Biochemical and Hepatoprotcetive Effects of Pure Cinnamic acid Against Cyclophosphamide in White Mice

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Abstract

The study was carried out to determine hepatotoxicity and hepatoprotcetive effects for Cinnamic acid in comparison with vitamin C against the mutagenic influence of cyclophosphamide, a chemical compound that damage hepatic cells and has mutagenic effects .The effect was studied in mammalian system *invivo* depended on evaluating the enzymatic activity of three hepatic enzymes: Alanine Transaminase (ALT), Aspartate Transaminase (AST), and Alkaline Phosphate (ALP). Two concentrations of pure cinnamic acid (5.6 and 2.8 mg/kg) were evaluated to choose the suitable concentration which remembered the negative control. An interaction experiments, included two types of treatments pre-cyclophosphamide and post-cyclophosphamide was carried out in order to determine the mechanisms of the pure cinnamic acid. Results showed no toxic and hepatotoxicity influence in biological system and instead it showed highly performance in preventing or reducing the hepatotoxicity of cyclophosphamide. Cinnamic acid increased the ALT, AST and ALP especially in dose 2.8 mg/kg. The positive effect was higher when pure cinnamic acid was used as post-cyclophosphamide treatments and to less extent in pre-cyclophosphamide treatments. Therefore, cinnamic acid can be considered as a cure hepatocytes from acute liver damage at first degree and responsible at the second degree as a cardiac, skeletal muscle and placental tissue protective.

Introduction

Hepatotoxicity is a general term for liver damage [1].The symptoms of hepatotoxicity can be sign in damage of the liver which reflected in liver enzyme levels in the blood,when the liver is damaged, there enzymes are released in to the blood stream,where the levels can be measured by blood tests, these are called Liver Function Tests enzymes(LFTs)[2]that are

routinely checked as part of LFTs include:

- Alanine Transaminase (ALT) also called Serum Glutamic Pyruvate Transaminase (SGPT)or Alanine aminotransferase (ALAT),is an enzyme present in hepatocytes (liver cells).When a cell is damage,it releases this enzyme into the blood, where it is measured ALT rises dramatically in acute liver damage, such as viral hepatitis or paracetamol overdose. Elevations are often measured in multiple of the upper limit of normal (ULM) [3,4].
- Aspartate Transaminase (AST) also called Serum Glutamic Oxaloacetic Transaminas e(SGOT)or Aspartate aminotransferase (ASAT) is similar to ALT in that it is another enzyme associated with liver parenchmal cells. Its raised in acute liver damage,but is also present in red blood cells and cardiac and skeletal muscle and is therefore, not specific to liver.The ratio of AST to ALT is sometimes useful in differentiating between causes of liver damage.Elevated AST levels are not specific for liver damage and AST has also been used as a cardiac marker[4,5].
- Alkaline Phosphatase (ALP)is an enzyme in the cells lining the billary ducts of the liver.ALP levels in plasma will rise with large bile duct obstruction,intrahepatic cholestasis or infiltrative diseases of the liver. ALP is also present in bone and placental tissue [5].

Most liver diseases cause only mild symptoms initially but it is vital that these diseases be detected early. Hepatic (liver)involvement in some diseases can be of crucial importance.The tasting AST, ALT and ALP are liver function tests (LFTs)is performed by a medical technologist on a patient serum or plasma sample obtained by phlebotomy. Some tests are associated with functionality (e.g., albumin),some with cellular integrity (e.g., transaminase)and some with conditions linked to the billary tract (ALP)[6].

Liver plays a central role in transforming and clearing chemicals and is susceptible to the toxicity from these agents [7].Certain medicinal agents,when taken in overdoses and sometimes even when introduced within therapeutic ranges,may injure the organ[8].Other chemicals agents,such as those used in laboratories and industries,natural chemicals and herbal remedies can also induced hepatotoxicity chemicals that cause liver injury are called hepatotoxins[9].

