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Influence of α-tocopherol, propolis and piperine on therapeutic potential of tiferron against beryllium induced toxic manifestations

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ABSTRACT: The therapeutic potential of the chelator tiferron (sodium-4,5-dihydroxy-1,3-benzene disulphonate; 300 mg kg^{-1}, i.p.) and adjuvants, i.e. α-tocopherol (25 mg kg^{-1}, p.o.), propolis (a honey-bee hive product; 200 mg kg^{-1}, p.o.) and piperine (10 mg kg^{-1}, p.o.) were evaluated individually and in combination against beryllium induced biochemical alterations and oxidative stress consequences. Female albino rats were exposed to beryllium nitrate (1 mg kg^{-1}, i.p.) daily for 28 days followed by treatment with the above mentioned therapeutic agents for 5 consecutive days. Administration of beryllium altered blood biochemical variables with significant depletion in hemoglobin, blood sugar, total serum protein, albumin and significant enhancement in the release of serum transaminases. A significantly increased lipid peroxidation and a decreased level of glutathione after beryllium exposure indicated oxidative stress in the liver and kidney. Beryllium exposure decreased total protein and glycogen contents, whereas triglycerides and cholesterol increased significantly in liver and kidney. Individual administration of all the four compounds showed significant therapeutic potential in reverse of some of the biochemical parameters mentioned above. Furthermore, the combination of tiferron with α-tocopherol, propolis or piperine, respectively, could reverse all the variables significantly more towards the control. None of the test compounds showed any significant change in choleretic activity (bile flow and bile solids), indicating that these compounds had no adverse effects at these dose levels. It was concluded that all the combinations of tiferron and adjuvants played a beneficial role in reducing beryllium induced systemic toxicity at relatively lower doses and the combination of tiferron and propolis showed a more pronounced therapeutic potential. Copyright © 2007 John Wiley & Sons, Ltd.

KEY WORDS: beryllium toxicity; biochemical alterations; tiferron; α-tocopherol; propolis; piperine; combined therapy

Introduction

Metal contamination and its health hazards are very common in the age of competitive industrialized and urbanized civilizations. Beryllium is one of the metals having a greyish silver color, lightest weight, bivalent, lower density and harder form (Stonehouse and Zenczak, 1991) with numerous applications in medical, aerospace, defense, electronics, nuclear and ceramic industries (Kolanz 2001; Weston *et al*., 2005). A distribution study in animals exposed to beryllium via different routes indicated that liver, kidney, skeleton, lymph nodes and lungs are the target organs for beryllium toxicity (Cikrt and Bencko, 1975; Bugryshev *et al*., 1976; Stiefel *et al*., 1980; Finch *et al*., 1990; Sakaguchi *et al*., 1993; ATSDR, 2002). Occupational exposure to beryllium may lead to symptoms of berylliosis, dermatitis, granulomatosis, pneumonia, cardiac failure, hepatomegaly, splenomegaly, cyanasis and finger clubbing (WHO, 1990). Chronic beryllium disease (CBD) is an occupationally acquired lung disease, which resembles sarcoidosis and tuberculosis (Finch *et al*., 1996) and occasionally involves other organs including the liver, spleen, lymph nodes and bone marrow. The continued prevalence of beryllium sensitization and CBD may also be due to unchecked skin exposure to beryllium containing particles (Day *et al*., 2006). Extensive work has been conducted on the detection and treatment of beryllium toxicity using immunological aspects, but less attention has been paid to chelation therapy accompanied by the simultaneous recovery of the altered biochemical variables. Several synthetic chelators, i.e. ATA (Schubert and Rosenthal, 1959), EDPPA (Arkhipova *et al*., 1964), DMPS (Mathur *et al*., 1994a), DMSA (Flora *et al*., 1995), DPA (Johri *et al*., 2004), DTPA (Mathur *et al*., 1993; 2004), EDTA (Cash *et al*., 1959), HEDTA (Mathur *et al*., 1993; Nirala *et al*., 2004)

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and tiron (Sharma *et al*., 2002; Mathur *et al*., 2004) have been shown to reduce beryllium toxicity but have achieved limited success. Some chelators have a lower half-life, thus are not bioavailable for binding toxic metal ions. In addition, administration at higher doses may impose certain adverse effects (Mathur *et al*., 1993; Ceci *et al*., 2002; Franchini and Veneri, 2004). Tiferron (sodium-4,5-dihydroxy-1,3-benzene disulphonate), a phenolic compound is commonly known as tiron. Being a hydrophilic chelator, it has been used for the treatment of a number of experimental metal poisoning including manganese (Sanchez *et al*., 1995), uranium (Bosque *et al*., 1993), vanadium (Domingo, 1995) and beryllium (Sharma *et al*., 2002). Tiferron is a superoxide dismutase (SOD) mimetic and a cell membrane permeable scavenger of superoxide anion (Krishna *et al*., 1992). Adverse effects of tiferron, however, at relatively higher doses have also been reported (Ortega *et al*., 1991; Ghosh *et al*., 2002).

However, α-tocopherol provides protection against the adverse side effects of tiferron administered at 471 mg kg^{-1} (Mathur *et al*., 2004) but the use of smaller doses of chelator should be preferred to avoid any unwanted effect. Any pharmacological intervention that enhances the bioavailability of smaller amounts of drugs and produces a sustained level in the body for a longer period should be advantageous. Thus, the use of chelators at relatively lower doses in combination with certain adjuvants may prove a better therapeutic approach against metal toxicity (Nirala *et al*., 2004). An adjuvant is simply a compound administered with a compound of defined therapeutic activity to enhance its effectiveness. Some natural compounds, especially phenolics, possess chelating as well as free radical scavenging capacity, which may be used as adjuvants for modulation of beryllium induced toxicity. α-Tocopherol is a major lipid soluble chain breaking antioxidant in nature, which can protect biological membranes and lipoproteins from metal induced oxidative damage (Abdel-Fattah *et al*., 1998; Fernandez-Cabezudo *et al*., 2003). Piperine (1-piperoyl piperidine) is a major alkaloid of black pepper (*Piper nigrum* Linn.) and long pepper (*Piper longum* Linn.). This compound is known for several pharmacological activities such as antifungal (Madhyastha and Bhat, 1984), anticancerous (Selvendiran *et al*., 2003), hepatoprotective and antioxidative effects (Koul and Kapil, 1993) as well as to enhance the bioavailability of various structurally and therapeutically diverse drugs (Khajuria *et al*., 1998). Propolis is a honey-bee hive product consisting of more than 300 compounds of different groups (Banskota *et al*., 2001; Simoes *et al*., 2004). It has been used as a herbal medicine due to its antioxidative (Isla *et al*., 2001), anticancer or cytotoxic (Banskota *et al*., 1998) and hepatoprotective activity (Shukla *et al*., 2005). The use of α-tocopherol as a drug delivery vehicle is well established (Constantinides *et al*., 2006) whereas adjuvant effects of propolis (Gebaraa *et al*., 2003; Khayyal *et al*., 2003; Chu, 2006) and piperine (Bano *et al*., 1991; Badmaev *et al*., 2000) have also been reported.

In the present investigation, the chelator tiferron and naturally occurring products, i.e. α-tocopherol, propolis and piperine as adjuvants were evaluated at relatively lower doses individually and in combination to validate them as a potent antidote against beryllium induced toxic manifestations.

Materials and Methods

Chemicals

Beryllium nitrate [$Be(NO_3)_2$] was purchased from Fluka (Switzerland); tiferron and α-tocopherol acetate were obtained from Himedia Laboratories Ltd, Mumbai, India and piperine was procured from Sigma Aldrich Company. Crude propolis was gifted by Professor O.P. Agrawal, Senior Entomologist of School of Studies in Zoology, Jiwaji University, Gwalior, India. All the therapeutic agents were stored refrigerated in a desiccator to avoid oxidation and thermal decomposition. All other chemicals used in the study were of pure and analytical grade and procured from standard chemical dealers.

