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# Modulation of Nitrosamine-Induced Liver Injury in Rats by Propolis Extract: Long-term Study

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Author JAK designed the study, performed the statistical analysis, wrote the protocol and first draft of the manuscript. Authors JAK and TMA managed the analyses of the study. Author JAK managed the literature searches. Both authors read and approved the final manuscript.*

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## ABSTRACT

This study investigated the prophylactic effect of propolis extract against nitrosamine-induced liver injury in experimental animals. Eighty male adult rats were grouped into 8; control, treated with propolis extract, 3 groups treated with 0.1, 0.2, 0.5 mg nitrosamine/kg b.w and 3 groups treated with nitrosamine plus 2.5 mg/kg propolis extract for 12 weeks. Data obtained showed that, propolis extract exert prophylactic activity against nitrosamine-induced hepatotoxicity. Administration of nitrosamine lead to, liver function tests, oxidative stress markers and inflammatory markers were significantly increased. Propolis extract administration resulted in normalizing of the elevated liver functions. The propolis extract lowered the oxidative stress and the inflammatory markers compared with untreated group. In addition, histological investigation of livers revealed that propolis extract reduced lymph infiltration, hepatic congestion and inflammation in nitrosamine-injected rats. It was deduced that, the propolis extract exert hepatoprotective effect against liver damage induced by nitrosamine and it possess anti-inflammatory action.

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## 1. INTRODUCTION

Different food additives are common in our life and play an important role in the human being's life. Previous studies showed that people especially children always use food containing colourant and additives with great amounts which attracts their attention. The use of many synthetic compounds that used as food additives should be either omitted completely or highly restricted to the lowest levels as a result of their known side effects [1-5].

Nitrate and nitrite are common in nature. The presence of them is very important for the soil fertility. Nitrosamine is used as international food additive which is added to the original food or a mixture of foods for specific aims [6]. Nitrites are known to be toxic to different mammalian cells. Nitrites are known to induce carcinogenesis, nephrotoxicity hepatotoxicity, impairment of reproductive system, growth retardation, disturbance of the endocrine system, the disease known as methaemoglobinaemia and the impairment of defence mechanisms [7]. Many new synthetic food colouring agents are synthesized by the modern organic chemistry. These colouring agents have been added to the list of food additives and are widely used by the food industries [8].

A previous study in 1970 showed that the body can theoretically metabolize the inorganic nitrates to the known N-nitroso derivatives. These N- nitroso compounds are carcinogenic. Recently, there is an evidence that nitrates can be converted in the body to nitrites and nitric oxide. This metabolic conversion plays a useful function to protect against infraction, to improve the performance of the exercise, to attenuate the diseases of the vascular system and to protect the stomach [9,10].

Propolis is a viscous liquid obtained from bee products. Propolis extract constitute functional foods that meet requirements of many cases [6]. Its chemical composition is rich with vital elements and compounds. Mainly it include vitamins, amino acids, minerals, enzymes. glucose, fructose, 4-5% prebiotic [11]. Propolis extract was widely used in the folk medicine [12,13]. Cumulative data showed that propolis extract possess a considerable anti-inflammatory, anti-oxidative and anti-tumour activity.

The current study aimed to evaluate the adverse effects of nitrosamine on the liver functions and oxidants/antioxidant enzymes of the liver of male rats as well as the prophylactic effect of propolis extract to attenuate these adverse effects.

## 2. MATERIALS AND METHODS

### 2.1 Animals

Propolis extract was obtained from GMC shop, Jeddah, Saudi Arabia. Adult male albino rats weighed (130±5 g) were used in this study. The animals were obtained from KFMRC, KAU, Jeddah. Rats were given normal diet and water ad libitum. Rats were grouped into 8 groups as follows:

Group 1: control.

Group 2: Rats received 2.5 g/kg b.w of propolis extract in an aqueous solution orally, daily.

Group 3-5: Rats were given nitrosamine either 0.1, 0.2 and 0.5 mg/kg b.w orally for12 weeks [11].

Group 6-8: Rats were given nitrosamine either 0.1, 0.2 and 0.5 mg/kg b.w and 2.5 g/kg body weight of natural propolis extract in an aqueous solution for 12 weeks.

