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Research paper

Liver malfunction of wistar rats induced by combination of benzoic and citric acids as food additive

Muawia I. A. Abdelgadir Elrrigieg [1] *, Mahmood.M. Hassan [2], Salah E. Idris [3], Murwan K. Sabahelkhier [4], Zeineb M. Ali [1], Mohamed A. Adris [5], Omer F. Idris [4]

[1] Department of chemistry, Faculty of Science, University of Bakht El – Ruda, Eld – Dueim, Sudan
[2] Department of Food technology, Faculty of Agricultural technology and Fish Sciences, El Neelain University, Khartoum, Sudan
[3] Department of Biochemistry, Faculty of Medicine and Health Sciences, University of Blue Nile, Sudan
[4] Department of Biochemistry and molecular biology, Faculty of Science and Technology, El Neelain University, Khartoum, Sudan
[5] Department of Biochemistry and molecular biology, Faculty of Medicine, Dungla University, Dungla, Sudan
**Corresponding author E-mail: elrrigieg.muawia@gmail.com*

Abstract

This study was aimed to investigate liver function of Wistar rats influenced by oral ingestion of different doses of combination of benzoic and citric acids as food additives. Administered doses were (100, 500 and 1250 mg/kg b.w). Rats' serum GGT and ALP were used as an indicators. Forty four Wistar rats were divided into four groups' eleven rats in each group, six males and five females. One group was put as control, and the remainder of the three groups received different doses of combination of benzoic and citric acids. Housing in Meck Nimir research center Khartoum. They were recived free tab water and prepared food systems liberally. Serum was taken from each rats and analyzed by spectrophotometer. Transverse sections of liver organs were used to prepare Histopathiological slides. A significant ($p \leq 0.05$) gradual increase according to increased treatment doses in serum GGT and alkaline phosphatase were observed in treated animals, compared with control. Also there were different signs of liver histopathological changes including plate hyperaemia, hemorhage, haemosiderosis,diffuse coagulative necrosis ,cytoplasmic vacuolation and sinusoidal dilatation. Change in nuclei. Compared to control all tested animals showed significant ($p \leq 0.05$) inccreased body weight. All animals survived till the end of the experiment. We concluded that oral ingestion of combination of benzoic and citric acid causes liver malfunction, so it is preferred to ingest foods and beverages containing combination of benzoic and citric acid with caution. This study was aimed to investigate rats' liver malfunction induced by oral ingestion of combination of benzoic with citric acid in ratio (1:1/ v: v).

Keywords: *Alkaline Phosphatase; Benzoic Acid; Citric Acid; Food Additives; Gamma-Glutamyltransferase; and Liver Histopathological Changes.*

1. Introduction

Benzoates are food additives with the following international numbering system (INS), benzoic acid E 210, sodium benzoate E 211, potassium benzoate E 212 and calcium benzoate E 213. They are used as preservative food. Metabolically, in mammals, benzoic acid is primarily metabolized to its glycine conjugate, hippuric acid, which is readily excreted via the renal organic anion transport system. Citric acid can be added to ice cream as an emulsifying agent to keep fats from separating, to caramel to prevent sucrose crystallization, or in recipes in place of fresh lemon juice. Citric acid is used with sodium bicarbonate in a wide range of effervescent formulae, both for ingestion (e.g., powders and tablets) and for personal care (e.g., bath salts, bath bombs, and cleaning of grease). Citric acid is also often used in cleaning products and sodas or fizzy drinks. Citric acid sold in a dry powdered form is commonly sold in markets and groceries as "sour salt", due to its physical resemblance to table salt. It has use in culinary applications where an acid is needed for either its chemical properties or for its sour flavor, but a dry ingredient is needed and additional flavors are unwanted (Frank 2005). One reasonable explanation is that citric acid entering the organism can be absorbed by the detoxifying organs such as liver and act as an adjuvant to complex with metal ions contained in the detoxification enzymes inactivating them. Furthermore, detoxifying this kind of xenobiotics leads to the generation of free radicals such as hydrogen peroxide (H_2O_2) by means of oxidation/reduction reaction, exerting a damaging impact on body's tissues. Although many parameters have been used to indicate the toxic effects of citric acid or citrate on living organisms, there still remains limited information on its detailed effects in the livers. It was reported from clinical biochemistry examination of drink poisoning in children that enlarged baby's liver was related to the addition of citric acid into drinks. In addition, by monitoring the short-term effect of single dose citric acid on mouse tissue, (Aktaç et al. 2003) discovered that citric acid treatment caused injury of hepatocyte membranes, cytoplasmic vacuolization in hepatocyte, karyopyknosis, suggesting the toxic effects of citric acid on mice. Toxicity of citric acid was performed through biochemical analysis on citric acid-treated mice and the result showed a significant decrease in the activities of many antioxidative enzymes and a series of pathological changes such as disorganized hepatocyte cords, blood clot in central veins, lymphocyte and neutrophil infiltrating (Zhang et al. 2011). However, there are still very few studies for exploring whether citric acid or citrate could induce apoptotic cell death in mouse liver. Recently, it found that necrotic changes caused by this xenobiotic substance, such as vacuolated cytoplasm in

