Evaluation of Toxic Oxidant Activity for Pure Cinnamic Acid in Albino Mice

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Abstract

The study was carried out to determine the toxic, oxidant and antioxidant effects of cinnamic acid in comparison with vitamin C against the mutagenic effect of Cyclophosphamide, which is a chemical compound that damage hepatic cells and has mutagenic effects. The effect was studied in mouse. This *invivo* system is depended on evaluating the enzymatic activity of two antioxidant enzymes: Catalase and Glutathione Reductase. Two concentrations of pure cinnamic acid 5.6 and 2.8 mg/kg were evaluated to choice the suitable concentration which remembered the negative control. In an interaction experiments, included two types of treatment pre-cyclophosphamide and post–cyclophosphamide was carried out to determine the mechanisms of pure cinnamic acid. Results showed no toxic and oxidant effects in biological system and instead it showed highly performance in preventing or reducing the oxidant stress influences of cyclophosphamide. It increased the Catalase and Glutathione reductase antioxidant activity, especially in dose 2.8 mg/kg. The positive effect was higher when pure cinnamic acid was used as pre-cyclophosphamide treatment and to less extent in post–cyclophosphamide treatment. Therefore, the cinnamic acid can be considered as an antioxidant compound and Desmutagens in the first degree and Bioantimutagens in the second degree.

Introductions

Oxidative stress is thought to contribute to the development of a wide range of diseases including Al-Zheimer's disease [1,2]. Parkinson' disease [3]. The pathologic is caused by diabetes [4,5], rheumatoid arthritis [6] and neuro degeneration in motor neuro-diseases [7]. It has been proposed that polymorphisms in

enzymes such as Catalase and Glutathione Reductase are associated with DNA damage and subsequently the individual's risk of cancer susceptibility [8].

Antioxidant molecular is capable of inhibiting the oxidation of other molecular,When oxidant compound produce free radicals and that radicals can start chain reaction which damage cells the antioxidant compounds terminate these chain reaction by removing free radicals intermediates and inhibit other oxidation reaction and that happen by being oxidized themselves,so they often reducing against such as thiols, ascorbic acid and Polyphenols [9]. Antioxidant are widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer,coronary heart disease and even attitude sickness, they work by chelating transition metals and preventing from catalyzing the production of free radicals in the cell or by scavenge reaction oxygen before they can damage cells[10].

The bioavaibilities of Polyphenols in plants such as cinnamic acid in cinnamon bark(with all kinds),grape fruit and others and their ability to inhibit and prevent tumor formation after entering blood circulation and absorbing by bowel[11]. They work directly to inhibitor by effect on protein or control factors which operate in active the system repairing cell and also because of motivate immunology system and increasing conformation natural killer cells and effect in the enzymes which responsible of process and complete the cell cycle by hyperexpression arrangement [12].The pure cinnamic acid is a white crystalline hydroxyl cinnamic acid,sli8ghtly soluble in water, it's a part of the biosynthetic shikimate and phenylpropanoid pathways. It is biosynthesis performed by action of the enzyme phenylalnine aminanie-lyse (PAL) on phenylalanine [13].

The derivatives of cinnamic acid such as ferulic acid, cinnamicaldehyde,caffic acid, chlorogenic acid and others showed ability to cure some disease[14]such as antioxidant in vitro and prevention of type 2-Diabetes Mellitus and cardio vascular diseases [15] 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 of albino mice.

Materials and Methods

Solution:

1.Phosphate Buffer Solution(PBS) [16].
2.Beta-Nicotinamide Adenine Dinucleotide Phosphate Sodium Salt (NADPH+H) (1Mm)[17].
3.Hydrogen peroxide(Catalase)(H_2O_2[18].
4.Bovine Serum Albumin (BSA)1%[18].
5.Colchicine Solution : Colchicine1mg (one tablet)and sterile distilled water 1ml.The solution was used immediately after preparing 2.5 to 3 hours.[19].
6. Biuret solution.made by 9g from Potassium tatrate dissolving in 500 ml from 0.2M from NaOH(8g/1L)and adding to it 3g from $CuSO_4.2H_2O$ and 5g from Potassium Iodine and the volume

completed to one liter by using the solution NaOH(0.2M)then kept in refrigerator 4Ċ till used.

Doses:

Two doses from the pure cinnamic acid (Riedel-de Haën company)which are(5.6,2.8) mg/ Kg(LD_{50} =1600mg/kg) [20], vitamin C(180 mg/ kg)as comparative groups and cyclophosphamide compound in (50 mg/kg) as a positive control and the PBS as a negative control.

