

PROTECTIVE EFFECT OF *TRIFOLIUM PRATENSE* EXTRACT ON OXIDATIVE STRESS INDUCED BY BISPHENOL A IN RATS

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Abstract

Bisphenol A (BPA), a food contaminant yielded by plastic packaging, is known as xenoestrogen and oxidative stress inductor. *Trifolium pratense* L. (TP) is a natural source of phytoestrogens as well as of polyphenols. The aim of the present study was to evaluate the antioxidant potential and the protective effect of a TP hydroalcoholic extract in female rats subchronically exposed to BPA. Plasma samples were analysed after 30, 60 and 90 days of exposure to BPA (50 mg/kg bw) and/or a TP diluted tincture (2 mg total isoflavones / kg bw). Malondialdehyde (MDA), a lipid peroxidation end-product, and 2,3-dihydroxybenzoic acid (2,3-DHBA), a marker of *in vivo* hydroxyl radical production, were used to evaluate the oxidative stress generation. BPA causes the increasing of total MDA plasma levels. The simultaneous administration of TP extract countered the effect of BPA on lipid peroxidation. BPA is not a significant source of free hydroxyl radicals *in vivo*. However, TP extract diminished *in vivo* hydroxyl radical generation compared with control group ($p < 0.05$ after 90 days). In conclusion, TP showed a protective antioxidant effect that increased with prolonged period of repeated administration.

Rezumat

Bisfenolul A (BPA), un contaminant alimentar cedat de ambalajele din plastic, are proprietăți xenoestrogene și induce stres oxidativ. *Trifolium pratense* L. (TP) este o sursă naturală de fitoestrogeni și polifenoli. S-a evaluat potențialul antioxidant și efectul protector al unui extract hidroalcoolic de TP la femele de șobolan expuse subcronic la BPA. S-au analizat probe de plasmă după 30, 60 și 90 de zile de expunere la BPA (50 mg/kg corp) și/sau tinctură diluată de TP (2 mg izoflavone totale / kg corp). Pentru evaluarea inducerii stresului oxidativ s-au determinat malondialdehida (MDA), un produs final al peroxidării lipidice, și acidul 2,3-dihidroxibenzoic (2,3-DHBA), un marker al producerii

radicalilor hidroxil *in vivo*. BPA a determinat creșterea nivelelor plasmatiche de MDA totală, dar administrarea simultană a extractului de TP a atenuat semnificativ efectul lui asupra peroxidării lipidice. BPA nu este o sursă semnificativă de radicali hidroxil liberi *in vivo*. Totuși, extractul de TP a diminuat formarea radicalilor hidroxil *in vivo* comparativ cu grupul de control ($p < 0.05$ după 90 de zile). În concluzie, TP prezintă un efect antioxidant protector, care crește în intensitate odată cu prelungirea perioadei de administrare.

Keywords: Xenoestrogens, bisphenol A, *Trifolium pratense* L. (TP), oxidative stress.

Introduction

Red clover (*Trifolium pratense* L.) (TP) is rich in isoflavones and polyphenolic substances which are known for their potential antioxidant properties [26]. The main isoflavones are daidzein and genistein, and their precursor methylated forms, formononetin and biochanin A. Recent studies have shown that isoflavones from red clover have besides estrogenic effects, a number of other effects such as inhibition of angiogenesis [9], neuroprotection [2], peroxisome proliferator-activated receptor gamma (PPAR γ) activation and amelioration of the metabolic syndrome [15], anti-inflammatory and antiatherogenic effects [23].

Bisphenol A (BPA) is known as a xenoestrogen molecule. It is used in the manufacturing of polycarbonate plastics, epoxy resins, food packaging and coatings, dental sealants, rubber chemicals and flame retardants. Exposure to BPA is almost ubiquitous in present; over 90% of Americans have detectable levels of BPA in their bodies [1]. BPA has a modest estrogenic activity, with a relative binding affinity for estrogenic receptors ~10,000 times smaller than estradiol [6]. BPA is not mutagenic but can exhibit genotoxic activity and can induce oxidative stress [25, 8, 5, 22]. The NOAEL (no-observed-adverse-effect-level) of BPA in rats and mice is 5 mg/kg bw/day, while the LOAEL (lowest-observed-adverse-effect-level) is 50 mg/kg bw/day [27].