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hepatocytes, and chromatin decrease in mouse liver (Aktaç et al. 2003). All doses were orally administered in mg/ kg body weight. A previous study revealed some pathological changes in liver of mice esposed to citric acid, such as vacuolisation and glassy cytoplasm in the hepatocyte, nuclear membrane invaginations, picnotic nuclei. Similarly, with the effect of sodium benzoate in the rats and mice, high vacuolisation and glassy appearance in hepatocyte cytoplasm was explained (Fujitani 1993).

2. Material and methods

2.1. Experimental animals

Forty four Wistar rats of different sex weight ranged from (200 - 250 g), were divided into four groups eleven rats for each (6 males and 5 females). All animals were provided the basal diet composed of: (beef meat 10.6%, sesame oil 46.6%, corn flour 42.4%, and table salt 0.29%). All rats were put in quarantine for seven days. All animals were freely accessed to prepared diet and tab water. 3 groups orally received 100, 500 and 1250 of combination of benzoic and citric acids in ratio (1:1/ v/v). The proportion of each acid (benzoic and citric), for the doses of 100 (50 of each acid), 500 (250 of each acid) and 1250 (625 of each acid). According to {(Xiaoguang et al. 2014), doses of citric acid of (120 mg/kg b.w), middle dose (240 mg/kg b.w) and high dose groups (480 mg/kg b.w). ADI of (up to 5 mg/kg b.w) for benzoic acid. In short-term studies with rats, high doses (>1800 mg/kg b.w) of (benzoic acid/sodium benzoate) were adminstered over 5-10 days (Wibbertmann et al. 2000). NOAEL of (1200 mg/kg b.w) was determined for citric acid (Coleman 1997)}, Oral doses of combination of benzoic with citric acids were calculated to be administered by Wistar rats to assess liver function. In this study the low dose approximately equal to (Xiaoguang et al. 2014), but the middle and high doses were double as in to (Xiaoguang et al. 2014). Doses were administered once daily for twenty eight days. Blood samples were taken from rats' eyes using capillary tubes, labeled then centrifuged at 30000 rpm and serum was kept at 5° C. Blood sampling was done: initially, on the 14th and on the 28th day. By the 28th day all animals were autopsied and liver organs were taken, labeled then kept in 10% formal/saline solution. Slides were prepared according to method described by (Bancroft & Gamble 2002).

2.2 Measurement of serum GGT and ALPase concentrations

An Automatic machine Elecsys 2010 Germany full automatic device was calibrated for measuring serum alkaline phosphatase concentrations for animal groups according to the method described by (Tate & Meister 1985) and (Friedman & Young 1997) respectively.

3. Statistical analysis

All values were express as Means ± Sd. The SPSS one – way Anova test was used for the evaluation of differences between Wistar rats groups according to dose and sex. The differences were considered significant if a P. value was less than 0.05.

4. Results and discussions

In this study, oral administration doses of (100, 500 and 1250 mg/kg b.w) of combination of benzoic and citric acids resulted in a significant ($p \leq 0.05$) gradual increase in rats' serum GGT and ALP, compared to control, according to increased doses orally ingested by each sex (Tables 1, 2, 3 and 4). A significant ($p \leq 0.05$) gradual increased activity of rats' serum gamma-glutamyltransferase (GGT) (Tables 1 and 2), confirmed liver disease,

Table 1: Effects of Different Doses of Combination of Benzoic with Citric Acid on Male Rats' Serum GGT in IU/L

Groups Time interval	Group 1: Control	Group 2: 100 mg/kg b w	Group 3: dose 500 mg/kg b w	Group 4: dose 1250 mg/kg bw
Initial	18.83 ± 5.27 $^{N.S}$	27.00 ± 4.52 $^{N.S}$	31.67 ± 4.27 $^{N.S}$	41.67 ± 8.76 $^{N.S}$
14th Day	27.00 ± 4.52 ^{a}	153.83 ± 15.21 ^{b}	210.5 ± 16.81 ^{c}	251.50 ± 8.26 ^{d}
28th Day	31.33 ± 7.50 ^{a}	221.00 ± 20.54 ^{c}	277.83 ± 6.12 ^{d}	305.83 ± 12.21 ^{d}

Values are means ± SD. Means with rows not sharing common letter (s) are significantly different (P < 0.05). N.S = non- significant.