After 12 weeks, the experimental animals were sacrificed after being fasted for 12 hours. Blood samples were collected, centrifuged and the serum was used for the various biochemical analysis (AST, ALT, GGT, t. bilirubin, t. protein, and serum albumin). The liver was immediately removed, rinsed with ice saline. Part of the liver was used to prepare the liver homogenate in ice-cold physiological saline solution (normal saline 0.9% sodium chloride in de-ionized water) and kept at -80ºC for further biochemical analysis. The rest of the liver was used for performing the histopathological examinations.

The level of reduced glutathione, the activity of glutathione peroxidase, glutathione s transferase, superoxide dismutase, levels of malondialdehyde, nitric oxide, interleukine-1B and tumour necrosis factor were determined by kites obtained from bio diagnostic. The concentration of the protein in the homogenate was measured by using the commercially available kit.

## 2.2 Histological Investigation

Liver tissues were fixed in 10% formalin.Sections of paraffin-embedded tissue were 5 µm thick were obtained using microtome [14].

## 2.3 Statistical Analysis

The values were expressed as mean ± SD. SPSS version 16 for Windows was used. A value of $P<0.05$ was considered significance

## 3. RESULTS

Data obtained were analyzed by statistical analysis and showed that rats administrated with nitrosoamines exert a hepatotoxic effect by elevated liver enzymes activities significantly compared with normal rats ($p<0.001$). However, rats treated with Propolis protected the liver against sodium nitrite-induced hepatotoxicity and attenuated the elevation of liver functions compared with untreated ($p<0.001$). Administration of sodium nitrite caused elevation of liver enzymes (ALT, AST, GGT and LDH), decreased protein and albumin levels, increased free radical production (Malondialdehyde), inflammatory mediator (NO, TNF, IL-6) release was dose-dependently elevated. Propolis administration protected against this liver functions by inhabiting release of inflammatory mediators and enhance antioxidant activities (SOD, GSH- Px, catalase).Lipid peroxidation marker as MDA revealed that Propolis at different doses significantly inhibits the formation of MDA in sodium nitrite-treated rats. After 5, 10 and 20 mg/kg sodium nitrite administration, the liver MDA level significantly increased significantly as compared with control. However, the oral administration of Propolis lowered the level of MDA significantly compared with untreated.

It was found that sodium nitrite lowerd reduced glutathione (GSH) level significantly compared with control and it was dose depended. Administration of Propolis statistically attenuated these changes by elevating the level of GSH compared with untreated. It was found that, a reduction in the hepatic glutathione s-transferase activity in sodium nitrite injected as compared to the control group and the action is dose depended. While it was significantly increased in the rat groups treated with different doses of Propolis as compared with the sodium nitrite treated groups ($P<0.01$).

In rats injected with nitrosoamine, the activities of SOD and GSH-Px in liver were statistically decreased relative to control group. The reduction of these enzymes was related to the dose of nitrosamine. The treatment of rats with Propolis extract inhibited the decrease of these enzymes activity when compared to the nitrosamine group ($P<0.001$). It was found that sodium nitrite administration caused a significant reduction in the activity of CAT compared with normal rats. Orally administrated Propolis improved these reduced activities to normalize it compared with untreated rats.

## 4. DISCUSSION

Nitrosamines are known to be toxic to different mammalian cells. Nitrosamines are known to induce carcinogenesis, nephrotoxicity hepatotoxicity, impairment of reproductive system, growth retardation, disturbance of the endocrine system, the disease known as methaemoglobinaemia and the impairment of defence mechanisms [15]. Many new synthetic food colouring agents are synthesized by the modern organic chemistry. These colouring agents have been added to the list of food additives and are widely used by the food industries [16]. One of the preservative compounds is nitrate which is converted to nitrosamine in the body causing toxicity.

The data obtained showed that, administration of different doses of nitrosamines induced a significant elevation in the activities of liver enzymes AST, ALT, ALP, GGT and LDH, the level of total bilirubin and a significant decrease of levels of serum protein and albumin as compared to normal control. The elevation in liver enzymes is due to damage of hepatocyte and release of intracellular enzymes. The effect of nitrosamines is dose-dependent. However, Propolis with different doses significantly improved these hepatic changes and tend to reach normal values.

