the hot plate (35-39°C) to dry.

**Statistical analysis:** The statistical analysis is done to get the means  $\pm$ SE and test the different significant among the means by using Duncan test (Duncan, 1955) then differences among the means in interaction experiments were compared between the Vitamin C, cinnamic extract and the endoxan by using T- test (Steel and Torrie, 1980).

## Results and Discussion

### Extraction and purification of cinnamic acid:

**Chemical Identification of cinnamic acid:** Cinnamic acid (partial and purification crystals) was tested for general phenolic tests (Table 1).

**High Performance Liquid Chromatography (HPLC) for Cinnamic acid:** HPLC for cinnamic acid detection was used the wave length: 280 nm for both cinnamic acid extract and the standard under the same conditions (Harbon, 1984). Table (2) appeared peak area and retention area and the corresponding retention time in both cinnamic acid standard and extraction. Results emphasized that the conditions for extraction and purification occur in dark. place, the study focus on qualitative specification of the cinnamic acid which is the proper way for extraction and application. Steps followed can be assumed for cinnamic acid qualitative study the isolated substance was determined by the specific  $\lambda$  max and HPLC technique. Measuring both standard and extracted cinnamic acid at maximum wave length absorption and the retention time explained the selectivity and accuracy of the applied method.

### Bioassay of cinnamic acid:

**The Histopathological effects (7 days):** Histopathological study showed focal infiltration of neutrophils cells in liver fibers of mice, which treated with endoxan component that led to congestion in blood vessels adjacent to the central vein, because Endoxan was a poison substances caused necrosis with dark places near the central vein and reached to the liver via blood vessels. Also the concentration (56 mg /kg) of the extracted and purified cinnamic acid, PBS treatment and vitamin C treatment as showed in figures 1, 3 and 4 respectively and concentration 28 mg/kg from the same extracted was showed in figure 2, with dark reddish spots of liver and gain cells, while there were no change appearing in the central vein.

Decreasing of AST, ALT and ALP enzymes activity in mice serum referred to the lowering of illness effects in many parts of the body such as: heart, liver, spleen,

bones and others, beside to prolonged intoxication due to damage liver (Jens and Ganne, 2002), while increasing of AST, ALT and ALP suggests that possibility of purified cinnamic acid to give protection against hepatic injury (Kansal *et al.*, 2011). The lowing in the liver glutathione and catalase which caused necrosis due to the rapid action of hydroxyl radical was repairing by the following mechanisms:

1. Avoid and prevent forming some compound as a result of hydrogen peroxide products which gave the first spark for starting the chemical interactions chain, such as lipid peroxidation (Valko *et al.*, 2004), Also avoid or prevent or repair the oxidation of DNA and proteins (Nakabeppu *et al.*, 2006). All these processes were depending on the cinnamic acid hydroxyl groups (Suanarunsaw *et al.*, 2009).
2. Present of lipid which lowing the effect and antioxidant activity (Synder *et al.*, 1993). Hepatocytes were arranged in trabecules running radiantly from the central vein and separated by sinusoids, they were regular and containing a large spherical nucleus and showed radically arranged of hepatic cords around the central vein with evident of sinusoids. The liver tissue restored most of its normal structure and able to diminish fibrosis, congestion incidence of inflammatory cells infiltration, centrilobular hepatocytes swelling, hepatocytes visualization, fatty changes and hemorrhagic clots (Figures 2 and 3). The central and portal veins were congested, considerable number of hepatic cells were damaged and repealed from their characteristic appearance while central vein and sinusoids between hepatocytes were dilated.

Endoxan is well known to induce hepatic injury (Synder *et al.*, 1993) and pathological changes which impaired liver function and interferes with the secretion of plasma proteins (Lapeyre *et al.*, 2006) caused decreased blood osmotic pressure, decreased drainage of tissue fluids, oedema and congestion in different tissue. The cytoplasmic vacuolation disturbance lipid and fat metabolism during pathological changes and accumulation of the injurious substances in the cells (Zhang and Wang, 1984). Other whose support that abundance of leucocytes in lymphocytes, with particular and a prominent response of body tissues can prevent any injury (El-Banhawy *et al.*, 1993).

These pathological changes were happened due to the presence of flavonoids and ascorbic acid, antioxidant property is one of the mechanism of Hepatoprotective drugs and suggested to acts as

antioxidants by free radical scavenging (Wenger and Fintel, 1999).

**Histopathological effects before and after endoxan treatment:** There are focal infiltration in neutrophil cells of liver fibers in mice when treating with PBS before and after cyclophosphamide treatment and congestion in blood vessels near the central vein due to appearing of lymphocytes and gain cells in necrosis areas with dark color specially near the central vein, which belong to effect of endoxan after reaching the liver via blood vessels, in addition PBS had no ability to prevent damage or to repair it.