The bioaviabilities of Polyphenols in plants such as cinnamic acid in cinnamon bark(with all kinds),grape fruit and other and their ability to inhibition and prevent tumor formation after entering blood circulation and absorbing by bowel. They work directly inhibitor by effect on protein or control factors which operate in active the system repairing cell[10]and also because of motivate immune system and increasing conformation natural killer cells and effect in the enzymes which responsible of process and complete the cell cycle by hyperexpression arrangement [11].The pure cinnamic acid is a white crystalline hydroxyl cinnamic acid,slightly soluble in water, it's a part of the biosynthetic shikimate and phenylpropanoid pathways. It is

biosynthesis performed by action of the enzyme phenylalnine aminanielyse (PAL)on phenylalanine [12].

The derivates of cinnamic acid such as ferulic acid,cinnamicaldehyde,caffeic acid,chlorogenic acid and others sowed ability to cure some disease[13]such as antioxidant in vitro and prevention of type 2-Diabetes Mellitus and cardio vascular diseases and because the scientific and locals tends to use the natural products specially the graces in medical and nutrition yields that made us to focus our immediately study to evaluated the antioxidant effect of pure cinnamic acid in one of the biosystem the white mice [14].

Material and Methods

Solution:

Phosphate Buffer Solution(PBS) [15].
NaOH(0.4N)prepared according to [16].
Colchicine Solution : Colchicine1mg (one tablet)and sterile distilled water 1ml.The solution was used immediately after preparing 2.5 to 3 hours [17].

Doses:

Two doses from the pure cinnamic acid (Riedel-de Haën company)which are(5.6,2.8) mg/ kg and vitamin C(180 mg/ kg)[18](as comparative groups and cyclophosphamide compound in (50 mg/ kg)[19]as a positive control and the PBS as a negative control.

Hepatoprotectivity effects:

To study the hepatotoxicity effect and the hepatoprotcetive in laboratory animals, the gulping was orally by syringe 1 ml size supplying with gulping instrument as thin plastic tube to turning shape and soft edge to avoid harm the mice and inserted to the digestive system of mouse,but the cyclophosphamide was injected Intraperitonially because it lost after (3-12) hours by urine [20]. The white mice was 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) Ċ and gave the water and integrated animal fed which manufacture locally.

The experiment:

Two concentration of from pure cinnamic acid (5.6,2.8) mg/ Kg,the concentration a count depended on the mouse weight.The experiment contains 40 mice divided in to 5 groups of 8 mice each (16 mice gulped with the two cinnamic acid concentration (5.6,2.8) mg/ Kg,8 mice gulped with PBS and depended as a negative control,8 mice injected with cyclophosphamide compound and depended as a positive control,8 mice gulped with vitamin C and depended as a comparative groups and from the two control) and the comparative groups can gain primary idea about the suitable concentrate to cinnamic acid.

The interaction between the cinnamic acid and cyclophosphamide:

After treated with cyclophosphamide compound,24 mice were used in this experiment,8 gulped with the perfect concentrate from the pure cinnamic acid 5.6 mg/ kg, other 8 gulped with vitamin C(180 mg/ kg) and the last 8 mice gulped with the PBS.

- 1st group:(positive control): Mice injected with cyclophosphamide

compound 50 mg/ kg in the Intraperitonial membrane in the first day with dose 0.1 ml and then gulped orally with the PBS for 7 days,mice dissected after24 h from the last dose.

- 2nd group: Mice injected with cyclophosphamide compound 50 mg/ kg in the Intraperitonial membrane in the first day with dose 0.1 ml and then gulped orally with the vitamin C (180 mg/ kg) for 7 days,mice dissected after24 h from the last dose.
- 3rd group: Mice injected with cyclophosphamide compound 50 mg/ kg in the Intraperitonial membrane in the first day with dose 0.1 ml and then gulped orally with the perfect concentrate of pure cinnamic acid (2.8 mg/kg), mice dissected after 24 h from the last dose.