Maintenance of Animals and their Feeding

Adult female albino rats of Sprague-Dawley strain (8–10 weeks old having 130 ± 10 g body weight for biochemical assays and 10–12 weeks old having 200 ± 10 g body weight for choleretic activity of test compounds) were randomly selected from the departmental animal facility where they were housed in polypropylene cages (3 animals per cage) under uniform husbandry conditions of light (14 h) and dark (10 h) at 25 ± 2 °C temperature with relative humidity of 60–70%. After being obtained from the animal facility, rats were acclimatized to laboratory conditions for one week and fed with standard commercially available pellets of animal diet (Pranav Agro Industries Ltd, New Delhi, India) and drinking water *ad libitum*. Experiments were conducted in accordance with the guidelines set by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Chennai, India and experimental protocols were approved by Institutional Ethical Committee (CPCSEA/501/01/A) of Jiwaji University, Gwalior, India.

Preparation of Propolis Extract

Propolis extract was obtained as described by Shukla *et al*. (2005) with some modifications. Crude propolis (10 g) was suspended in 100 ml of 90% ethanol overnight

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with constant stirring and the suspension was decanted at room temperature. This process was repeated twice and finally the suspension was filtered. The filtrate was concentrated under reduced pressure to yield 65.38% (w/w) of propolis extract and kept at 4 °C for further use.

Preparation of Doses and Treatments

Beryllium nitrate was dissolved in triple distilled water making up doses of 1 mg 2 ml^{-1} kg^{-1} and administered intraperitoneally. The dose of tiferron (300 mg 2 ml^{-1} kg^{-1}) was prepared in 0.9% saline and administered intraperitoneally. α-Tocopherol was dissolved in olive oil making doses of 25 mg 5 ml^{-1} kg^{-1}, whereas aqueous suspension of piperine (10 mg 5 ml^{-1} kg^{-1}) and propolis (200 mg 5 ml^{-1} kg^{-1}) were prepared in gum acacia and administered orally with the help of an intragastric rubber catheter. The selection of doses of toxicant, therapeutic agents and duration of treatments were based on earlier works (Dalvi and Dalvi, 1991; Sharma *et al*., 2002; Johri *et al*., 2004; Shukla *et al*., 2005) as well as our previous studies (Mathur *et al*., 2004; Nirala *et al*., 2004). Fifty four adult female rats were divided into nine groups of six each as follows:

Group 1: received sodium nitrate (1 mg kg^{-1}, i.p.) once a day daily for 28 days followed by saline (2 ml kg^{-1}, i.p.) for 5 days and served as the normal control. Group 2: received beryllium nitrate once a day daily for 28 days followed by saline for 5 days and served as the experimental control. Group 3: received toxicant as in group 2 and was treated with tiferron for 5 consecutive days after toxicant administration. Group 4: received toxicant as in group 2 and was treated with α-tocopherol for 5 consecutive days after toxicant administration. Group 5: received toxicant as in group 2 and was treated with propolis for 5 consecutive days after toxicant administration. Group 6: received toxicant as in group 2 and was treated with piperine for 5 consecutive days after toxicant administration. Group 7: received toxicant as in group 2 and concomitantly was treated with tiferron and α-tocopherol for 5 consecutive days after toxicant administration. Group 8: received toxicant as in group 2 and concomitantly was treated with tiferron and propolis for 5 consecutive days after toxicant administration. Group 9: received toxicant as in group 2 and concomitantly was treated with tiferron and piperine for 5 consecutive days after toxicant administration.

Twenty four hours after the final administration, the animals were killed under mild ether anesthesia, withdrawing blood by puncturing the retro-orbital venous sinus to isolate serum. The liver and kidney were immediately excised, blotted free of adhering fluid and processed for biochemical studies. Standard techniques were applied to assay various blood and tissue biochemical parameters.

Biochemical Estimations

Hemoglobin

Sahli's acid hematin method (Swarup *et al*., 1992) was adopted to estimate hemoglobin. Blood was sucked into a hemoglobin pipette up to the 0.5 mark and was poured into a graduated tube containing N/10 HCl up to 2 marks and mixed well with a glass rod. After 10 min, distilled water was added drop by drop with simultaneous shaking and produced a color that was matched with the standard colored tube, which directly showed the hemoglobin concentration in the blood.

Blood Sugar

Blood sugar was measured according to the method of Asatoor and King (1954). 0.1 ml of blood was taken in 3.8 ml of isotonic solution and 0.1 ml of sodium tungstate (10%) was added immediately followed by centrifugation at 450 *g* for 10 min. Supernatant and alkaline tartarate solutions of 1 ml each were added and incubated in boiling water bath for 10 min. After cooling, 3 ml each of phosphomolybdic acid and distilled water were added, mixed well and the absorbance was recorded at λ 680 nm against the blank.

Serum Transaminases

Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum were estimated by the method of Reitman and Frankel (1957). 0.5 ml of AST/ALT substrates were incubated for 5 min, followed by adding 0.1 ml of serum (for experimental tubes) and further incubated for 60 and 30 min for AST and ALT, respectively, at 37 °C. 0.1 ml of serum was added to the control tubes immediately after incubation. Standard and blank tubes contained 0.1 ml of working standard and distilled water, respectively. 2,4-Dinitrophenylhydrazine (DNPH) was allowed to react at room temperature in all the tubes for 20 min. 5 ml of NaOH (0.4N) was added, followed by incubation for 10 min at room temperature and the absorbance was measured at λ 510 nm against the blank.

Serum Albumin and Total Protein Contents

Serum albumin was measured by kit method using a Merck auto-analyser (Micro Lab 200) according to the directions given in the manual of the diagnostic kit (E-Merck India Ltd). Total proteins in serum and tissues (homogenate prepared in chilled hypotonic solution) were measured as described by Lowry *et al*. (1951). Briefly, total proteins were precipitated in 0.2 ml homogenates/serum by adding 0.2 ml chilled TCA (10%) and incubated for 15 min at room temperature followed by centrifugation

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at 450 *g* for 15 min. The precipitate was dissolved in 1 ml NaOH (0.2 N) and 0.1 ml aliquots were taken and the volume was made up to 0.5 ml with distilled water. For standard and blank, 0.1 ml of BSA and distilled water were taken, respectively, in place of aliquots and incubated for 10 min at room temperature after adding 5 ml of alkaline copper reagent. 0.5 ml of Folin Ciocalteau reagent (diluted with distilled water) was added and incubated for 30 min in the dark and the absorbance was recorded at λ 625 nm against blank.

Glycogen Contents

Glycogen was measured according to the method of Seifter *et al*. (1950). Fresh tissues of liver and kidney were dropped in 1 ml KOH (30%) followed by digestion in a water bath for 10 min. The extraction process involved repetitive addition of 1.25 ml of 95% ethanol then brought to boil in a water bath and centrifuged at 1000 *g* for 20 min. Sedimented glycogen was dissolved in 5 ml of distilled water and 10 ml of freshly prepared anthrone reagent (0.2%) was added; incubated for 10 min at 100 °C and the absorbance was recorded at λ 620 nm against the blank.

Thiobarbituric Acid Reactive Substances (TBARS)

The amount of total TBARS formed was quantitated with thiobarbituric acid (TBA) reaction by the method of Sharma and Krishna Murti (1968) and used as an index of lipid peroxidation (LPO). 1 ml of homogenates, prepared in KCl (0.15%) were incubated at 37 °C for 30 min and the proteins were precipitated by adding 1 ml chilled TCA (10%) then centrifuged at 450 *g* for 15 min. Supernatant and TBA solution (0.67%) (1 ml each) were kept in a boiling water bath for 10 min and after cooling, the optical density was noted at λ 535 nm.