Oxidative stress generation may increase the vulnerability of the exposed tissues to injury. The aim of the present study was to evaluate the antioxidant potential and the protective effect of *Trifolium pratense* L. in nonpregnant adult female rats subchronically exposed to BPA. Plasma levels of two biomarkers were determined in order to evaluate the oxidative stress generation: malondialdehyde (MDA), a lipid peroxidation end-product [19, 7], and 2,3-dihydroxybenzoic acid (2,3-DHBA), a marker of *in vivo* hydroxyl radical production [20, 11]. In many studies, lipid peroxidation is evaluated after MDA derivatization with thiobarbituric acid (TBA), but this assay has low specificity and can lead to overestimated results. MDA measurement by high performance liquid chromatography

(HPLC) after derivatization with 2,4-dinitrophenylhydrazine (DNPH) should be preferred to TBA techniques (spectrophotometric TBA test or HPLC methods after derivatization with TBA) [12]. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) offers considerable advantages for 2,3-DHBA level assay through its powerful performances: speed, selectivity, sensitivity and robustness, and simple and rapid sample preparation [20].

Materials and Methods

Chemicals. Bisphenol A (2,2-bis(4-hydroxyphenyl)propane) of analytical-reagent grade ($\geq 99\%$) was purchased from Sigma-Aldrich (USA). HPLC-grade acetonitrile, methanol, formic acid, ammonium acetate, and hexane were purchased from Merck (Darmstadt, Germany). All other chemicals were analytical reagent grade and they were obtained from Merck (sodium hydroxide, hydrochloric acid, 2,4-dinitrophenylhydrazine (DNPH), acetaldehyde, sodium salicylate). Deionised water was obtained using a Milli-Q water purification system (Millipore, Milford, MA, USA).

Preparation of the tincture of *Trifolium pratense* L (TP). Plants of red clover (*Trifolium pratense* L., fam. *Fabaceae*) were collected from organic culture near Cluj-Napoca, România, in July 2008, from S.C. PlantExtrakt S.R.L., Rădaia-Cluj, România, and authenticated by the Quality Control Department from S.C. PlantExtrakt S.R.L. (batch 073108A). Voucher specimens were deposited in the Herbarium of the S.C. PlantExtrakt S.R.L. The mother tincture for homeopathic preparations was prepared from fresh herba of TP using 14 g of 70% (v/v) ethanol for 10 g of fresh plant material [3]. The active compounds were extracted by cold extraction (maceration). The vegetal product and solvent were let to stand for 10 days in a dark place, with a short gentle stirring once daily. The tincture was obtained by pressing the plant-ethanol mixture, followed by filtration.

Animals. Female Wistar albino rats (body weight 146 ± 18 g) were supplied from the Practical Skills and Experimental Medicine Center of “Iuliu Hațieganu” University of Medicine and Pharmacy Cluj-Napoca (România). The rats were housed in large polypropylene cages (six animals per cage) and maintained under standard conditions ($25 \pm 3^\circ\text{C}$, 35-60% humidity, 12 h light/12 h dark cycle). They had access to standard dry pellet diet and water *ad libitum* throughout the experiment.

Experimental protocol. The experimental protocol was in compliance with the EEC Directive of 1986 (86/609/EEC) for laboratory animal experiments, being approved by the Ethics Committee of the “Iuliu

Hațieganu” University of Medicine and Pharmacy Cluj-Napoca (Romania). The protocol included three experimental groups and a negative control group, each consisting of 12 rats. The substances were administered daily for 90 days: BPA (50 mg/kg bw) in food and TP tincture diluted in water by gastric intubation (2 mg total isoflavones / kg bw), as following: group I – only BPA; group II – TP hydroalcoholic extract and BPA; group III – only TP diluted tincture. Group IV served as negative control group and received standard food without substances.