Comparing between perfect cinnamic acid extract before and after endoxan treatment (Figure 6) found that results after endoxan treatment was the best and with low damage in liver cells and enzymes, while results before endoxan treatment were showed necrosis and high enzymes leak, comparison with both Vitamin C before and after endoxan treatment (Figures 7 and 8), mechanism for repair necrosis by cinnamic acid were:

1. Avoid and prevent hydroxyl radical as a product of hydrogen peroxide and gave the first spark for

start the chemical interaction such as lipid peroxidation (Lertlakana *et al.*, 2011).

2. Avoid or prevent or repair oxidation of DNA and protein, which depend on the hydroxyl groups of cinnamic acid (Fernarunsaw *et al.*, 2009; Valko *et al.*, 2004).
3. Cinnamic acid was suppressed hepatic fibrosis and protected liver against damage (Yamamoto *et al.*, 2005).
4. Cinnamic acid have anti-hyperlipidemic action (Suanarunsaw *et al.*, 2009).
5. Release of inflammatory mediators such as cytokines, histamine, prostaglandins and leukotrenes to protect hepatocyte (Suanarunsaw *et al.*, 2009).
6. The liver cytochrome p-450 system converts endoxan to 4- hydroxyEndoxan, which is an equilibrium with aldophosphamide. phosphoramidate mustard and acrolien were yielded from cleavage aldophosphamide. These two compounds are highly cytotoxic (Aubrey, 1970). Endoxan is uncommon hepatic toxin and its effect was due to an idiosyncratic reaction (Shaunal *et al.*, 1988).

**Table (1): General phenolic compound test**

Test		Result
NaOH 5% solution (detected oxidation phenolic compounds)	+ve	Soluble
Sodium carbonate 5% solution (detected antioxidation phenolic compounds)	+ve	Insoluble
Ferric chloride 1% solution (detection phenolic compounds)	+ve	Green color
Liebermann reaction (detection of phenolic compound with para position)	+ ve	Red
Phthalein reaction (phenolic compounds)	+ ve	Red colour
Aluminum chloride test (Friedle graft) (for the aromatic ring)	+ve	Yellow to orange colour
Bromine decolourisation test ( for the double bond)	+ ve	Discharge of reddish – brown colour
Baeyer test ( for the double bond)	+ve	Disappears of the purple colour

**Table (2): HPLC results for purification extracted and standard cinnamic acid (128 min).**

Sample	Wave length	Concentration (ppm)	Retention area	Peak area
Cinnamic acid standard	280 nm	100 ppm	8.40	2564135
		25ppm	8.91	6618661
Cinnamic acid extract	280 nm	100 ppm	8.13	4611420
		25ppm	8.88	5803060



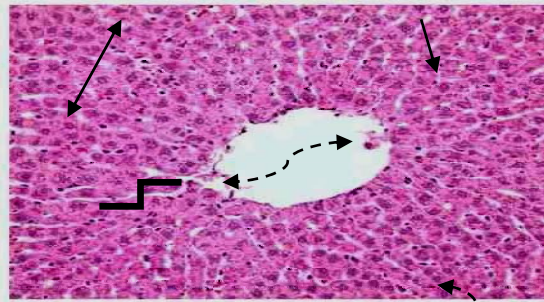


Figure (1): Tissue part of PBS treatment ( ) central vein ( ) gain cells ( ) hepatocytes, ( ) sinusoids.

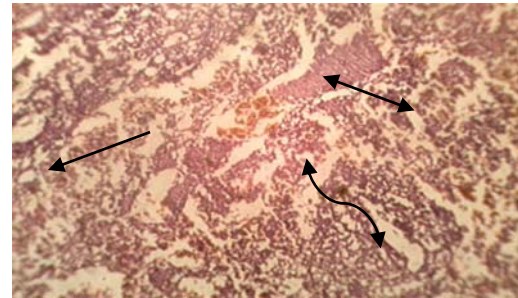


Figure (2): Tissue part of endoxan treatment ( ) central vein, ( ) gain cells, ( ) hepatocytes necrosis.

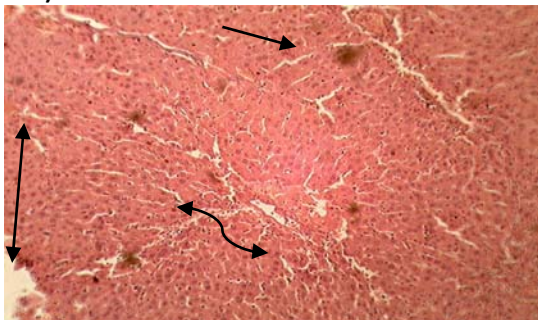


Figure (4): Tissue part of Vitamin C treatment ( ) central vein , ( ) gain cells, ( ) hepatocytes .