The obtained results showed a marked increase in serum ALP and γGT enzymes activity in rats receive nitrosoamine and dose-dependent. This may correlate with the damage of liver cell membranes and hence liver dysfunction which may be due to in part to the effect of nitric oxide (NO) free radical production induced by nitrites [17]. This is in agreement with the finding of [18] who found that nitrosoamine toxicity resulted in an increase in the release of these enzymes from the liver to circulation as a marker of liver

**Table 1. liver enzymes (ALT, AST, ALP, GGT), total proteins, albumin and total bilirubin in all studied groups(Mean ±SD)**

| Groups / Parameters | (Gpl) Control | (Gpll) Propolis | (Gplll) nitrosamine 0.1mg/kg | (GplV) nitrosamine 0.2mg/kg | (GpV) nitrosamine 0.5mg/kg | (Gpvi) nitrosamine 0.1mg/kg propolis | (Gpvii) nitrosamine 0.2mg/kg propolis | (Gpviii) nitrosamine 0.5mg/kg propolis |
|---|---|---|---|---|---|---|---|---|
| ASP-T(U/L) | 20±2.3 | 22±5.0 | 45±2.5 | 68±2.6 | 97±3[a,b,c] | 59±2.5 | 47±2.6 | 30±3 |
| ALA-T(U/L) | 18±2.1 | 21±1.1 | 41±2.2 | 56±2.5 | 70±4[a,b,c] | 60±2.2 | 48±2.5 | 21±3.3 |
| ALK-P (mg/dl) | 90±13.2 | 84±16.8 | 180±20 | 190±13 | 196±2.6[a,b,c] | 130±20.0 | 110±13 | 100±2.6 |
| GGT(mg/dl) | 40±8 | 37±10 | 88±12 | 96±13[a,b,c] | 105 ± 9.7[a,b,c] | 87 ±12.3[a,b,c] | 55±±13[a,b,c] | 49±7[a,b,c] |
| ALP (mg/dl) | 138±11.22 | 11±14.8 | 231±23 | 192±16[a,b,c] | 173±15[a,b,c] | 221±22[a,b,c] | 194±14 | 151±17[a,b,c] |
| T. protein (g/dl) | 8.9±0.88 | 8.1±0.6 | 6.3±0.54 | 5±0.51[a,b,c] | 5.4±0.4[a,b,c] | 6±0.5[a,b,c] | 6.1±0.7[a,b,c] | 7±0.5[a,b,c] |
| Albumin (g/dl) | 4.9±0.9 | 4.6±0.6 | 3.3±0.5 | 3.1±0.8[a,b,c] | 4.3±0.11[a,b,c] | 3.9±0.5[a,b,c] | 3.8±0.61[a,b,c] | 4±0.7[a,b,c] |
| Total bilirubin (mg/dl) | 1.0±0.01 | 1.1±0.02 | 1.5±0.13 | 2±0.19[a,b,c] | 1.71 ±0.41[a,b,c] | 1.7±0.21 | 1.0±0.18[a,b,c] | 1.0±0.05[a,b,c] |