Plasma samples. Sodium salicylate (equivalent dose of 500 mg salicylic acid / kg bw) was administered orally (by gastric intubation) to all animals as *in vivo* hydroxyl radical trap on the 30th, 60th and 90th days of the experiment, respectively. The tested substances were administered after 30 min and the blood was collected from the retro-orbital sinus in the presence of sodium fluoride as anticoagulant after other 60 min. Plasma sampling was performed by a veterinary specialist at the Practical Skills and Experimental Medicine Center, using tetracaine drops for local anaesthesia. Plasma separated by centrifugation (3000 g / 15 min) was stored until the analysis at -20°C.

Determination of total MDA levels. Plasmatic MDA bound to proteins was hydrolysed with 6M NaOH and derivatized with 5mM DNPH in 2M HCl at room temperature, protected from light. The resulted hydrazone was extracted in hexane and the residues obtained after evaporation were dissolved in mobile phase and injected into HPLC system. Chromatographic separation was achieved on a Spherisorb ODS column (250mmx4mm, 5µm) with a Spherisorb ODS (20mmx4mm, 3µm) guard column, maintained at 25°C, and with a mixture of 1% formic acid/acetonitrile (62/38, v/v) as the mobile phase. The flow rate was 1mL/min and the absorbance of the eluent was monitored at 307 nm [7].

Determination of 2,3-DHBA levels. Plasma samples were deproteinized with acetonitrile. After vortex-mixing and centrifugation, the supernatants were transferred in autosampler vials and analysed. Chromatographic separation was performed on a Luna HILIC (100 x 2.0 mm, 4 µm) column maintained at 15°C, using a mixture of 50 mM ammonium acetate in water (pH 4.5)/acetonitrile (6:94, v/v) with a flow rate of 0.5 mL/min. The detection was performed in multiple reaction monitoring (MRM) mode using an ion trap mass spectrometer equipped with an electrospray ionization ion source (ESI), operated in negative mode. The ion transition monitored was m/z 153 → m/z 109 [20].

Statistical analysis. The results are presented as mean values ± standard deviation and the experimental groups were compared with the

control group and between them by ANOVA analysis of variance using Origin 4.1 computer software. The difference showing a p level of 0.05 or lower was considered to be statistically significant.

Results and Discussion

A mother tincture for homeopathic preparations was prepared from fresh herbs of *Trifolium pratense* L. (TP), according to the 6th edition of the European Pharmacopoeia – mother tinctures for homeopathic preparation [3]. The obtained *Trifolium pratense* L. tincture was analysed in order to determine the main quality parameters. The extract had 2.06 % dry residue, a relative density of 0.925 and 54 % volume ethanol content. The dry residue was obtained by drying in a thermostat oven. The relative density was determined using an Anton Paar DMA35 density meter. The ethanol content was obtained by distillation followed by determination of relative density of the distilled ethanol, according to the 7th edition of European Pharmacopoeia method [4].

The total isoflavones content of TP tincture (the sum of three glycosides: daidzin, genistin, ononin, and four aglycones: daidzein, glycitein, genistein, and formononetin) determined by a LC-MS/MS method was of 32.08 mg per 100 g fresh vegetable material, with a very high content in formononetin (79.4 %) [21]. Hyperoside (9.766 µg/mL) and isoquercetin (11.206 µg/mL) were determined as main polyphenols. The presence of p-coumaric acid and ferulic acid was observed only in hydrolysed extract due to the fact that these compounds are found natively bounded. The main aglycones found in *Trifolium pratense* L. by other authors are quercetin and kaempferol. However, in Romanian *Trifolium pratense* L. (TP) only the presence of quercetin (5.581 µg/mL) was remarked [17].