Preparing of tissue extract from Liver mouse:

One gram from the mouse liver were cutting in to very small pieces by sharp knife in 1 ml from PBS and using in the same time the pressure of hand used to crush the liver tissue till be sticky solution then move the attain to the centrifuge with (5000 round/ second) speed for one hour.Remove the upper layer and let the remainder in the bottom of the test tubes,, keep in freezer (-20) Ċ until evaluate or use directly to measure the activity of enzyme [21].

Enzymatic assay:

Measured the enzyme activity of Alanine transaminase (ALT), Aspartate transaminase (AST).

It was used the test instrument belonging to measure the activity of AST enzyme (Aspartate transaminase), ALT (Alanine transaminase), which imported from SYRBIO company get the two test tubes for all par ten, the first one containing blank reagent and the other the sample which need to measure the enzyme activity [16].

	Reagent (blank)	Sample
Sample	---	0.1 ml
Solution 1 (ALT or AST)	0.5 ml	0.5 ml
Distilled Water	0.1 ml	--
Shaken gently and keep Incubate 37 Ċ for 30 minute		
Solution 2 (ALT or AST)	0.5 ml	0.5 ml
Shaken gently and keep in 20 Ċ for 20 minute.		
NaOH (0.4 N)	5 ml	5 ml
Shaken tubes,read absorbance after 5-10 minutes at wavelength 530 – 550 (546) nm		

-Measured the Enzyme activity of Alkaline Phosphatase (ALP):

Four test tubes prepared for all part ten,the first containing the sample, The second is the sample blank, The third containing the standard sample,The fourth containing the detection blank, [22].

Contains	First tube (sample)	Second tube (sample blank)	Third tube (standard sample)	Fourth tube (detection blank)
Detection reagent	2 ml	2 ml	2 ml	2ml
Incubate for 5 minute in 37 Ċ				
Serum	50 µl	___	___	___
Reagent 2	----	---	50 µl	___
Incubate for 51 minute exacting in 37 Ċ				
Reagent 3	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Shaken the tubes handily or by vortex				
Reagent4	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Serum	---	50 µl	---	---
D.W.	---	---	---	50 µl
Shaken tubes, read absorbance after 10 minutes, in wavelength 520 nm				

$$\text{The absorbance measured} = \frac{\text{OD of sample serum} - \text{OD Serum blank}}{\text{Standard absorbance}} \times n$$

Statistical analysis

The statistical analysis is done to get the means ±SE and test the different significant among the means by using Duncan test [23] then differences among the means in interaction experiments were compared between the Vit. C, cinnamic extract and the cyclophosphamide by using T- test [24].

Results and Discussion

Select the perfect concentrate from the pure cinnamic acid in antioxidant activity:

The changes of liver function tests (LFTs) in the serum of white mice

The liver carries out numerous synthetic, excretion and detoxification functions[25,26], however only a minority of these can be measured by levels of products in the blood. Liver Function Testes (LFTs) measure the concentration of various different protein and enzyme in the blood that are either produced by liver cell or released when liver cells are damaged [25.26]

Aspartate Transaminase Enzyme (AST):

Table (1) expressed that the position treatment with cyclophosphamide related to low value of AST enzyme in the serum (27.2U/L) with significant ($p \leq 0.05$) comparing with the negative treatment (30.75U/L), while comparative group showed high value in enzyme level (40.2U/L) and increasing with different significant when compared with both the negative and positive treatment. The gulping with cinnamic acid (2.8mg/kg) showed that the value reached to (30.77U/L) when comparison with both negative and positive treatment and no significant with the comparative group ($p \leq 0.05$), but when gulping with the concentrate (2.8mg/kg) of cinnamic acid showed significant when compared with the positive treatment and high significant (40.69U/L) when compare with both cinnamic acid (5.6 mg/kg) and also with the comparative group, there is no significant in compared with the negative treatment.

The result indicated that (2.8 mg/kg) was the best concentration when gulping for seven days, which showed in the result of AST enzyme and referred to caused heart attack, infectious mononucleosis, liver disease hepatitis and trauma when the level of this enzyme was increasing [25,26].