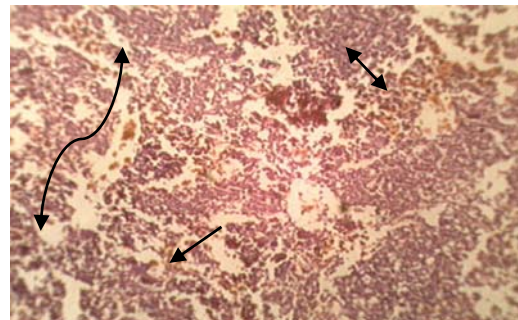


Figure (3): Tissue part of cinamic acid of (56 mg /kg) treatment ( ) central vein, ( ) gain cells, ( ) liver cells necrosis

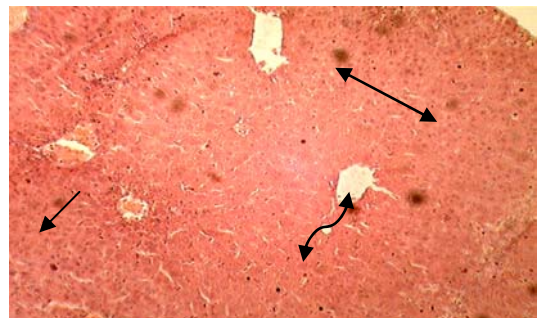


Figure (6):Tissue part of cinamic acid (28 mg/kg) after Endoxan treatment ( ) central vein and enzyme leak ( ) gain cells, ( ) hepatocytes

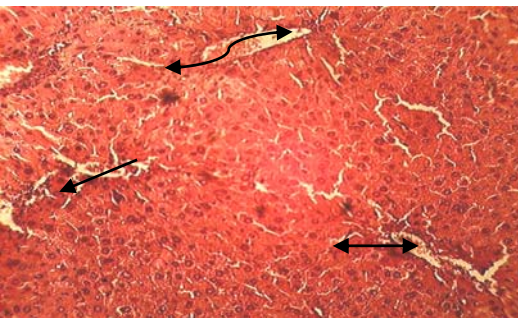


Figure (5): Tissue part of Cinamic acid of (28mg / kg) treatment ( ) central vein, ( ) gain cells, ( ) hepatocytes

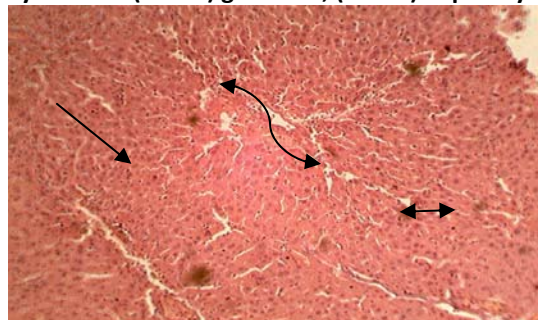


Figure (8): Tissue part of Vitamin C after Endoxan treatment ( ) central vein and enzyme leak, ( ) gain cells, ( ) hepatocytes

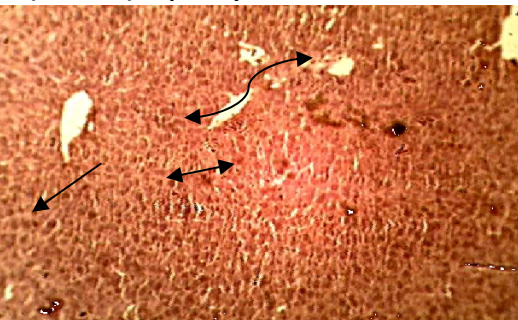


Figure (7): Tissue part of Vitamin C before Endoxan treatment ( ) central vein, ( ) gain cells, ( ) hepatocytes

### Conclusions

1. The yield extraction from cinnamon bark showed 96% pure cinnamic acid by high performers liquid chromatography.
2. Histopathological results for endoxan, cinnamic acid dose 56 mg/kg and pre-endoxan treatment showed fibrosis, cirrhosis that liver begin to shrunk and become hard, liver failure, zonal necrosis that showed a large zone of liver lobules because of high level of ALT and leading to acute liver failure.
3. Histopathological results of cinnamic acid dose 28 mg/kg and post-endoxan treatment showed remove and cure hepatocytes from fibrosis, cirrhosis, liver Failure, zonal necrosis and acute liver failure when compared with each the vitamin C treatment and the negative control.

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