Total Reduced Glutathione (GSH)

Homogenates were prepared in sucrose solution (1%) for measuring reduced GSH according to Brehe and Burch (1976). 0.1 ml of homogenate and 0.9 ml of distilled water was added with 1.0 ml sulfosalicylic acid (10%) followed by centrifugation at 1000 *g* for 10 min. Blank and standards were prepared by taking 0.5 ml of distilled water and 0.5 ml of GSH standard, respectively. 0.5 ml of supernatant was added with 4.5 ml of Tris buffer (pH 8.23). Color was developed by adding 0.5 ml of dithiobis-2-nitro-benzoic acid solution and the optical density was recorded at λ 412 nm.

Triglycerides

Triglyceride contents were estimated by the method of Neri and Frings (1973). 800 mg zeolite and 5 ml isopropanol were added to 0.2 ml of homogenate (homogenate prepared in chilled hypotonic solution) and centrifuged at 1000 *g* for 10 min. Distilled water and triolein were used as blank and standard, respectively. 2 ml of supernatant was added with 0.5 ml KOH (1 N) then incubated for 5 min at 60 °C. Sodium-m-periodate solution (0.5 ml) and coloring reagent (3.0 ml) were added to each tube and incubated at 60 °C for 30 min. The optical density of the yellow colored complex was recorded after cooling at λ 410 nm against blank.

Total and Esterified Cholesterol

The method of Zlatkis *et al*. (1953) was followed for the estimation of total and esterified cholesterol. Freshly prepared 2 ml of homogenate (homogenate prepared in chilled hypotonic solution) was centrifuged at 450 *g* for 10 min. The obtained pellet was extracted in 1 ml ethyl alcohol and diethyl ether mixture (3 : 1) and centrifuged for 10 min to collect the supernatant for estimation of total cholesterol. The residue was re-extracted with 1 ml of chloroform and methanol mixture (2 : 1) and the supernatant was used to measure esterified cholesterol. For both total and esterified cholesterol, 0.5 ml of filtrate, 2.5 ml of glacial acetic acid and 0.1 ml of distilled water were used. Color was developed by adding 2 ml of $FeCl_3$ (0.1%) and optical density was noted at λ 560 nm after 30 min.

Choleretic Activity (Effect of Therapeutic Agents on Bile Flow and Bile Solids)

In order to estimate the choleretic activities of tiferron, α-tocopherol, propolis and piperine, the rate of bile flow and excretion of bile solids between untreated and treated animals were measured and compared over an interval of time according to Cook *et al*. (1953). Rats were divided into six groups of six each and were anesthetized with 25% urethane (6 ml kg^{-1}, i.p.) following an overnight fasting period. The bile duct was surgically exposed followed by a midline incision (25 mm) and cannulated with PE-10 tubing (Klassen and Plaa, 1969). Bile was collected for 1 h in glass tubes. At the end of 1 h, normal saline, dehydrocholic acid as standard (DHC, 50 mg kg^{-1}), tiferron (300 mg kg^{-1}), α-tocopherol (25 mg kg^{-1}), propolis (200 mg kg^{-1}) and piperine (10 mg kg^{-1}), respectively, were injected intraduodenally (i.d.) in animals of groups 1–6. Bile was collected for the next 4 h (2–5 h periods) following administration of the compounds. Total bile solids were estimated by evaporating samples to dryness and weighing the residue. The criterion for the choleretic activity of a test drug is based on the increase in bile volume (ml rat^{-1}) and in bile solid (mg rat^{-1}) excretion between 2–5 h of collection periods in treatment groups compared with the control. The first hour (control) bile flow was measured as a high bile output compared with 2–5 h period. An increase in bile flow and bile solid were designated as *B* and calculated by given equation.

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$$B = Bo - (\text{ratio})\ C$$

where B is the calculated bile volume (ml) or bile solids (mg); Bo is the observed bile volume (ml) or bile solids (mg) obtained between 2–5 h and C is the observed bile volume (ml) or bile solids (mg) during control h.

$$\text{Percent in increase in bile volume/solids} = \frac{B}{C} \times 100$$

Statistical Analysis

The results are presented as mean ± SEM of six animals used in each group. Significance of differences between mean values was determined by one-way analysis of variance ($P \leq 0.05$) followed by Student's t-test ($P \leq 0.01$ and $P \leq 0.05$) for comparison between two groups (Snedecor and Cochran, 1994). Tukey's honestly significant difference *post hoc* test was used for comparison among groups of combination treatments ($P \leq 0.05$).

Results

The therapeutic effects of tiferron, α-tocopherol, propolis and piperine individually and in combination on blood and serum variables after beryllium intoxication are presented in Table 1. Exposure to beryllium for 28 days caused significant depletion in blood sugar and serum albumin ($P \leq 0.01$) and significant decrease in hemoglobin and serum protein contents ($P \leq 0.05$). Individual administration of tiferron, α-tocopherol, propolis and piperine were significantly effective ($P \leq 0.05$) in bringing the value of blood sugar and albumin towards the control, except individual treatment of tiferron in the case of albumin. Combination therapy of tiferron along with α-tocopherol, propolis and piperine, respectively, were significantly effective in increasing the level of blood sugar and albumin more towards the control ($P \leq 0.01$). The release of AST and ALT was significantly enhanced after beryllium exposure ($P \leq 0.01$). All the therapeutic groups significantly lowered the release of AST ($P \leq 0.01$), while piperine was able to lower its activity significantly at $P \leq 0.05$. Individual administration of propolis and piperine lowered the enhanced release of ALT significantly ($P \leq 0.05$) towards control, whereas the other therapeutic groups showed significant inhibition in ALT even at $P \leq 0.01$. Table 1 revealed a better percentage protection by the combination of tiferron and propolis over the other two combinations.

A significant enhancement in LPO and a significant depletion in GSH indicated beryllium induced oxidative stress in liver and kidney ($P \leq 0.01$; Table 2). Increased LPO in liver and kidney declined in all the therapeutic groups significantly ($P \leq 0.01$), except piperine, which was effective in lowering LPO in kidney at $P \leq 0.05$. Combined administration of tiferron along with propolis and piperine, respectively, showed significant therapeutic potential ($P \leq 0.05$) bringing the GSH level towards the control in liver, whereas the combination of tiferron and propolis was significantly effective in kidney ($P \leq 0.01$). Combined therapy of tiferron and propolis offered maximum percentage protection in hepatic LPO and renal GSH. Co-treatment of tiferron along with α-tocopherol and piperine were maximally effective in renal LPO and hepatic GSH, respectively, thus, suppressing oxidative stress induced by the toxicant. Beryllium intoxication decreased total protein and glycogen contents in liver and

Table 1. Effectiveness of tiferron along with α-tocopherol, propolis and piperine against beryllium induced alteration in blood biochemical variables (values are mean ± SEM from 6 rats in each group)

