Original Article

In vivo Studies on the Protective Effect of Propolis on Doxorubicin-Induced Toxicity in Liver of Male Rats

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ABSTRACT

Objective: Since anticancer drugs are to be administered for long durations of time and are associated with systemic toxicities, the present studies were conducted to evaluate the protective potential of honey bee propolis against a widely used anticancer drug, doxorubicin (DXR) induced toxicity and oxidative damage in liver tissues of rats. **Materials and Methods:** Sixteen male Sprague Dawley rats, weighing between 200-220 g, were used and were divided into four equal groups. Propolis was given orally to rats [250 mg/kg body weight (bw) for 14 consecutive days] and DXR [25 mg/kg bw; intraperitoneally (i.p) was administered on 12th, 13th and 14th day of the experiment. All the animals were sacrificed on day 15th day by decapitation. Blood and tissue samples were collected for measurement of toxicity and oxidative damage parameters (enzymatic assays and biochemical estimations). **Results:** Administration of DXR for 3 days at a cumulative dose of 25 mg/kg bw, induced toxicity and oxidative stress in rats as significantly decreased activity of catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione peroxidase (GSH-Px) and glutathione reductase (GR) were observed in rat liver supernatants when compared to control group. Increased activity of serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) was obtained in DXR administered rats. Also there are significantly increased levels of lipid peroxides (measured as malondialdehyde formation) and significantly decreased level of glutathione (GSH) in doxorubicin treated rat liver supernatants as compared to healthy controls. On the other hand, administration of animals with propolis prior to DXR treatment led to significant modulation of the oxidative damage related parameters in liver and hepatotoxicity parameters in blood, when compared to doxorubicin treated group. However results were still not comparable to control group or only propolis group indicating partial protection by propolis at the concentration used against anticancer drug toxicity. **Conclusion:** Propolis extract was found to have a protective effect against doxorubicin-induced toxicity in rat liver though it was still not normalized. It can be concluded that propolis provides partial protection from toxicity of anticancer drug.

Key words: Antioxidant, doxorubicin, liver toxicity, oxidative stress, propolis

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INTRODUCTION

Adriamycin (ADR) or Doxorubicin (DXR) belonging to anthracycline class of antibiotics is widely used in the treatment of various cancers.[1] Its cumulative dose-dependent cardiac toxicity has been a major concern for oncologists in cancer therapeutic practice for decades.

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It also exhibits generalized toxicity that limits the extent to which this life saving agent can safely be used.[2] DXR is reported to cause cardiotoxicity,[3] nephrotoxicity[4] and hepatotoxicity.[5] This toxicity seems to be due to the generation of reactive oxygen species (ROS) including hydroxyl radicals and superoxide anions thus leading to lipid peroxidation and tissue damage.[6] Proposed mechanism of DXR induced oxidative stress is formation of an anthracycline-iron (Fe2+) free radical complex.[7] ROS are chemically reactive molecules containing oxygen. During times of stress ROS levels can increase. This may result in significant damage to cell structures, known as oxidative stress. Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage.[8] The imbalance can result from a lack of antioxidant capacity caused by disturbance in production, distribution, or by an over-abundance of ROS from environmental toxicants. ROS can impair lipids, proteins, carbohydrates and nucleotides, which are important parts of cellular constituents, including membranes, enzymes and DNA.

Natural components, such as honey bee propolis and related polyphenolic compounds including flavonoid aglycones, phenolic acid and their esters, phenolic aldehydes, ketones, terpenes, sterols, vitamins and amino acids[9,10] have recently been reported to have some antioxidant properties.[11,12] DXR induced toxicity,[13] free radicals and other toxic intermediates generated by this anthracycline can be reduced by nutritional supplements and exogenous antioxidants.

Therefore the present work was proposed to systematically evaluate the protective role of propolis on the toxicity and oxidative stress related enzymes and molecules in doxorubicin treated Sprague Dawley (S.D.) rats.

MATERIALS AND METHODS

Chemicals

DXR was obtained as doxorubicin hydrochloride (50mg/vial) from Pfizer Pharma. All other chemicals used in this study were of analytical grade. 2- thiobarbituric acid (TBA) and enzyme glutathione reductase (GR) was procured from Sigma-Aldrich Chemical Company (St. Lous, MO, USA). NADPH, CDNB, glutathione oxidized, glutathione reduced, hydroxylamine HCl, Triton X-100, EDTA, NBT and DTNB were purchased from SRL Chemicals (India). serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT) and Bilirubin assay kits were procured from Reckon Diagnostics private Ltd. India.