Antioxidant effects:

To study the oxidant effect and the antioxidant 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 solution was injected Intraperitonially because it lost after (3-12)hours by urine[21].The albino mice was used in the experiments which is *Mus muscles*(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:

The experiment performed by using 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,8mice 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.

Interaction between the cinnamic acid and cyclophosphamide:

Forty-eight mice used in studying 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): The 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 anatomy happened after24 h from the last dose.
- 2nd group: The 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 anatomy happened after24 h from the last dose.
- 3rd group: The 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 anatomy happened after24 h from the last dose.

Preparing of tissue extract from Liver mouse:

Weight 1 g from the mouse liver and cut it to very small pieces by sharp knife in 1 ml from PBS and using in the same time the Mechanism pressure of hand to crush the liver tissue till be sticky solution then move the attain to the centrifuge with(5000 round/ second)speed for one hour.Get the upper layer and let the remainder in the bottom of the test tubes,avoid the fatty layer above it,store in freezer(-20)Ċ until evaluate or use directly to measure the activity of enzyme [23].

Value the activity of Glutathione Reductase:

To evaluate the activity of enzyme in endly volume 1 ml, contains interaction mixture: 0.1 ml from BSA 1%., 0.4 ml from oxidation Glutathione., 0.4 ml from 1mM NADPH+H., 0.1 ml from the enzyme extraction, then read the interaction mixture in spectrophotometer in 340 nm and recorded the reading every 30 second for half an hour. The Glutathione Reductase value in the liver extract based on the hydrogen move from $NADPH_2$ to the oxidation Glutathione and then measure the interaction by spectrophotometer,the continuous of interaction led to reduced the absorbance as a result to change the oxidation Glutathione to Glutathione Reductase. The unit of enzyme evaluation based on reduces the amount of absorbance through one minute under the standard interaction and the unit of enzyme known that it is the change in absorbance 0.001/1 second under interaction elements[17,18]. To evaluate the enzyme activity (unit/milliliter) flow the rule:

$$\text{Enzyme activity} = \frac{\text{The whole volume of interaction}}{\text{0.001 x1xenzyme extract volume}} \times \frac{\Delta^{\circ}}{T2 - T1}$$

Δ°= referred the reduce mount in absorbance.

$T_2 - T_1$ = referred to the change in time.

Value Catalase activity:

Dissolved 1 mg from the standard Catalase which contain 60 U/mg in 10 ml from PBS. A chain of concentrate made (0.6, 0.3, 0.15, 0.075, 0.0375) U/ml and that to gain standard curve Fig(1).

Input 3 milliliter from the physiology hydrogen peroxide (H_2O_2) in quartz cell of spectrophotometer and let in for 3 minutes to get stability, after that 0.1 ml from standard Catalase added and mixed in the same time. The highly reading in spectrophotometer must be in wavelength 240nm in absorbance 0.5,the educe in absorbance followed every 20 second,the first reading was at 10 second from adding enzyme,and the second reading be after 30 second,to draw the standard curve.

The standard Catalase curve drawing from the absorbance reading against the standard enzyme concentrated valuated with unites (Unit/milliliter),So can get the mount of enzyme in strange substance from count (Catalase) standard enzyme curve line. Specific activity express about the enzyme units numbers for all mg/protein in enzyme extract,using by Annion (1964) in

evaluating the protein quality for enzyme specific activity,exampled in reduced the amount of absorbance 1 ml in enzyme extract through one minute for all milligram from protein under interaction elements.

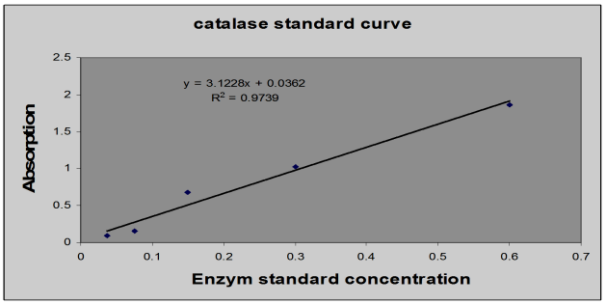

Fig (1) the activity of standard Enzyme

Evaluate protein mount:

Three test tubes get, the first example, the blank, the second example the standard and the third referred to the test sample.