Treatment	Hb (mg%)	Blood sugar (mg 100 ml^{-1})	S. protein (mg 100 ml^{-1})	Albumin (IU l^{-1})	AST (IU l^{-1})	ALT (IU l^{-1})
Control	15.18 ± 0.837	109 ± 6.02	38.2 ± 2.11	5.65 ± 0.31	68.8 ± 3.80	43.2 ± 2.38
Be	12.14 ± 0.67[a]	71.2 ± 3.93[b]	31.5 ± 1.74[a]	3.31 ± 0.18[b]	122 ± 6.74[b]	82.0 ± 4.53[b]
Be + Tiferron	13.70 ± 0.75	91.2 ± 5.04[c]	33.1 ± 1.83	3.90 ± 0.21	83.1 ± 4.59[d]	56.3 ± 3.11[d]
% Protection	***51.31%***	***52.96%***	***24.33%***	***25.21%***	***73.12%***	***66.10%***
Be + α-toco	13.55 ± 0.74	87.7 ± 4.81[c]	34.8 ± 1.92	4.10 ± 0.22[c]	91.3 ± 5.05[d]	60.4 ± 3.34[d]
% Protection	***46.38%***	***42.30%***	***48.96%***	***33.76%***	***57.59%***	***55.54%***
Be + Propolis	14.00 ± 0.77	89.7 ± 4.96[c]	35.8 ± 1.98	3.94 ± 0.22[c]	81.2 ± 4.49[d]	64.6 ± 3.57[c]
% Protection	***61.18%***	***49.07%***	***64.24%***	***28.63%***	***76.63%***	***44.84%***
Be + Piperine	13.80 ± 0.76	93.9 ± 5.18[d]	34.7 ± 1.92	4.20 ± 0.23[c]	100 ± 5.52[c]	65.7 ± 3.63[c]
% Protection	***54.60%***	***60.00%***	***48.07%***	***38.03%***	***41.35%***	***41.90%***
Be + Tif + α-toco	14.2 ± 0.78	97.2 ± 5.37[d]	34.9 ± 1.92	4.60 ± 0.25[d]	74.16 ± 4.09[d]	55.2 ± 3.05[d]
% Protection	***67.76%***	***68.78%***	***50.44%***	***55.12%***	***89.92%***	***68.99%***
Be + Tif + Propolis	14.8 ± 0.81[c]	108 ± 6.00[d]	37.5 ± 2.07	4.90 ± 0.27[d]	71.28 ± 3.94[d]	52.8 ± 2.92[d]
% Protection	***87.50%***	***98.94%***	***89.02%***	***67.94%***	***95.33%***	***75.10%***
Be + Tif + Piperine	14.6 ± 0.80[c]	96.4 ± 5.33[d]	35.7 ± 1.97	4.80 ± 0.26[d]	73.58 ± 4.06[d]	53.1 ± 2.93[d]
% Protection	***80.92%***	***66.82%***	***63.20%***	***63.67%***	***91.01%***	***74.27%***
F Variance	1.554ns	5.756*	1.331ns	9.391*	15.393*	12.872*

Anova at 5% level: * Significant; ns, not significant; Be vs control at [a] $P \leq 0.05$, [b] $P \leq 0.01$; Drugs vs Be at [c] $P \leq 0.05$, [d] $P \leq 0.01$.
Hb, hemoglobin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Be, beryllium; Tif, tiferron; α-toco, α-tocopherol.

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Table 2. Therapeutic influence α-tocopherol, propolis and piperine on tiferron against beryllium induced deviation in markers of oxidative stress, protein and glycogen contents (values are mean ± SEM from 6 rats in each group)

	Lipid peroxidation (nmol MDA mg protein^{-1})		Reduced glutathione (μmol g^{-1})		Total proteins (mg 100 mg^{-1})		Glycogen (mg 100 g^{-1})	
Treatment	Hepatic	Renal	Hepatic	Renal	Hepatic	Renal	Hepatic	Renal
Control	0.28 ± 0.01	0.21 ± 0.001	8.40 ± 0.46	4.81 ± 0.26	15.8 ± 0.87	14.2 ± 0.78	2792 ± 154	86.2 ± 4.76
Be	0.68 ± 0.03^{b}	0.93 ± 0.05^{b}	6.30 ± 0.34^{b}	3.26 ± 0.18^{b}	12.1 ± 0.66^{b}	8.94 ± 0.49^{b}	1929 ± 106^{b}	42.8 ± 2.36^{b}
Be + Tiferron	0.45 ± 0.02^{d}	0.58 ± 0.03^{d}	7.10 ± 0.39	3.76 ± 0.20	13.9 ± 0.76	12.1 ± 0.67^{d}	2230 ± 123	61.2 ± 3.38^{d}
% Protection	***57.78%***	***48.14%***	***38.09%***	***32.25%***	***48.64%***	***60.45%***	***34.87%***	***42.39%***
Be + α-toco	0.41 ± 0.02^{d}	0.67 ± 0.03^{d}	7.10 ± 0.39	3.36 ± 0.20	13.3 ± 0.73	11.7 ± 0.64^{c}	2313 ± 127	58.7 ± 3.24^{d}
% Protection	***67.83%***	***36.17%***	***38.09%***	***6.45%***	***34.05%***	***52.47%***	***44.49%***	***36.72%***
Be + Propolis	0.37 ± 0.02^{d}	0.59 ± 0.03^{d}	6.96 ± 0.38	3.84 ± 0.18	13.8 ± 0.76	11.9 ± 0.65^{d}	2258 ± 124	66.3 ± 3.66^{d}
% Protection	***77.88%***	***46.90%***	***31.42%***	***37.41%***	***48.37%***	***56.65%***	***38.12%***	***54.28%***
Be + Piperine	0.47 ± 0.02^{d}	0.76 ± 0.04^{c}	6.89 ± 0.38	3.63 ± 0.20	13.91 ± 0.76	12.6 ± 0.69^{d}	2340 ± 129^{c}	61.3 ± 3.39^{d}
% Protection	***52.26%***	***23.65%***	***28.09%***	***23.87%***	***48.91%***	***69.58%***	***47.62%***	***42.81%***
Be + Tif + α-toco	0.36 ± 0.02^{d}	0.38 ± 0.02$^{d\$}$	7.30 ± 0.40	3.79 ± 0.20	14.1 ± 0.78	13.6 ± 0.75^{d}	2370 ± 131^{c}	62.0 ± 3.42^{d}
% Protection	***78.14%***	***75.65%***	***47.85%***	***36.22%***	***56.21%***	***90.11%***	***51.10%***	***44.23%***
Be + Tif + Propolis	0.29 ± 0.01$^{d@\#}$	0.42 ± 0.02$^{d\#}$	7.68 ± 0.42^{c}	4.47 ± 0.24$^{d@\#}$	14.8 ± 0.81^{c}	13.9 ± 0.77^{d}	2622 ± 144^{d}	74.2 ± 3.93$^{d@}$
% Protection	***95.72%***	***70.28%***	***65.71%***	***78.06%***	***73.78%***	***96.00%***	***80.30%***	***65.46%***
Be + Tif + Piperine	0.35 ± 0.01^{d}	0.52 ± 0.02^{d}	8.16 ± 0.45^{c}	3.82 ± 0.21	13.97 ± 0.77	12.8 ± 0.71^{d}	2455 ± 135^{c}	67.1 ± 3.71^{d}
% Protection	***88.90%***	***57.08%***	***88.57%***	***36.12%***	***50.54%***	***74.33%***	***60.95%***	***56.03%***
F Variance	30.710*	49.821*	3.140*	6.345*	1.999ns	6.372*	4.159*	12.258*

Anova at 5% level: * Significant, ns, not significant; Be vs control at ^{b} $P \leq 0.01$; Drugs vs Be at ^{c} $P \leq 0.05$, ^{d} $P \leq 0.01$.
Be, beryllium; Tif, tiferron; α-toco, α-tocopherol.
$^{@}$ Tif + α-toco vs Tif + Propolis; $^{\$}$ Tif + α-toco vs Tif + Piperine; $^{\#}$ Tif + Propolis vs Tif + Piperine for Tukey's HSD *post hoc* test ($P \leq 0.05$).

kidney significantly at $P \leq 0.01$ (Table 2). The combination of tiferron and propolis significantly increased protein level in liver ($P \leq 0.05$), whereas all the therapeutic groups depicted a significant increase in kidney protein. Tiferron with propolis was the most effective combination in recouping glycogen contents of both liver and kidney in a significant manner at 1% level of probability, which is indicative of improved physiological functions of both organs.