Animals

Male S.D. rats weighing between 200-220 g were procured from the Central Animal House of Panjab University, Chandigarh and were used in the present study. Animals were housed in polypropylene cages in a 12 h light/dark schedule at a temperature of 22 ± 3ºC under hygienic conditions in the department animal house before experimental use. Animals were fed *ad libtium* with standard laboratory pellet chow diet (Ashirwaad Industries, Tirpari, Punjab) and had free access to water throughout the period of experimentation. Animals were weighed before the experiment. All experiments and protocols reported here strictly followed the principals as laid down by the Institutional Animal Ethical Committee (IAEC), Panjab University, Chandigarh and met the standards laid down by Govt. of India.

Extract preparation

Propolis was collected from an apiary at village Tierra near Chandigarh. Ethanolic extract of propolis was prepared by following the method of Mani *et al*.[14] Propolis collected from bee hive should be clean and free of wax, paint, wood etc., It was ground into fine pieces. Placed the proper amount of propolis and alcohol (30 g propolis and 70% ethanol to make 100 ml volume) in the container and sealed the top, did moderate shaking and kept for 2 weeks at room temperature. The propolis extract was filtered twice, dried and stored in sealed bottles at 4°C until use. The dried form was dissolved in water just before oral administration according to required dosage.

Experimental design

Dose: The cumulative dose of doxorubicin used in present studies is 25 mg/kg body weight (bw). The chosen dose of DXR in normal saline was given in three equal intra peritoneal injections to rats. Final dried form of propolis was adjusted to 250 mg/kg bw in distilled water and given orally for 14 days during the 15 days experimental period. Following groups were formed.

Control group (C): Animals in this group were given distilled water orally by intragastric gavage for 14 days.

Doxorubicin treated group (T1): Animals were given three equal intra peritoneal injections (cumulative dose 25 mg/kg bw) on day 12th, 13th and 14th during the experiment.

Propolis-doxorubicin group (T2): Propolis exract was given to animals for 14 days at a dose of 250 mg/kg bw/day and on 12th, 13th and 14th day DXR was administered as per above mentioned dosage of T1 group.

Propolis only group (T3): These animals were given propolis extract (PE) at a dose of 250 mg/kg bw orally by intragastric gavage for 14 days.

All the animals were sacrificed on day 15th by decapitation. Blood sample was collected from jugular vein of each animal in a centrifuge tube and left to clot at room temperature for 45 mins. Serum was separated by centrifugation at 3000 r.p.m. at 30°C for 15 min. Serum biochemical assays and enzyme activities were measured on the same day. Liver samples were rapidly excised after dissection, washed with ice-cold saline and 10% liver homogenates were prepared in ice cold phosphate buffer saline (PBS, pH 7.4) using mechanically driven Teflon fitted Potter- Elvehjem homogenizer for 2 mins at 3000 r.p.m in ice till total disruption of cells. Post mitochondrial supernatant was prepared by centrifugation at 10,000 g for 20 min at 4°C.

Biochemical analysis

Catalase (CAT) activity was determined according to method of Luck[15] based on determination of the H_2O_2 decomposition rate at 240 nm and values were expressed as U/mg protein. Superoxide dismutase (SOD) activity was determined by following the method of Kono[16] at 560 nm. Values were expressed as U/mg protein. Lipid peroxidation product was determined by measuring malondialdehyde (MDA) content in tissue homogenates according to the method of Begue and Aust[17] spectrophotometrically at 532 nm. Values were expressed as nM/mg protein. The level of reduced glutathione (GSH) was measured as protein-free sulfhydryl content by the method of Sedlak and Lindsay[18] at 412 nm and values were expressed as µM/mg protein. Acitivity of glutathione-S-transferase (GST) was determined using spectrophotometer by the method of Habig *et al*.,[19] at 340 nm and expressed as µM of GSH-CDNB conjugate formed/min/mg protein. Glutathione peroxidase (GSH-Px) was assessed according to the method of Pagila and Valentine[20] at 340 nm and expressed as U/mg protein. GR activity was determined by the method of Carlberg and Mannervick.[21] Activity of Serum Glutamate Pyruvate Transaminases (GPT/ALT) and Serum Glutamate Oxaloacetate Transaminases (GOT/AST) was determined spectrophotometrically at 340 nm and bilirubin level was measured at 578 nm using kits from Reckon Diagnostics P. ltd. India.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). The statistical significance of data was evaluated by one way analysis of variance (ANOVA), using SPSS software version 9.0 for windows. The data was analyzed by post-hoc analysis with Least Square Difference. A value of $P < 0.05$ was considered to indicate a significant difference between groups.