Table 2: Effects of Different Doses of Combination of Benzoic with Citric Acid on Female Rats' Serum GGT in IU/L

Groups Time intervals	Group 1: Control	Group 2: dose100 mg/kg b w	Group 3: dose 500 mg/kg b w	Group 4: dose 1250 mg/kg bw
Initial	33.83 ± 4.40 $^{N.S}$	37.67 ± 17.17 $^{N.S}$	41.67 ± 8.76 $^{N.S}$	35.67 ± 9.97 $^{N.S}$
14th Day	32.67 ± 5.16 ^{a}	181.00 ± 3.90 ^{b}	251.50 ± 8.26 ^{c}	291.67 ± 7.87 ^{d}
28th Day	33.50 ± 5.32 ^{a}	257.83 ± 4.55 ^{c}	277.83 ± 6.18 ^{c}	435.33 ± 18.95 ^{e}

Values are means ± SD. Means with rows not sharing common letter (s) are significantly different (P < 0.05). N.S = non- significant.

Table 3: Effects of Different Doses of Combination of Benzoic with Citric Acid on Male Rats' Serum Alp in Iu/L

Groups Time intervals	Group 1: Control	Group 2: dose100 mg/kg b w	Group 3: dose 500 mg/kg b w	Group 4: dose 1250 mg/kg bw
Initial	15.00 ±3.16 $^{N.S}$	16.80 ±3.03 $^{N.S}$	18.00 ± 3.54 $^{N.S}$	21.80 ± 7.89 $^{N.S}$
14th Day	23.00 ± 3.87 ^{a}	52.40 ± 5.60 ^{b}	131.40 ± 3.21 ^{c}	144.40 ± 2.07 ^{c}
28th Day	24.80 ± 4.21 ^{a}	89.80 ± 3.00 ^{b}	189.00 ± 15.91 ^{d}	199.40 ± 1.81 ^{d}

Values are means ± SD. Means with rows not sharing common letter (s) are significantly different (P < 0.05). N.S = non- significant.

Table 4: Effects of Different Doses of Combination of Benzoic with Citric Acid on Female Rats' Serum ALP in IU/L

Groups Time intervals	Group 1: Control	Group 2: dose100 mg/kg b w	Group 3: dose 500 mg/kg b w	Group 4: dose 1250 mg/kg bw
Initial	23.83 ± 4.40 $^{N.S}$	31.67 ± 4.27 $^{N.S}$	31.40 ± 3.05 $^{N.S}$	47.8.80 ± 7.12 $^{N.S}$
14th Day	38.00 ± 5.48 ^{a}	210.50 ± 16.81 ^{b}	279 ± 10.10 ^{c}	306.40 ± 7.57 ^{c}
28th Day	39.00 ± 7.65 ^{a}	305.83 ± 12.21 ^{c}	398.80 ± 9.63 ^{d}	511.20 ± 9.20 ^{f}

Values are means ± SD. Means with rows not sharing common letter (s) are significantly different (P < 0.05). N.S = non- significant.

As mentioned by (Tietz Textbook of Clinical Chemistry 1994). The source of elevated rats' serum ALP levels can be deduced by obtaining serum levels of gamma glutamyltransferase (GGT). Concomitant increases of ALP with GGT should raise the suspicion of hepatobiliary disease (Vroon. "Clinical Methods ") (Aach 1981). Increases in serum ALP also indicated liver disease in ex-

perimental rats (Berk & Korenblat 2007). Liver abnormalities confirmed by elevations of both rats' serum GGT and ALP. Changes in blood chemistry in this study are in agreement with (Coleman 1997). Also changes in serum parameters, and liver histopathological changes in this study, are in agreement with (Wibbertmann et al. 2000). Liver histopathological changes in this study, are in agreement with (Aktaç et al. 2003) and (Zhang et al. 2011) and (Fujitani 1993). Liver histopathological changes included: Plate B indicated that there is excessive hyperaemia, slight hemorrhage, haemosiderosis black to brown material inside kupflers, cells, diffuse cogitative necrosis, cytoplasmic vacuolation and simusoidal dilatation. Plate C showed that changes in nuclei, cytoplasmic vacuolation and no haemosiderosis.