1. The first tube, add to it 2ml from sterilizing distiller water.
2. The second tube, add 2ml from the standard solution of protein(BSA).
3. The third tube, add 0.2 ml from the test (the liver extract from mouse liver)and 1.8 ml from sterilizing distilled water.
4. Five milliliter from Biuret solution[18] to the three tubes and mixed well,then put in the water bath in 37Ċ for 10 minutes after that let to cool and read in wavelength 50nm,the calculate of protein mount happen by:

$$\text{Protein mount g/100 ml=test absorbance} = \frac{\text{Standard concentrate of protein}}{\text{Standard absorbance of protein}}$$

Statistical analysis

The statistical analysis is done to get the means ±SE and test the different significant among the means by using Duncan test [24] then differences among the means in interaction experiments were compared between the Vit. C, cinnamic extract and the cyclophosphamide by using T- test [25].

Results and Discussion

Select the perfect concentrate from the pure cinnamic acid in Antioxidant activity: Specific activity to Catalase enzyme (CAT).

To determine the value of enzyme concentrate with unknown component by calculating the value of absorption via spectrophotometer, comparison with the value of catalase standard curve (Figure 1).

The results in (Table 1) showed denoted with lowing in specific activity of catalase when treated with cyclophosphamide, the treatment with comparative group showed that there was statistically significant of specific activity (0.91U/ mg protein) in comparison with negative and positive treatment with p value ($p \leq 0.05$).

The cinnamic acid extract concentration (5.6mg/kg) showed that different significantly (594U/mg protein) comparison with positive treatment and comparative group while there is no significant with negative treatment. So, the cinnamic acid extract concentration (2.8mg/kg) showed no significant comparison with comparative group (0.965 and 0.91 U/mg protein) respectively.

Specific activity of Glutathione Reductase enzyme (CR):

Treating with positive group as cyclophosphamide component led to low value of specific activity with significant for Glutathione Reductase (3.755U/mg protein), The comparative group gave significant differences of enzyme activity comparison with negative and positive treatment (7.096, 4.132 and 3.75U/mg protein) respectively. Results of gulping the mice with concentrate (2.8mg/kg) of cinnamic acid extract showed significant (5.126U/mg protein) comparing with other treatment, while gulping the mice with concentrate (2.8mg/kg) of cinnamic acid showed elevating in significantly (7.77U/ mg protein) and no significant when compared with the comparative group ($p \leq 0.05$) (Table 1).

The pure cinnamic acid dose 5.6 mg /Kg showed increasing and with significant in antioxidant activity by increasing the liver Glutathione and Catalase enzyme which caused lowing in oxidant pressure that confirmed with the results of [26].The oxidant and damaging cells happened because the Cyclophosphamide[22]attaches the guanine base in the 7 nitrogen at one of the imidazole ring and stopping the cell divided, The main effect of it by metabolize the phosphoramide mustard and this formed only in cell that have low of aldehyde -dehydrogen (ALDH). Phospharamide mustard forms DNA crosslinks between (interstrand crosslinkages) and within (intrastrand crosslinkages) DAN strands at guanine N-7 position that is irreversible and leads to cell dead. Dead cell by cell caused “hyper acute Liver Failure “if the failure happen in 7 days and with decreased production of protein [27].

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

After make sure from no oxidative 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.

Specific activity to Catalase enzyme (CAT).

Table 2 showed that treating with Vit. C after cyclophosphamide treatment increasing in specific activity (0.434)U/mg protein comparison with control treatment (0.193)U/mg protein. When gulping with concentration (2.8mg /kg) of cinnamic acid after cyclophosphamide treatment showed increasing in

Specific activity of Glutathione Reductase enzyme (CR).

Table 2 showed that treating with vitamin C after cyclophosphamide treatment increasing in specific activity of glutathione reductase (2.905) U/mg protein comparison with control treatment (0.903) U/mg protein. When gulping with (2.8 mg/kg) of cinnamic acid after the cyclophosphamide treatment showed increasing in specific activity (5.085) U/ mg protein when

compared with both control and Vit.C treatment with p value ($p \leq 0.05$).

The results revealed that the pure cinnamic acid able to act as antioxidant [26] in a number of ways**:**

1. Phenolic hydroxyl groups in cinnamic acid are a good hydrogen donors[27], hydrogen donating antioxidants can react with react oxygen and reactive nitrogen species [28, 29] and breaks the cycle of generation of new radicals [30].
2. Following interaction with the initial reactive species, a radical form of the antioxidant was produced and had a greater chemical stability than the initial radical [30,31].
3. Interaction of phenol hydroxyl groups with π-electrons of benzene ring gave molecules with special properties, the ability to generate free radicals where stabilized by delocalization [28]. Formation of these long-lived free radicals is able to modify radical-mediated oxidation processes [30].
4. Antioxidant capacity of phenolic compounds is also attributed to ability chelate metal ions involved in production of free radicals [33]. However, phenolic compounds can acts as pro-oxidants by chelating metals in manner that maintains or increases their catalytic activity or by reducing metals, thus increasing their ability to form free radicals [34].