TP tincture diluted in water (2 mg total isoflavones / kg bw) and / or BPA (50 mg/kg bw) were administered daily to nonpregnant adult female rats for 90 days. A negative control group was used in parallel.

Plasma samples were collected on the 30th, 60th and 90th days of the experiment after oral administration of sodium salicylate as *in vivo* hydroxyl radical scavenger and were stored until the time of analysis at -20°C.

Effect on oxidative stress induced by lipid peroxidation

Total MDA levels were quantified by a HPLC method [7]. Oral exposure of nonpregnant adult female rats to a dose of 50 mg BPA/kg bw/day increased the lipid peroxidation process with the increase of total MDA plasma levels, statistically significant after 30 days ($p < 0.05$) (Figure 1). There are numerous reports on the *in vivo* toxicity of BPA mediated by

reactive oxygen species (ROS) induction [22, 21, 5]. The main metabolic pathway of BPA is detoxification by glucuronidation. A minor route is the oxidation by hydroxylation to a catechol, 3-hydroxy-bisphenol A, followed by further transformation to an o-quinone, bisphenol A-3,4-quinone. The catechol-o-quinone couple is capable of redox cycling with generation of ROS and oxidative stress [8].

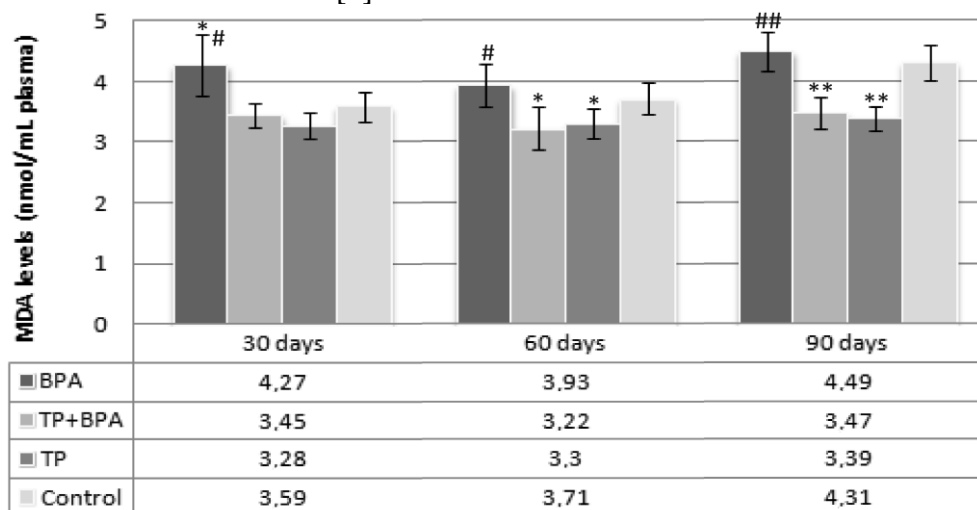


Figure 1.

Effect of bisphenol A, *Trifolium pratense L.* (TP) extract and their association on total MDA level (nmol/mL) in rat plasma. The results are presented as the mean \pm SD (n = 6): * $p < 0.05$ and ** $p < 0.01$ compared with the control group; # $p < 0.01$ and ### $p < 0.001$ compared with the TP+BPA group

Subchronic administration of TP diluted tincture (2 mg total isoflavones/kg bw/day) reduced the MDA level compared with the control group, the effect becoming more pronounced with increasing exposure time (statistically significant after 60 days, $p < 0.05$, and 90 days of exposure, $p < 0.01$) (Figure 1). In the case of simultaneous exposure to BPA and TP extract, the lipid peroxidation level diminished compared to TP group. The simultaneous administration of TP extract cancelled the effect of BPA on lipid peroxidation (statistically significant change between BPA group and TP+BPA group: $p < 0.01$ after 30 and 60 days of exposure, and $p < 0.001$ after 90 days, respectively) (Figure 1). The results prove the protective antioxidant effect of tested TP extract and they are in accordance with other studies.