Plate A

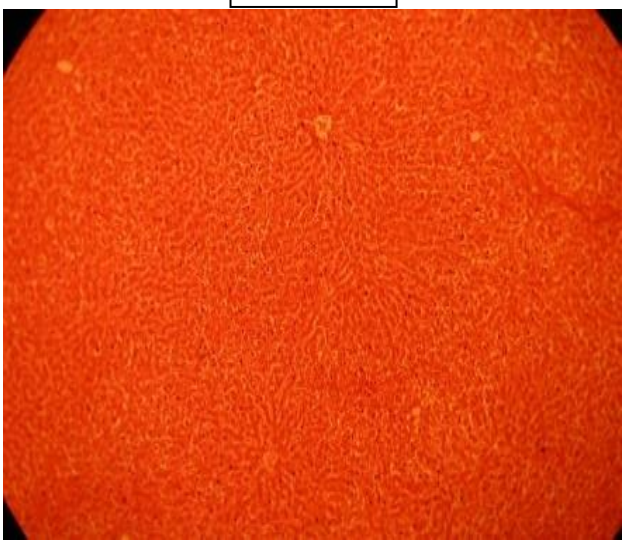
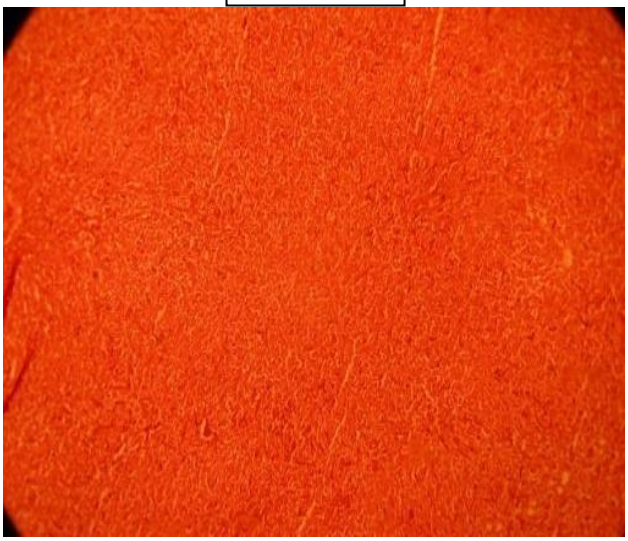


Plate B



(Eosin hematoxillin × 1000) (Eosin hematoxillin × 1000)

Plate D

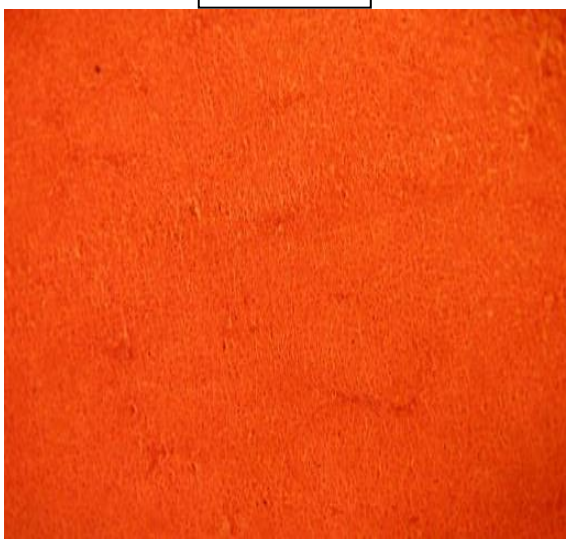
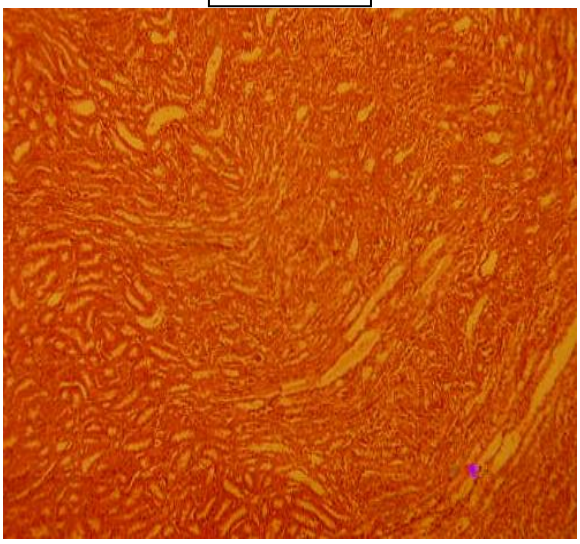


Plate C



(Eosin hematoxillin × 1000) (Eosin hematoxillin × 1000)