Alanine Transaminase Enzyme (ALT):

Table (1) showed that the positive treatment as a result by using cyclophosphamide was lowing in ALA concentration reached to (61.4U/L) and this result indicated to different significant in comparing with negative treatment (63.6U/L) and comparative group (70.36U/L) with p value ($p \leq 0.05$).The cinnamic acid (5.6mg/kg) showed (75.62U/L) when compared with positive and negative treatment and no significant when compared with comparative group, then the present study showed that the mean of cinnamic acid (2.8mg/kg) and comparative group significantly from other treatment with p value ($p \leq 0.05$)

In comparison with other study, results referred to increasing in significant between them and the best result related to concentrate (2.8 mg/kg) of cinnamic acid in value of ALA enzyme results which caused hepatitis, cirrhosis and infectious mononucleosis [25,26]

Alkaline phosphates Enzyme (ALP).

This enzyme is mainly implicated in the diagnosis of billary abstraction and was normally found in small bile tracts in the liver, it is also found in the liver, bone, placenta and the evaluated levels may be due to a problem outside the liver such as a malignancy (cancer) [25,26].Mice treatment with cyclophosphamide was showed significantly elevated (256.6U/L), comparison with negative treatment (148.38U/L) and Vit.C with p value ($p \leq 0.05$). The cinnamic acid concentration (5.6 mg/kg) showed significantly elevated (438.7U/L) in mice serum with p value ($p \leq 0.05$) comparative with other treatment, while cinnamic acid extract concentration (2.8mg/kg) showed increased with significant reached (395.7U/L) in mice serum with p value ($p \leq 0.05$) comparative with other treatment (Table 1).

Interaction between the cyclophosphamide and the pure cinnamic acid dose 2.8 mg /kg:

After make sure from no hepatotoxicity effects to the perfect concentrate of the pure cinnamic acid which depended in this study,the interaction between the pure cinnamic acid and cyclophosphamide which caused toxicity and mutation influences because it prevent the cell from divided by damaging the DNA itself and the interaction contain giving the pure cinnamic acid with dose2.8 mg /Kg after the mutation factor.

The changes of liver function tests (LFTs) in the serum of white mice after the cyclophasphamide treatment.

Aspartate Transaminase Enzyme (AST).

Table (2) showed that treating with Vit. C after cyclophosphamide increased the rate of AST concentrated in the serum of mice (33.01)U/L comparison with control (28.05)U/L. When gulping with (2.8mg/Kg) of cinnamic acid after cyclophosphamide was showed no significant (31.09) U/L when compared with the Vit. C with p value ($p \leq 0.05$)

Alanine Transaminase Enzyme (ALT).

Results showed increasing in the rate of ALT concentrated when treating with Vit.C after cyclophosphamide (59.89)U/L comparison with the control treatment (52.52)U/L. When gulping with (2.8mg/Kg) of cinnamic acid extract after the cyclophosphamide showed increasing in the rate of ALT comparing with control and no significant when it compared with the Vit.C with p value ($p \leq 0.05$), as showed in table (2).

Alkaline phosphates Enzyme (ALP).

Table (2) was appeared that Vit.C treatment after the cyclophosphamide increased in ALP concentrated in serum of mice (478.09) U/L comparison with control treatment (456.32)U/L. When gulping with (2.8mg/kg) of cinnamic acid after cyclophosphamide showed lowing Lin ALP concentrate (236.74) U/L when compared with both control and Vit. C treatment with p value ($p \leq 0.05$).

The above results showed that the pure cinnamic acid dose 2.8 mg/Kg have hepatoprotcetive activity and with activity more than the vitamin C, mechanisms of cinnamic acid to repair hepatocytes were:

- 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 [27].
- Avoid or prevent or repair oxidation of DNA and protein, which depend on the hydroxyl groups of cinnamic acid [28.29].
- Cinnamic acid was suppressed hepatic fibrosis and protected liver against damage [30].
- Cinnamic acid have anti-hyperlipidemic action [31].
- Release of inflammatory mediators such as cytokines, histamine, prostaglandins and leukotrenes to protect hepatocyte [31].
- The liver cytochrome p-450 system converts cyclophosphamide to 4-hydroxycyclophosphamide, which is a equilibrium with aldophosphamide. phosphoramide mustard and acrolien were yielded from cleavage aldophosphamide. These two compounds are highly cytotoxic.Cyclophosphamide is uncommon hepatic toxin and its

effect was due to an idiosyncratic reaction [32].