Hydrophobic benzenoid rings and hydrogen bonding potential of phenolic hydroxyl groups interact with protein and gave cinnamic acid capacity to inhibit some enzymes involved in radical generation [32; 33]

The above results showed that the pure cinnamic acid dose(2.8 mg/kg) have antioxidant activity and with activity mire than the vitamin C and Can consider the pure cinnamic acid from Bioantimutagen because it work to remove the toxicity influence of mutation factor,so we recommendated to study the effect of pH on the antioxidant activity and study other sides such as the Antimutagenic activity of pure cinnamic acid and the anticancer activity and the ability of cinnamic acid to protect the immunology and mutation system from the Mutants damage that caused.

Table (1): The average of Antioxidant enzymes results for 7 days.

Treat Test	**Negative treat (PBS)**	**Comparative groups Vit. C. (180mg/kg)**	**Positive treat Cyclophosphamide (50mg/kg)**	**Cinnamic acid (5.6 mg /kg)**	**Cinnamic acid(2.8 mg /kg)**
Mean ±SE (U/ mg protein)					
Catalase	0.53±0.13 b	0.91±0.31 a	0.162±0.13 c	0.594±0.18 b	0.965±0.21 a
Glutathione Reductase	4.132±0.24 c	7.096±0.52 a	3.75±0.21 c	5.126±0.22 b	7.77±0.10 a

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

* The means within any column with different letters are of significant differences**.**

Table (2): The average of Antioxidant enzymes results after the Cyclophasphamide treated for 7 days.

Treatment Test	Cyclophosphamide and phosphate buffer solution	Cyclophosphamide and Vit. C	Cyclophosphamide and Perfect concentrate of Cinnamic acid (2.8 mg/kg)
Mean ±SE (U/ mg protein)			
Catalase	0.193±0.25 c	0.434 ± 0.34 b	0.746 ± 0.33 a
Glutathione reductase	0.903 ± 0.32 c	2.905 ± 0.81 b	5.085 ± 0.85 a

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

* The means within any column with different letters are of significant differences.

تقييـــم فعاليــة السمية المؤكسدة لحامض السينـــاميك النقـي في الفئـران البيض

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الخلاصــة

أجريت الدراسة للكشف عن التأثير السمي المؤكسد والمضاد للأكسدة لحامض السينماميك النقي cinnamic acid ومقارنته بفيتامين C تجاه السايكلوفوسفومايد Cyclophosphamide والذي يعد مركب كيمائي يسبب تلف خلايا الكبد وتمتلك تأثيرا سمي مؤكسد ،وباستخدام نظام اللبائن في in vivo وبالاعتماد على تقييم الفعالية الإنزيمية للمضادين للأكسدة: الكاتليز وكلوتاثايون المختزل لمجانس خلايا الكبد. استخدم تركيزين للسينماميك اسد النقي (5.6، 2.8 ملغم/كغم) وكل على انفراد لاختبار فعلها المؤكسد والمضاد للاكسدة في الفئران البيض، وتم انتخاب التركيز الأمثل للمركب والذي أعطى نتائج افضل من الحالة الطبيعية السيطرة السالبة،بعد ذلك اجري التداخل مابين التركيز الامثل والمؤكسد السايكلوفوسفومايد وبشكل معاملتان قبل وبعد العامل المؤكسدلمعرفة الاليةالتي يعمل بها هذا المركب في منع او تقليل الاثر التاكسدي السايكلوفوسفومايد فقد عمل على رفع قيمة انزيمي الماتاليز والكلوتاثايون المختزل المضادين للاكسدة وقد كان الفعل الاكثر ايجابية عند استعمال حامض السينماميك النقي بجرعة 2.8 ملغم /كغم قبل العامل المؤكسد وبدرجة اقل عند معاملة الحيوانات بحامض االسينماميك النقي بعد العامل المؤكسد وبالتالي يمكن تصنيف فعل هذا المركب في نظام اللبائن كونه مضاد للاكسدة ومثبط مباشر Desmutagens بالدرجة الاولى ومثبطات حيوية Bioantimutagens بالدرجة الثانية.

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