*a,b,c are p value <0.05*

**Table 2. Malondialdehyde (MDA) , glutathione levels, and activities of antioxidants enzymes (Mean± SD)**

| Group / Parameter | (Gpl) Control | (Gpll) propolis | (Gplll) Nitrosoamine 0.1mg/kg | (GplV) Nitrosoamine 0.2mg/kg | (GpV) Nitrosoamine 0.5mg/kg | (Gpvi) Nitrosoamine 0.1mg/kg propolis | (Gpvii) Nitrosoamine 0.2mg/kg propolis | (Gpviii) Nitrosoamine 0.5mg/kg propolis |
|---|---|---|---|---|---|---|---|---|
| MDA( µmol/mg) Mean ±SD | 2.5±0.1 | 2.3±0.2 | 16±1.6 | 18±1.1[a,b,c] | 24.0±0.8[a,b,c] | 15.5±0.5[a,b,c] | 9.0 ± 0.66[a,b,c] | 4± 0.41[a,b,c] |
| GSH( µmol/mg) Mean± SD | 68±13.2 | 70±10.2 | 29±2.8 | 30±6.0[a,b,c] | 32± 3[a,b,c] | 45±8[a,b,c] | 55± 3[a,b,c] | 62±8[a,b,c] |
| GST( µmol/mg) Mean± SD | 19±1.3 | 22 ±2.5 | 14 ±3.5 | 15±1.0[a,b,c] | 14± 1.0[a,b,c] | 16±2.0[a,b,c] | 18± 1.0[a,b,c] | 21±2.0[a,b,c] |
| SOD( µmol/mg) Mean± SD | 27.8±2.6 | 20±1.4 | 14±1.2 | 11.97±1.5[a,b,c] | 9.2±1.3[a,b,c] | 10.25±1[a,b,c] | 16.2±1.3[a,b,c] | 15.5±1[a,b,c] |
| GSH-Px( U /mg protein) | 25.95±2.0 | 25.5±2.3 | 15±1.5 | 12±0.8[a,b,c] | 12±0.9[a,b,c] | 16±1.3[a,b,c] | 18±1.2[a,b,c] | 22±1.1[a,b,c] |

*a,b,c are p value <0.05*

**Table 3. Serum nitric oxide and liver IL6, TNF- α among studied groups (Mean±SD)**

| Group / Parameters | (Gpl) Control Normal | (Gpll) propolis | (Gplll) Nitrosoamine 0.1 mg/kg | (GplV) Nitrosoamine 0.2 mg/kg | (GpV) Nitrosoamine 0.5 mg/kg | (Gpvi) Nitrosoamine 0.1 mg/kg propolis | (Gpvii) Nitrosoamine 0.2 mg/kg propolis | (Gpviii) Nitrosoamine 0.5 mg/kg propolis |
|---|---|---|---|---|---|---|---|---|
| NO(µmol/mg) | **3.2 ±0.12** | **2.3±0.2** | **16±1.6[a]** | **18±1.1**[a,b] | **24.0±0.8[a,b,c]** | **15.5±0.5[a,b,c]** | **9.0±0.56[a,b,c]** | **4±0.43[a,b,c]** |
| IL-6 (µmol/mg) | **68±9** | **70±10** | **129±2.8** | **130±6[a,b,c]** | **142± 11[a,b,c]** | **45±8[a,b,c]** | **55± 3[a,b,c]** | **62±8[a,b,c]** |
| TNF- α (µmol/mg) | 19±1.3 | 22 ±2.5 | 44 ±3.5 | 45±1.0[a,b,c] | 56± 1.0 a,b,c | 36±2.0 a,b,c | 48±1.0 a,b,c | 29±2.0 a,b,c |