RESULTS

Oxidative stress related parameters

Generation of ROS upon administration of doxorubicin leads a series of derangements measured in terms of related enzymes and molecules in rat liver post mitochondrial supernatants as shown in Table 1. No significant difference was found in control and propolis alone group for all the parameters studied except for GST activity, which was found to be higher in propolis alone group. Decreased activity of catalase was found in DXR administered rat liver supernatants as compared to control animals, indicating depletion/inhibition of the most important antioxidant enzyme. Administration of animals with propolis prior to DXR treatment was found to increase ($P < 0.01$) the CAT activity highly significantly as compared to the corresponding DXR treated T1 group ($P < 0.001$) but was significantly less than control group and propolis alone group [Table 1]. The Activity of superoxide dismutase significantly decreased (1.8 ± 0.65 ^{a1} IU/mg protein) in DXR treated rats, indicating that the endogenous antioxidant system

Table 1: Levels of various antioxidant molecules and enzymes in differently treated animal groups as explained in methodology. Effect of DXR (T1), combination of DXR plus propolis (T2) and propolis alone (T3) on activity of Catalase, glutathione-S-tranferase, glutathione peroxidase, glutathione reductase, superoxide dismutase and level of glutathione and malondiadehyde in liver tissue of male SD rats compared to control (C) group. Values are expressed as the mean±SD (*n*=5)

Parameters	Control group (C)	Doxorubicin treated group (T1)	Doxorubicin+Propolis treated group (T2)	Propolis alone group (T3)
Catalase (µmoles of H_2O_2 decomposed/min/mg protein)	0.140±0.015	0.064±0.010^{a1}	0.086±0.013^{a1b2}	0.128±0.011^{b1c1}
Superoxide dismutase (IU/mg protein)	6.07±0.84	1.8±0.65^{a1}	3.5±0.60^{a1b3}	6.2±0.71^{b1c1}
Lipid peroxidaton (nmol of malondiadehyde/mg protein)	6.95±0.93	14.22±1.06^{a1}	11.72±0.74^{a1b1}	6.42±0.63^{b1c1}
Reduced Glutathione (µmol/mg protein)	4.075±0.221	1.1±0.294^{a1}	2.45±0.31^{a1b1}	3.7±0.163^{a3b1c1}
Glutathione-S-transferase (µmoles of CDNB conjugates/min/mg protein)	0.882±0.022	0.538±0.023^{a1}	0.688±0.021^{a1b1}	0.916±0.023^{a2b1c1}
Glutathione peroxidase (µmol of NADPH oxidized/min/mg protein)	0.447±0.021	0.279±0.021^{a1}	0.335±0.017^{a1b1}	0.404±0.016^{b1c1}
Glutathione reductase (µmol of NADPH oxidized/min/mg protein)	0.378±0.024	0.154±0.020^{a1}	0.234±0.026^{a1b1}	0.367±0.025^{b1c1}

SD = Standard deviaton, DXR = Drug, doxorubicin, CDNB= 1 chloro 2,4 dinitrobenzene, NADPH= Nicotnamide adenine dinucleotde phosphate, $^{a1}P < 0.001$; $^{a2}P < 0.01$; $^{a3}P < 0.05$: Statstcally signifcant difference from control group $^{b1}P < 0.001$; $^{b2}P < 0.01$; $^{b3}P < 0.05$: Statstcally signifcant difference from doxorubicin group $^{c1}P < 0.001$; $^{c2}P < 0.01$; $^{c3}P < 0.05$: Statstcally signifcant difference from doxorubicin plus propolis tretead group

was damaged by DXR. In DXR and propolis treated rats, values were higher (3.5 ± 0.60 ^{a1b3} IU/mg protein) as compared to T1 but were significantly less than control values of (6.07 ± 0.84 IU/mg protein). The level of lipid peroxides was assessed by estimation of formation of MDA, an indicator of lipid peroxidation. Animals treated with DXR (T1) showed a significant increase ($P < 0.001$) in the level of MDA concentration compared to control group (14.22 ± 1.06 ^{a1} nM/mg and 6.95 ± 0.93 nM/mg respectively). Prior administration with propolis extract (T2) significantly eliminated the effect of DXR on hepatic lipid peroxidation (11.72 ± 0.74 ^{a1b1} nM/mg), though still much higher than their respective controls. Similarly, treatment of rats with DXR alone caused a significant decrease ($P < 0.001$) in liver reduced glutathione level when compared with control group. GSH status improved on propolis co-treatment ($P < 0.01$).

GST consists of a large family of GSH utilizing enzymes that play an important role in detoxification of xenobiotics in mammalian systems. DXR was found to inhibit GST activity significantly in liver ($P < 0.001$). However, propolis co-treatment significantly improved enzyme activity in male rats (0.688 ± 0.021 ^{a1b1} μM/min/mg protein) as compared to T1 (0.538 ± 0.023 ^{a1} μM/min/mg protein). GSH-Px and GR activities were significantly elevated in propolis treated groups (T2 and T3; $P < 0.01$) in comparison to DXR treated group that produced a significant decrease in the activities of GSH-Px and GR ($P < 0.001$).