The effects of individual and combined treatments of therapeutics on beryllium induced disturbed lipid profile are presented in Table 3. Subchronic beryllium exposure significantly elevated triglycerides and total and esterified cholesterol in liver and kidney ($P \leq 0.01$). All three combinations of tiferron were significantly effective in lowering triglycerides in liver and kidney after beryllium induced deviation ($P \leq 0.01$). Single therapy of tiferron and propolis and all the three combinations significantly lowered total cholesterol in liver ($P \leq 0.01$). Analysis of variance showed significant lowering in renal total cholesterol and maximum percentage protection was found by co-therapy of tiferron and propolis. Individual administration of tiferron and α-tocopherol were able to decrease esterified cholesterol significantly ($P \leq 0.05$) towards the control in liver, while all the other therapeutic groups declined esterified cholesterol significantly at $P \leq 0.01$. All the therapeutic groups, except individual treatment with α-tocopherol, were effective in decreasing renal esterified cholesterol towards the control significantly at the 1% level of probability.

The rate of bile flow and bile solid contents, which relates to the choleretic activity of tiferron, α-tocopherol, propolis and piperine is presented in Table 4. The therapies stimulated liver activity moderately to excrete more bile than normal animals, the same as DHC (standard drug) yet there was no significant change in the excretory

Table 3. Efficacy of tiferron along with α-tocopherol, propolis and piperine on triglycerides and cholesterol contents after beryllium exposure (values are mean ± SEM from 6 rats in each group)

	Triglycerides (mg 100 mg^{-1})		Total cholesterol (mg 100 mg^{-1})		Esterified cholesterol (mg 100 mg^{-1})	
Treatment	Hepatic	Renal	Hepatic	Renal	Hepatic	Renal
Control	12.8 ± 0.70	10.4 ± 0.57	0.136 ± 0.007	0.127 ± 0.007	0.070 ± 0.003	0.022 ± 0.001
Be	32.2 ± 1.78^{b}	28.3 ± 1.56^{b}	0.249 ± 0.013^{b}	0.228 ± 0.012^{b}	0.164 ± 0.009^{b}	0.104 ± 0.005^{b}
Be + Tiferron	26.2 ± 1.44^{c}	23.8 ± 1.31	0.177 ± 0.009^{d}	0.190 ± 0.010^{c}	0.128 ± 0.007^{c}	0.075 ± 0.004^{d}
% Protection	***30.92%***	***25.13%***	***63.71%***	***37.62%***	***38.29%***	***35.36%***
Be + α-toco	27.1 ± 1.49	23.2 ± 1.28^{c}	0.217 ± 0.011	0.198 ± 0.010	0.138 ± 0.007^{c}	0.098 ± 0.005
% Protection	***26.28%***	***28.49%***	***28.31%***	***29.70%***	***27.65%***	***7.31%***
Be + Propolis	23.2 ± 1.28^{d}	21.9 ± 1.21^{c}	0.186 ± 0.010^{d}	0.187 ± 0.010^{c}	0.124 ± 0.006^{d}	0.068 ± 0.003^{d}
% Protection	***46.39%***	***35.75%***	***55.75%***	***40.59%***	***42.55%***	***43.90%***
Be + Piperine	24.7 ± 1.36^{c}	23.9 ± 1.32^{c}	0.227 ± 0.012	0.202 ± 0.011	0.105 ± 0.005^{d}	0.077 ± 0.004^{d}
% Protection	***38.65%***	***24.58%***	***19.46%***	***25.72%***	***62.76%***	***32.92%***
Be + Tif + α-toco	21.6 ± 1.19^{d}	18.5 ± 1.02^{d}	0.168 ± 0.009^{d}	0.177 ± 0.009^{c}	0.122 ± 0.006^{d}	0.071 ± 0.003^{d}
% Protection	***54.63%***	***54.74%***	***71.68%***	***50.49%***	***44.68%***	***40.24%***
Be + Tif + Propolis	18.8 ± 1.03$^{d@}$	17.5 ± 0.96^{d}	0.149 ± 0.008$^{d@\#}$	0.156 ± 0.008$^{d@}$	0.123 ± 0.006^{d}	0.058 ± 0.003$^{d@}$
% Protection	***69.07%***	***60.33%***	***88.49%***	***71.28%***	***43.61%***	***56.09%***
Be + Tif + Piperine	17.9 ± 0.98$^{d\$}$	17.3 ± 0.95^{d}	0.171 ± 0.008^{d}	0.161 ± 0.008^{d}	0.101 ± 0.005$^{d\$\#}$	0.061 ± 0.003$^{d\$}$
% Protection	***73.71%***	***61.45%***	***69.02%***	***66.33%***	***67.02%***	***52.43%***
F Variance	23.712*	23.977*	15.109*	10.293*	17.943*	40.812*

Anova at 5% level: * Significant; Be vs control at ^{b} $P \leq 0.01$; Drugs vs Be at ^{c} $P \leq 0.05$, ^{d} $P \leq 0.01$.
Be, beryllium; Tif, tiferron; α-toco, α-tocopherol.
$^{@}$ Tif + α-toco vs Tif + Propolis; $^{\$}$ Tif + α-toco vs Tif + Piperine; $^{\#}$ Tif + Propolis vs Tif + Piperine for Tukey's HSD *post hoc* test ($P \leq 0.05$).

Table 4. Choleretic activity (bile flow and bile solids) of tiferron, α-tocopherol, propolis and piperine in normal rats (values are mean ± SEM from 6 rats in each group)

	Bile flow (ml)			Bile solid (mg)		
Treatment	1 h	2–5 h	Ratio	1 h	2–5 h	Ratio
Control	0.64 ± 0.035	1.93 ± 0.106	1: 3.01	22.9 ± 1.26	71.9 ± 3.59	1: 3.13
Dehydrocholic acid	0.61 ± 0.033	2.28 ± 0.13	1: 3.73	22.7 ± 1.25	84.1 ± 4.64	1: 3.70
Tiferron	0.62 ± 0.034	1.99 ± 0.11	1: 3.20	23.4 ± 1.29	78.4 ± 4.13	1: 3.19
α-Tocopherol	0.64 ± 0.035	2.02 ± 0.11	1: 3.15	23.7 ± 1.31	76.5 ± 4.22	1: 3.22
Propolis	0.62 ± 0.034	2.12 ± 0.11	1: 3.41	23.1 ± 1.27	79.3 ± 4.38	1: 3.43
Piperine	0.63 ± 0.034	2.03 ± 0.11	1: 3.22	22.9 ± 1.26	75.2 ± 4.15	1: 3.28
F variance	0.143ns	1.405ns		0.101ns	1.190ns	

Anova at 5% level: ns, not significant.

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capacity, indicating no adverse effect of these therapeutic agents on liver at these doses.

Discussion

Observations of the present investigation revealed severe alterations in the blood and tissue biochemical variables after subchronic exposure to beryllium. A significant fall in hemoglobin as observed in the present study might be due to diminishment in the synthesis of heme and globin proteins, which consequently decreased the hemoglobin content of the erythrocytes (Venugopal and Luckey, 1978; Mathur *et al*., 1994b). Beryllium suppresses the activity of δ-amino levulinic acid synthetase (ALAS) and δ-amino levulinic acid dehydratase (ALAD) (Sakaguchi *et al*., 1997). It is assumed that besides the chelating effect of tiferron, adjuvants appear to play a role in heme synthesis by increasing the levels of ALAS and ALAD to maintain hemoglobin in erythrocytes. This may also be due to the regulation of iron metabolism through a potent antioxidant action of flavonoids present in propolis (Beutler, 1988; Jaiswal *et al*., 1997). Elevation in serum AST and ALT after beryllium exposure were used as biomarkers of hepatic injury. Tiferron may combine with toxic beryllium ions or reactive metabolites and lead to inactivate them. Simultaneously, α-tocopherol, propolis and piperine also counteract free radicals. Thus, combination therapy might prevent cellular injury and organ dysfunction more prominently thereby, inhibiting the rapid leakage of these enzymes.