Table (1) Liver function tests (LFTs) in the serum of white mice.

Treat / Test	Negative treat (PBS)	Comparative groups, Vit.C (180mg/kg)	Positive treatment Cyclophosphamide (50 mg /Kg)	Cinnamic acid (5.6 mg /kg)	Cinnamic acid (2.8 mg /kg)
Mean ±SE (U/L)					
AST	30.75±0.99 b	40.2±0.81 a	27.2± 0.96 c	30.77±0.75 a	40.69±0.14 b
ALT	63.6± 0.11 b	70.36±0.34 a	52.5± 0.17 c	61.4±0.89 a	75.62±0.91 b
ALP	148.30±0.85 a	385.125±0.92 b	256.6± 0.34 c	438.7±0.68 a	395.7±0.99 b

*****Values are presented as means ±SE (n= 8 mice /group). *Probability($p \leq 0.05$).

* a, b, c within any column significant differences.

Table (2): Liver function tests (LFTs) in the serum of white mice after with the Cyclophasphamide component treated(7 days).

Treat / Test	Cyclophosphamide after Phosphate Buffer solution	Cyclophosphamide after Vit. C	Cyclophosphamide after perfect concentrate of Cinnamic acid (2.8 mg/kg)
Mean ±SE (U/L)			
AST	28.05 ± 0.91 b	33.01 ± 0.83 a	31.812±0.25 a
ALA	52.52 ± 0.93 b	59.89 ± 0.91 a	61.23± 0.93 a
ALP	456.32 ± 0.62 b	478.09 ± 0.83 a	236.74±0.48 b

*****Values are presented as means ±SE (n= 8 mice /group). *Probability($p \leq 0.05$).

* a, b, c within any column significant differences.

التأثيرات الكيموحيوية لحامض السينـاميـك النقـي لحماية الكبد ضد السايكلوفوسفومايد في الفئـــران البيض

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الخلاصـــة

أجريت الدراسة للكشف عن التأثير السمي للكبد لحامض السيناميك النقي cinnamic acid ومقارنته بفيتامين C تجاه السايكلوفوسفومايد Cyclophosphamide والذي يعد مركب كيمائي يسبب تلف خلايا الكبد وباستخدام نظام اللبائن *invivo* وبالاعتماد على تقييم ثلاثة من إنزيمات وظائف الكبد: Alanine Transaminase (ALT),Aspartate Transaminase (AST),Alkaline Phosphate (ALP). استخدم تركيزيين لحامض السيناميك النقي (2.8 و 5.6 ملغم /كغم) كل على انفراد لانتخاب التركيز الأمثل للمركب والذي أعطى نتائج أفضل من الحالة الطبيعية السيطرة السالبة،. بعد ذلك اجري التداخل مابين التركيز الأمثل السايكلوفوسفومايد وبشكل معاملتان قبل وبعد لمعرفة الآلية التي يعمل بها حامض السيناميك في منع و تقليل الأثر السمي للكبد للسايكلوفوسفومايد فقد عمل على رفع قيمة إنزيمات وظائف الكبد LFTs (ALT,AST,ALP) وقد كان الفعل الأكثر إيجابية عند استعمال حامض السيناميك النقي بجرعة 2.8 ملغم /كغم بعد السايكلوفوسفومايد وبدرجة اقل عند معاملة الحيوانات بحامض السيناميك النقي قبل السايكلوفوسفومايد. يمكن بالتالي تصنيف فعل هذا المركب في نظام اللبائن كونه علاج بالدرجة الأولى لحماية خلايا الكبد من الضرر الحاد وبالدرجة الثانية وقائيا للعضلات الهيكلية والقلبية وخلايا المشيمة .

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