Polyphenols are strong antioxidants that can neutralize free radicals, suppress the generation of the free radicals and act as direct radical scavengers of the lipid peroxidation chain reactions [26]. Thus, oral

administration of formononetin isolated from red clover for 6 months increased the antioxidant enzymes activities and reduced MDA level in ovariectomized mice [14]. Genistein, an another isoflavone present in red clover, decreased ROS and lipoperoxide level in different animal models [13, 28]. More recent studies highlight the protective effect of quercetin, the main flavonol aglycone from red clover, against lipid peroxidation induced *in vivo* by various xenobiotics [16, 24, 18].

Effect on oxidative stress induced by hydroxyl radical generation

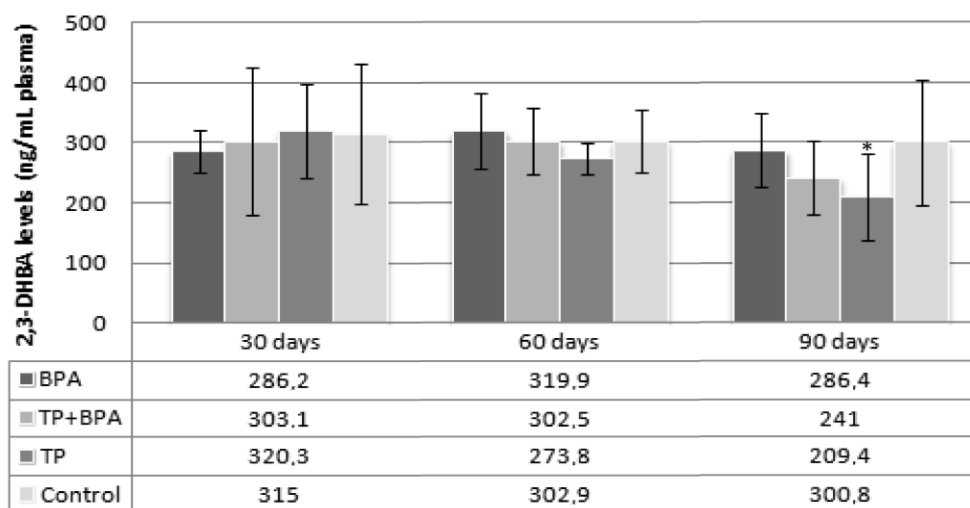


Figure 2.

Effect of bisphenol A, *Trifolium pratense L.* (TP) extract and their association on 2,3-DHBA level (ng/mL) in rat plasma. The results are presented as the mean \pm SD (n = 6): * $p < 0.05$ compared with the control group

2,3-DHBA is a metabolite of salicylic acid formed *in vivo* only in the presence of free hydroxyl radicals and it is determined as marker of *in vivo* hydroxyl radical generation. 2,3-DHBA levels were determined by a LC-MS/MS method [20]. The obtained results are shown in Figure 2. No significant changes were observed for 2,3-DHBA levels compared to the control group in case of animals treated only with BPA. Therefore, BPA is not a significant source of free hydroxyl radicals. However, TP extract diminished *in vivo* hydroxyl radical generation compared with the control group, with statistical significance after 90 days of exposure ($p < 0.05$). This suggests an antioxidant protective effect of TP extract by inhibiting the formation of free hydroxyl radicals, but obviously only after prolonged repeated administration. Kroyer *et al* also found a pronounced radical

scavenging capacity of red clover and its correlation with a remarkably high level of total polyphenols [10].

Conclusions

In conclusion, *Trifolium pratense* L. (TP) tincture proved a protective effect on oxidative stress induced by BPA in rats. The effect was proved by a significant decrease of lipid peroxidation level. Even if BPA is not a source of *in vivo* free hydroxyl radical generation, the results suggest a potential capacity of TP to inhibit the hydroxyl radical induction. The intensity of protective effect of TP tincture increased with prolonged period of repeated administration.

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