Plate D Showed excessive necrosis and hyperaemia. Gradual histopathological changes were observed through plates B, C and D, compared to plate A, the control. Plates B, C and D represented slides made from animals treated by (100, 500 and 1250 mg/kg b.w) of combination of benzoic with citric acid respectively. Benzoic acid and its salts (benzoate) may naturally be found in food, but according to their antimicrobial properties, they have long been used as food preservatives. Their use as food additives was limited by Joint FAO/WHO Expert Committee on Food Additives (JECFA). According to JECFA acceptable daily intakes (ADI) of (0 – 5 mg/kg b.w) for benzoic acid and benzoate have been established (World Health Organization 1997). Benzoic acid is relatively nontoxic. It is excreted as hippuric acid (Bindu 2001). Benzoic acid is metabolized by butyrate-CoA ligase into an intermediate product, benzoyl-CoA, which is then metabolized by glycine N-acyltransferase into hippuric acid. Benzoic acid is present as part of hippuric acid (N-benzoylglycine) in urine of mammals, especially herbivores. Humans produce about 0.44 g/L hippuric acid per day in their urine, and in case of toluene or benzoic acid exposure, it can rise above that level (Krebs et al. 1983). As a weak acid, exposure to pure citric acid can cause adverse effects: inhalation may cause cough, shortness of breath, or sore throat; ingestion may cause abdominal pain and sore throat; exposure to skin or

eyes may cause redness or pain. Long-term or repeated consumption may cause erosion of tooth enamel (Zheng et al. 2009). In short-term studies with rats, disorders of the central nervous system (benzoic acid/sodium benzoate) as well as histopathological changes in the brain (benzoic acid) were seen after feeding high doses (>1800 mg/kg b.w) over 5-10 days. Other effects included changes in serum parameters, or liver histopathological changes. The information concerning long-term oral exposure of experimental animals to benzoic acid is very limited, and there is no study available dealing specifically with possible carcinogenic effects. From a limited four-generation study, only a preliminary no-observed-(adverse-) effect level (NO (A) EL) of about 500 mg/kg b.w per day can be derived. With sodium benzoate, two long-term studies with rats and mice gave no indication of a carcinogenic effect. However, the documentation of effects is inadequate in most of these studies; therefore, no reliable NO (A) EL values can be derived. Data on its precursors support the notion that benzoic acid is unlikely to be carcinogenic (Wibbertmann et al. 2000). The average daily intake of citric acid from natural sources in the diet and food additiveswas estimated at about 40 mg/kg b.w for women, 130 mg/kg b.w for infants and 400 mg/kg b.w for individuals on slimming diets; maximum daily intake is reported to reach levels of 500 mg/kg b.w. No formal ADI (acceptable daily intake) level has been specified for citric acid and its common salts by the Joint FAO/WHO Expert Committee on Food Additives nor by the EC Scientific Committee for Food. Ingestion of a single dose of 25000 mg of citric acid by a woman (corresponding to approx. 417 mg/kg b.w) caused vomiting and nearly dying in one reported case. Volunteers given oral doses of potassium or magnesium citrate corresponding to approx. 4700 mg (corresponding to approx. 78.4 mg/kg b.w) of citric acid did not suffer any overt gastrointestinal effects. Based on wide spectrum of data relating to experimental animals and on human experience citric acid has a low acute toxicity; only one case of near fatal human intoxication was found. In a repeated dose study with rats a NOAEL of 1200 mg/kg b.w/day and a LOAEL of 2000 mg/kg b.w/day have been determined. The major subchronic and chronic toxic effects seem to be limited to changes in blood chemistry (Coleman 1997). Increased rats' weight is confirmed by (Heo et al. 2013) reporting that addition of citric acid or its salts can enhance the growth and the feed to gain ratio of weaned and growing-finishing pigs. {ADI of (up to 5 mg/kg b.w) for benzoic acid. (Wibbertmann et al. 2000) conducted short term toxicity studies in rats, using greater than (1800 mg/kg b.w) of benzoic acid. NOAEL of (1200 mg/kg b.w) for citric acid}, can justify the chosen doses administered by experimental rats in this study.

5. Conclusions and recommendations

According to the significant increase in rats' serum GGT and ALP, accompanied by the gradual observed histopathological changes in plates B, C and D, compared to control, it is almost concluded that combination of benzoic and citric acid results in rats' liver dysfunction. Since human daily - ingested doses of citric acid are almost below the NOAEL, I recommend that foods and beverages containing this chemical should be consumed with caution. As GGT and ALP are markers of cell damage with leakage of the cytoplasm, indicating cell damage, confirmed by the histopathological findings of necrosis, especially in group (1250 mg / kg b.w), I also recommend for further studies concerning more than a cellular damage analysis, thus increasing the robustness of the results.

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