Liver toxicity enzymes in serum

Estimations done in serum to check the extent of hepatic toxicity and tissue damage revealed that activity of GPT, GOT and level of bilirubin was significantly higher ($P < 0.001$) in DXR treated group as compared to control animals indicating liver dysfunction [Figure 1]. In DXR and propolis treated rats, activities of GPT, GOT and levels of bilirubin were significantly lower as compared to T1 but were still significantly higher ($P < 0.01$) than control and propolis alone group.

DISCUSSION

H_2O_2 is decomposed into water and oxygen molecules by the enzyme catalase present in cytoplasm, thus protecting the cells from hydroperoxide mediated damage. Recently, much attention has been focused on the protective role of polyphenols and their mechanism of action. Propolis has arisen as a promising agent with the recognition of its antioxidant effects. DXR induced cytotoxicity and oxidative stress relationship has been reported previously. Mechanism of drug toxicity is through production of free radical and their capability to induce apoptosis by production of ROS, DNA intercalation, lipid peroxidation, cell membrane damage.[22,23] In the present study, statistically significant differences were observed in the oxidative stress markers in liver tissues and in the serum biochemical parameters of male S.D rats treated with DXR as compared to control group rats, also statistically

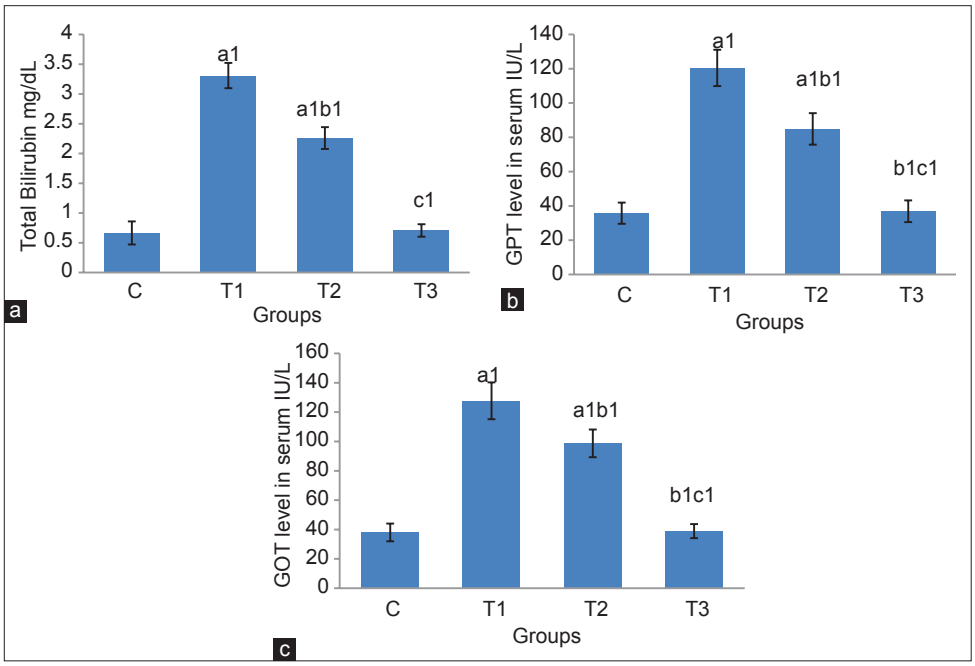

Figure 1: Effect of DXR (T1), combination of DXR plus propolis (T2) and propolis alone (T3) on level of (a) bilirubin and activity of (b) GPT and (c) GOT in serum of male SD rats compared to control (C) group. Values are expressed as the mean +/- SD ($n = 5$). a1 $P < 0.001$; a2 $P < 0.01$; a3 $P < 0.05$: Statistically significant difference from control group b1 $P < 0.001$; b2 $P < 0.01$; b3 $P < 0.05$: Statistically significant difference from doxorubicin group $P < 0.001$; c2 $P < 0.01$; c3 $P < 0.05$: Statistically significant difference from doxorubicin plus propolis tretead group c1

significant amelioration of toxicity was found in DXR plus propolis treated group as compared to DXR alone. The results suggested that when administered at the indicated dose and for the given time period to rats, bee propolis caused protective influence on the parameters investigated. Propolis has been found to have some protective effect against cypermethrin induced toxicity.[24] A recent study showed that the endogenous antioxidant system of the whole body was damaged by ADR.[25] In our results it is clearly reflected that ingestion of propolis in animals has antioxidant and protective effect when given prior to DXR. In healthy animals and propolis alone group, no significant difference could be observed.

It is thus concluded that propolis has strong potential to provide protection against DXR-induced toxicity in rats as evidenced by improved serum biochemical parameters and restoration of the antioxidant/oxidative status.

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