The present investigation showed hypoglycemia after beryllium exposure as an indication of hepatic injury. Hypoglycemia from a toxic dosage of beryllium salt has also been reported due to inactivation of phosphoglucomutase, hexokinase and many other key enzymes involved in carbohydrate metabolism (Aldridge and Thomas, 1966; Reiner, 1971). The major reason for hypoglycemia appears to be due to a decline in hepatic glycogen. The significant fall in renal and hepatic glycogen as observed in this study may also be due to disturbances in the carbohydrate metabolic pathway. Tiferron, as a chelator, probably reactivates the enzymatic activity by removing beryllium ions from their sites. Propolis and piperine enhance the bioavailability of a number of diverse drugs through various metabolic processes (Khayyal *et al*., 2003; Khajuria *et al*., 1998). It is, therefore, assumed that co-administration of these adjuvants might be helpful to enhance the effectiveness of tiferron by increasing its bioavailability, which could maintain hepatic and renal glycogen storage as well as synthesis for physiological functions of the hepatocytes and nephrones that eventually up-regulates the glucose status in blood. Serum albumin level has been used as a test for liver function because its concentration is affected by disturbance in hepatic protein synthesis (Thirunavukkarasu and Sakthisekaran, 2003). Hypoalbuminemia may also result from increased catabolism (Landel *et al*., 1985), which may happen as a toxic response to beryllium exposure. A decrease in total proteins in liver, kidney and serum might be due to a loss in cytosolic proteins because of LPO in cell membranes (Yeh *et al*., 2003) or reduction in protein synthesis. The synergistic antioxidant potential of tiferron and the adjuvants might stimulate protein synthesis as a contributory hepatoprotective mechanism, which consequently accelerates the regeneration process and production of liver cells.

Lipid peroxidation is a molecular mechanism of cell injury leading to the generation of cytotoxic products such as malondialdehyde (MDA) and 4-hydroxynonenal (Roy *et al*., 2000). Metal toxicity is attributed to generate reactive oxygen species (ROS), which causes peroxidation of membrane lipids (Hermes-Lima and Zenteno-Savin, 2002) and induces a plethora of alterations in structure and function of cellular membranes. Increased TBARS after beryllium administration indicated enhanced LPO due to failure of the antioxidant defense mechanism. Findings of the present study demonstrated a more pronounced inhibition in the LPO, which might be due to the dual function of combined therapy. One may be due to the indirect antioxidant effect of tiferron as it scavenges toxic beryllium ions from the tissue by donating a hydrogen to alkoxyl and peroxyl radicals, which are chain initiating and chain propagating intermediates. Simultaneously, the antioxidative property of α-tocopherol, propolis and piperine directly inhibits the formation of free radicals. Propolis has the capacity to pass through the membrane and be accumulated in both hydrophilic and hydrophobic environments, thereby, protecting cells against oxidative stress by scavenging free radicals (Sumathi *et al*., 1996). GSH is recognized as a protective compound within the body, detoxifying many xenobiotics or their metabolites through several mechanisms (Liu *et al*., 2002; Hwang *et al*., 2002) and an inverse relationship exists between peroxidative decomposition of membrane polyunsaturated fatty acids (PUFA) and GSH level (Gutteridge and Halliwell, 1990). Subchronic exposure to beryllium depleted hepatic and renal GSH that in turn, enhanced oxidative stress due to a loss of control on the production of ROS, which caused target tissue injury as a result of macromolecular damage. Combined treatment of tiferron along with α-tocopherol, propolis and piperine, respectively, up-regulated the GSH level. It is proposed that adjuvants may react with free radicals induced by beryllium and form adjuvant radical adducts in addition to the formation of a tiferron–beryllium complex, which consequently decreased the utilization of GSH.

Beryllium is evidenced as a pro-oxidant leading to peroxidative damage to cellular membranes. The beryllium induced increase in triglycerides and cholesterol in the liver and kidney may be either due to a breakdown of fatty acids or decreased utilization of triglycerides, which

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are synthesized in the liver by peripheral tissues or due to the lack of metabolic regulation. Combined administration of tiferron along with adjuvants lowered the triglycerides and cholesterol content, which might be due to their direct or indirect antioxidative influence on LPO that subsequently provided an improvement in the metabolic process.

Based on the present data, it seems quite reasonable to assume that besides the antioxidative activity, the adjuvants also exhibited synergistic effects through increasing the bioavailability of tiferron either by (i) promoting absorption from the gastrointestinal tract (Khajuria *et al*., 2002), (ii) protecting the drug from being metabolized or oxidized in the first passage through the liver after being absorbed (Khajuria *et al*., 1998), (iii) a combination of these two mechanisms or (iv) causing increased production of bile. A beneficial role of propolis in combination with certain drugs has also been reported (Nostro *et al*., 2006). Propolis is also supposed to be helpful in the absorption and utilization of various minerals due to the presence of acid derivatives in it, which in turn improved physiological functions by regulating ion dependent enzymatic activities (Haro *et al*., 2000) and thus, afforded better protection.

Bile is partially an excretory product of the liver, which plays an important role in the digestion and removal of drugs, toxins, pigments and various inorganic substances either derived from diet or synthesized by the body as cholesterol or cholic acid (Chandan *et al*., 1991). Increased bile flow indicates strong stimulatory action on the secretory activity of the liver. The results of bile flow revealed that none of the therapeutic agents increased the choleretic activity appreciably. This demonstrates that there is no adverse effect at these doses. Thus, the doses up to the level used in this study are relatively safe and can be used for further studies.

In conclusion, the combined administration of tiferron along with α-tocopherol, propolis or piperine, respectively, provides more pronounced therapeutic potential at relatively lower doses compared with using them individually. Furthermore, co-treatment of tiferron and propolis offers maximum therapeutic potential in regulating oxidative stress and cellular dysfunction. This may be useful in developing a suitable antidote against beryllium induced toxic manifestations.

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References

Abdel-Fattah AA, al-Yousef HM, al-Bekairi AM, al-Sawaf HA. 1998. Vitamin E protects the brain against oxidative injury stimulated by excessive aluminum intake. *Biochem. Mol. Biol. Int.* **46**: 1175–1180.

Aldridge WN, Thomas M. 1966. The inhibition of phosphoglucomutase by beryllium. *Biochem. J.* **98**: 100–104.

Arkhipova OG, Kochetkova TA, Rudomino MV, Merved TV, Kobachni MI. 1964. Vlianiya aminol kilfosfinovikh kislet na ekspovimentala noye extravlenive beryllium. *Dmkl. Akad. Nauk.* USSR **58**: 1235 (in Russian).

Asatoor AM, King E. 1954. Simplified colorimetric blood sugar method. *Process Biochem.* Jan 16; xliv 56 (325th Meeting).

ATSDR 2002. *Toxicological Profile for Beryllium*. Agency for Toxic Substances and Disease Registry (ATSDR), US Department of Health and Human Services, Public Health Service: Atlanta, GA.

Badmaev V, Majeed M, Prakash L. 2000. Piperine derived from black pepper increases the plasma levels of coenzyme q10 following oral supplementation. *J. Nutr. Biochem.* **11**: 109–113.

Bano G, Raina RK, Zutshi U, Bedi KL, Johri RK, Sharma SC. 1991. Effect of piperine on bioavailability and pharmacokinetics of propranolol and theophylline in healthy volunteers. *Eur. J. Clin. Pharmacol.* **41**: 615–617.

Banskota AH, Tezuka Y, Kodata S. 2001. Recent progress in pharmacological research of propolis. *Phytother. Res.* **15**: 561–571.

Banskota AH, Tezuka Y, Prasain JK, Matsushige K, Sakai I, Kodata S. 1998. Chemical constituents of Brazilian propolis and their cytotoxic activity. *J. Nat. Prod.* **61**: 828–900.