*a,b,c are p value <0.05*

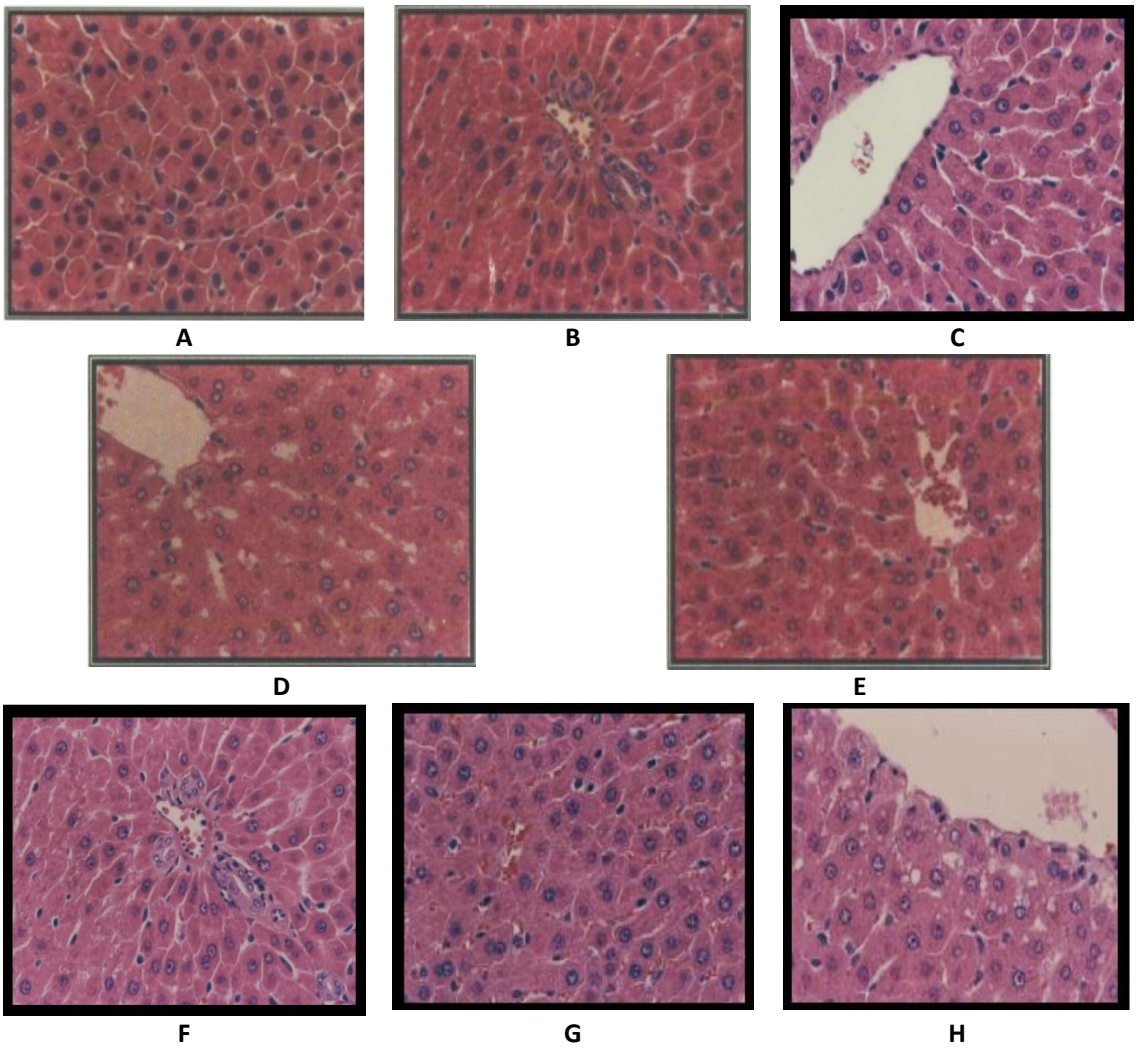


**Fig. 1. Sections of liver tissue with hematoxylin-eosin (X 200). A, control group; B, Propolis treated group; C-E, rats injected with 5, 10, and 20 mg nitrosamine/ kg; F-H, rat groups treated with Propolis for 12 weeks**

damage. Oral supplementation of Propolis induced significant amelioration in the observed abnormalities resulted from sodium nitrite as achieved by a marked improvement in the examined biochemical parameters indicating kidney functions.

These observations may be attributed to the antioxidant properties of honey which contain zinc and selenium [19,20], in addition to many forms of flavonoid compounds [21].

In the current study, it was found that nitrosamine caused a significant increase in inflammatory markers as IL-6, TN-F and NO compared with control group. Propolis attenuated the toxic effect of nitrosamine by reduction these mediators. A previous study [22,23], showed the similar results by inhibiting the bee honey toxic effect of nitrate, this explained by the active ingredients of flavonoids and prebiotic that suppress the release of the inflammatory mediators.

Nitrosamines injection in rats caused oxidative stress by elevation malondialdehyde and reduced antioxidant activities (GPx, SOD, CAT) and reduced glutathione compared with control. However, Propolis exert powerful antioxidants by enhancement antioxidant enzymes activates and prevention free radicals release. This is in accordance with the study of [24,25], who reported the propolis constitute super food due to

its high content of functional food and used as complementary medicine and prophylactic of many diseases.

## 5. CONCLUSION

In conclusion, propolis extract inhibits the production of free radicals and downregulate the inflammatory mediators in nitrosamine-induced liver injuries. The propolis extract is exerted protection against oxidative damage and enhancement of antioxidant potential. It is recommended that, utilization of propolis as complementary and prophylactic supplement against toxicity of nitroso compounds.

## CONSENT

It is not applicable

## ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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