Beutler E. 1988. *Modern Nutrition in Health and Disease*, 6th edn, Ruckenbush Y, Thivend P (eds). Salvat: Barcelona; 298–326.

Bosque MA, Domingo JL, Llobett JM, Corbella J. 1993. Effectiveness of sodium-4,5-dihydroxy-1,3-benzene disulphonate (Tiron) in protecting against uranium induced developmental toxicity in mice. *Toxicology* **79**: 149–156.

Brehe JE, Burch HB. 1976. Enzymatic assay for glutathione. *Anal Biochem* **74**: 189–197.

Bugryshev PF, Moskalev IuI, Kuznetsov AA, Nazarova VA. 1976. Distribution of beryllium in the organs of rats. *Farmakol. Toksikol.* **39**: 615–618 (in Russian).

Cash R, Shapiro RI, Levy SH, Hopkins SM. 1959. Chelating agent in the therapy of beryllium poisoning. *N. Engl. J. Med.* **260**: 683–686.

Ceci A, Baiardi P, Felisi M, Cappellini MD, Carnelli V, De Sanctis V, Galanello R, Maggio A, Masera G, Piga A, Schettini F, Stefano I, Tricta F. 2002. The safety and effectiveness of deferiprone in a large-scale, 3-year study in Italian patients. *Br. J. Haematol.* **118**: 330–336.

Chandan BK, Sharma AK, Anand KK. 1991. *Boerhhavia diffusa*: a study of its hepatoprotective activity. *J. Ethnopharmacol.* **31**: 299–307.

Chu WH. 2006. Adjuvant effect of propolis on immunisation by inactivated *Aeromonas hydrophila* in carp (*Carassius auratus* gibelo). *Fish Shellfish Immunol.* **21**: 113–117.

Cikrt M, Bencko V. 1975. Biliary excretion of 7Be and its distribution after intravenous administration of $7BeCl_2$ in rats. *Arch. Toxicol.* **34**: 53–60.

Constantinides PP, Han J, Davis SS. 2006. Advances in the use of tocols as drug delivery vehicles. *Pharm. Res.* **23**: 243–255.

Cook CL, Calvin LD, Kalm MA. 1953. The use of the cat in the bioassay of choleretic agents. *J. Pharmacol. Exp. Ther.* **109**: 167–174.

Dalvi RR, Dalvi PS. 1991. Differences in the effects of piperine and pipronyl butoxide on hepatic drug metabolizing system in rats. *Drug. Chem. Toxicol.* **14**: 219–229.

Day GA, Stefaniak AB, Weston A, Tinkle SS. 2006. Beryllium exposure: dermal and immunological considerations. *Int. Arch. Occup. Environ. Health* **79**: 161–164.

Domingo JL. 1995. Prevention by chelating agents of metal induced developmental toxicity. *Reprod. Toxicol.* **9**: 105–113.

Fernandez-Cabezudo MJ, Hasan MY, Mustafa N, El-Sharkawy RT, Fahim MA, Al-Ramadi BK. 2003. Alpha tocopherol protects against immunosuppressive and immunotoxic effects of lead. *Free Radic. Res.* **37**: 437–445.

Finch GL, Hoover MD, Hahn FF, Nikula KJ, Belinsky SA, Haley PJ, Griffith WC. 1996. Animal models of beryllium induced lung disease. *Environ. Health Perspect.* **104**: 973–979.

Finch GL, Mewhinney JA, Hoover MD, Eidson AR, Haley PJ, Bice DE. 1990. Clearance, translocation, and excretion of beryllium following acute inhalation of beryllium oxide by beagle dogs. *Fundam. Appl. Toxicol.* **15**: 231–241.

Flora SJS, Mathur S, Mathur R. 1995. Effects of meso 2,3-dimercapto succinic acid or 2,3-dimercapto propane-1-sulfonate on beryllium induced biochemical alterations and metal concentration in male rats. *Toxicology* **95**: 167–175.

Franchini M, Veneri D. 2004. Iron-chelation therapy: an update. *Hematol. J.* **5**: 287–292.

Gebaraa EC, Pustiglioni AN, de Lima LA, Mayer MP. 2003. Propolis extract as an adjuvant to periodontal treatment. *Oral Health Prev. Dent.* **1**: 29–35.

Ghosh M, Wang HD, McNeill JR. 2002. Tiron exerts effects unrelated to its role as a scavenger of superoxide anion: effects on calcium binding and vascular responses. *Can. J. Physiol. Pharmacol.* **80**: 755–760.

Gutteridge JMC, Halliwell B. 1990. The measurement and mechanism of lipid peroxidation in biological system. *Trends Biochem. Sci.* **15**: 129–135.

Haro A, Lopez-Aliaga I, Lisbona F, Barrionuevo M, Alferez MJM, Campos MS. 2000. Beneficial effect of pollen and/or propolis on the metabolism of iron, calcium, phosphorus, and magnesium in rats with nutritional ferropenic anemia. *J. Agric. Food Chem.* **48**: 5715–5722.

Hermes-Lima M, Zenteno-Savin T. 2002. Animal response to drastic changes in oxygen availability and physiological oxidative stress. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **133**: 537–556.

Hwang JM, Wang CJ, Chou FP, Tseng TH, Hsieh YS, Lin WL, Chu CY. 2002. Inhibitory effect of berberine on ter-butyl hydroperoxide induced oxidative damage in rat liver. *Arch. Toxicol.* **76**: 29205–29210.

Isla MI, Nieva-Moreno MI, Sampietro AR, Vattuone MA. 2001. Antioxidant activity of Argentine propolis extracts. *J. Ethnopharmacol.* **76**: 165–170.

Jaiswal AK, Venugopal R, Mucha J, Carothers AM, Grunberger D. 1997. Caffeic acid phenethyl ester stimulates human antioxidant response element-mediated expression of the NAD(P)H: Quinone oxidoreductase (NQO1) gene. *Cancer Res.* **57**: 440–446.

Johri S, Srivastava S, Sharma P, Shukla S. 2004. Analysis of time dependent recovery from beryllium toxicity following chelation therapy and antioxidant supplementation. *Indian J. Exp. Biol.* **42**: 798–802.

Khajuria A, Thusu N, Zutshi U. 2002. Piperine modulates permeability characteristics of intestine by inducing alterations in membrane dynamics: influence on brush border membrane fluidity, ultrastructure and enzyme kinetics. *Phytomedicine* **9**: 224–231.

Khajuria A, Zutshi U, Bedi KL. 1998. Permeability characteristics of piperine on oral absorption — an active alkaloid from peppers and a bioavailability enhancer. *Indian J. Exp. Biol.* **36**: 46–50.

Khayyal MT, El-Ghazalyb MA, El-Khatiba AS, Hatemc AM, de Vriesd PJF, El-Shafeic S, Khattaba MM. 2003. A clinical pharmacological study of the potential beneficial effects of a propolis food product as an adjuvant in asthmatic patients. *Fundam. Clin. Pharmacol.* **17**: 93–102.

Klaassen CD, Plaa GL. 1969. Biliary flow after microsomal enzyme induction. *J. Pharmacol. Exp. Ther.* **168**: 218–223.

Kolanz M. 2001. Introduction to beryllium: uses, regulatory history, and disease. *Appl. Occup. Environ. Hyg. J.* **16**: 559–567.

Koul IB, Kapil A. 1993. Evaluation of the liver protective potential of piperine, an active principle of black and long peppers. *Planta Med.* **59**: 413–417.

Krishna CM, Liebmann JE, Kaufman D, DeGraff W, Hahn SM, McMurry T, Mitchell JB, Russo A. 1992. The catecholic metal sequestering agent 1,2-dihydroxybenzene-3,5-disulfonate confers protection against oxidative cell damage. *Arch. Biochem. Biophys.* **294**: 98–106.

Landel AM, Hammond WG, Meguid MM. 1985. Aspects of amino acids and protein metabolism in cancer bearing cells. *Cancer* **55**: 230–237.

Liu CL, Wang JM, Chu CY, Cheng MT, Tseng TH. 2002. *In vivo* protective effect of protecatechuic acid on ter-butyl hydroperoxide induced rat hepatotoxicity. *Food Chem. Toxicol.* **40**: 635–641.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with Folin's phenol reagent. *J. Biol. Chem.* **193**: 265–275.

Madhyastha MS, Bhat RV. 1984. *Aspergillus parasiticus* growth and aflatoxin production on black and white pepper and the inhibitory action of their chemical constituents. *Appl. Environ. Microbiol.* **48**: 376–379.

Mathur R, Nirala SK, Mathur A. 2004. Comparative effectiveness of $CaNa_3DTPA$ and Tiron along with α-tocopherol against beryllium induced biochemical alterations in rats. *Indian J. Exp. Biol.* **42**: 570–574.

Mathur S, Flora SJS, Mathur R, DasGupta S. 1993. Mobilization and distribution of beryllium over the course of chelation therapy with some polyaminocarboxylic acids in rats. *Hum. Exp. Toxicol.* **12**: 19–24.

Mathur S, Flora SJS, Mathur R, Kanan GM, Das Gupta S. 1994a. Beryllium induced biochemical alterations and their prevention following co-administration of meso-2,3-dimercapto succinic acid and 2,3-dimercapto propane sulphonate in rats. *J. Appl. Toxicol.* **14**: 263–267.

Mathur S, Mathur R, Prakash AO. 1994b. Protective action of Liv. 52: A herbal preparation against beryllium toxicity in rats. *Biomed. Environ. Sci.* **25**: 180–197.

Neri BP, Frings CS. 1973. Improved method for determination for triglycerides in serum. *Clin. Chem.* **19**: 1201–1202.

Nirala SK, Mathur R, Mathur A. 2004. Amelioration of beryllium induced biochemical alterations by combined treatment of propolis and chelator. *J. Ecophysiol. Occup. Health* **4**: 45–54.

Nostro A , Cellini L, Di Bartolomeo S, Cannatelli MA, Di Campli E, Procopio F, Grande R, Marzio L, Alonzo V. 2006. Effects of combining extracts (from propolis or *Zingiber officinale*) with clarithromycin on *Helicobacter pylori*. *Phytother. Res.* **20**: 187–190.

Ortega A, Sanchez DJ, Domingo JL, Llobet JM, Corbella J. 1991. Developmental toxicity evaluation of tiron (sodium 4,5-dihydroxybenzene-1,3-disulfonate) in mice. *Res. Commun. Chem. Pathol. Pharmacol.* **73**: 97–106.

Reiner E. 1971. Binding of beryllium to proteins. In *Symposium on Mechanism of Toxicity*, Aldridge WN (ed.) Macmillan: London, 111.

Reitman S, Frankel S. 1957. A colorimetric method for determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. *Am. J. Clin. Pathol.* **28**: 56–63.

Roy K, De AU, Sengupta C. 2000. Evaluation of glutathione and ascorbic acid as suppressors of drug induced lipid peroxidation. *Indian J. Exp. Biol.* **38**: 580–586.

Sakaguchi S, Sakaguchi T, Nakamura I, Aminaka M, Tanaka T, Kudo Y. 1997. Effect of beryllium chloride on porphyrin metabolism in pregnant mice administered by subcutaneous injection. *J. Toxicol. Environ. Health* **50**: 507–517.

Sakaguchi T, Sakaguchi S, Nakamura I, Kudo Y. 1993. Distribution of radioisotopic beryllium in mice after administration by various routes of injection. *J. Toxicol. Environ. Health* **39**: 517–526.

Sanchez DJ, Gomez M, Domingo JL, Llobet JM, Corbella J. 1995. Relative efficacy of chelating agents on excretion and tissue distribution of manganese in mice. *J. Appl. Toxicol.* **15**: 285–288.

Schubert J, Rosenthal MW. 1959. Chemical approaches to the treatment of beryllium poisoning. *Am. Med. Assoc. Arch. Ind. Health* **19**: 169–178.

Seifter S, Dayton S, Novic B, Muintwyler E. 1950. The estimation of glycogen with anthrone reagent. *Arch. Biochem.* **25**: 191–200.

Selvendiran K, Singh JP, Krishnan KB, Sakthisekaran D. 2003. Cytoprotective effect of piperine against benzo(a)pyrene induced lung cancer with reference to lipid peroxidation and antioxidant system in Swiss albino mice. *Fitoterapia* **74**: 109–115.

Sharma P, Shah Z, Shukla S. 2002. Protective effect of Tiron (4,5-dihydroxybenzene-1,3-disulphonic acid disodium salt) against beryllium induced maternal and fetal toxicity in rats. *Arch. Toxicol.* **76**: 442–448.

Sharma SK, Krishna Murti CR. 1968. Production of lipid peroxides by brain. *J. Neurochem.* **15**: 147–149.

Shukla S, Bhadauria M, Jadon A. 2005. Evaluation of hepatoprotective potential of propolis extract in carbon tetrachloride induced liver injury in rats. *Indian J. Biochem. Biophys.* **42**: 321–325.

Simoes LMC, Gregorio LE, Da Silva, Filho AA, de Souza ML, Azzolini AECS, Bastos JK, Lucisano-Valim YM. 2004. Effect of Brazilian propolis on the production of reactive oxygen species by stimulated neutrophils. *J. Ethnopharmacol.* **94**: 59–65.

Snedecor GW, Cochran WG. 1994. *Statistical Method*, 8th edn. Iowa State University Press: Ames, Iowa; 217–236.

Stiefel T, Schulze K, Zorn H, Tolg G. 1980. Toxicokinetic and toxicodynamic studies of beryllium. *Arch. Toxicol.* **45**: 81–92.

Stonehouse AJ, Zenczak S. 1991. Properties, production process and application. In *Beryllium: Biomedical and Environmental Aspects*, Rossman MD, Preuses OP, Powers MB (eds). Williams and Wilkins: Baltimore.

Sumathi R, Bhaskaran G, Varalakshmi P. 1996. Effect of DL-α-lipoic acid on tissue redox state on acute cadmium-challenged tissues. *J. Nutr. Biochem.* **7**: 85–92.

Swarup H, Arora S, Pathak SC. 1992. Sahli's acid haematin method for haemoglobin. In *Laboratory Techniques in Modern Biology*. Kalyani Publishers: New Delhi; 187–189.

Thirunavukkarasu C, Sakthisekaran D. 2003. Influence of sodium selenite on glycoprotein contents in normal and N-nitrosodiethylamine initiated and phenobarbital promoted rat liver tumors. *Pharmacol. Res.* **48**: 167–173.

Venugopal B, Luckey TD. 1978. Toxicity of group II metals. In *Metal Toxicity in Mammals*. Plenum Press: New York; 43–58.

Weston A, Snyder J, McCanlies EC, Schuler CR, Kreiss K, Demchuk E. 2005. Immunogenetic factors in beryllium sensitization and chronic beryllium disease. *Mutat. Res.* **592**: 68–78.

WHO (World Health Organization). 1990. *Environmental Health Criteria: 106 'Beryllium'. International Programme on Chemical Safety*. World Health Organization: Geneva.

Yeh ML, Liu CF, Huang CL, Huang TC. 2003. Hepatoprotective effect of *Angelica archangelica* in chronically ethanol treated mice. *Pharmacology* **68**: 70–73.

Zlatkis A, Zak B, Boyle AJ. 1953. A new method for direct determination of serum cholesterol. *J. Lab. Clin. Med.* **41**: 486–492.

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