The Running Title: Yanka Karamalakova et al., TINOSPORA CORDIFOLIA…

INFLUENCE OF OCHRATOXIN-A AND AN EXTRACT OF TINOSPORA CORDIFOLIA AGAINST BIOCHEMICAL AND OXIDATIVE CHANGES IN MICE SPLEEN

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INTRODUCTION

Ochratoxin A (OTA) is a secondary mycotoxic metabolite produced mainly by strains of *Aspergillus ochraceus* and *Penicillium verrucosum* species, encountered mainly in stored forages with increased humidity. The aim of the present investigation was to study the combined effect of OTA and *Tinospora Cordifolia* (TC) on body weight, changes in some biochemical parameters, and changes in "real time" levels of Ascorbate radicals (Asc.), $NO^{\cdot}$ radicals and ROS production and lipid peroxidation in spleen of Balb/c mice, as well as to elucidate the protective efficacy of toxins induced by OTA by extract of TC.

A variety of previous epidemiological studies have shown that OTA could be involved in tumor formation in animals, in the pathogenesis of a large number of human nephropathies, renal cancer etc. OTA has been classified as a possible carcinogen and recent studies have shown that oxidative pathways/ changes and genotoxicity are the key points for renal toxicity and carcinogenicity (Pfohl-Leszkowicz and Manderville 2007; Denli and Perez, 2010). Pharmacokinetics studies revealed consumption of orally given OTA-contaminated feed showed faster uptake in the blood through the gastrointestinal tract which was followed by relatively slow elimination in urine and feces (Denli and Perez, 2010; Mantle, 2008). OTA, due to its polar

composition, is easily distributed in the body fluids along with different organs (*liver*, *kidneys*, *spleen*, *muscles*) and tissues of mice, rats, pigs, rabbits, chickens (Denli and Perez, 2010; Zepnik et al., 2009; Milićević et al., 2009). Some part of OTA also distributed and found in muscles and in the eggs of hens and chickens (Denli and Perez, 2010; Piskorska-Pliszczyńka and Juszkiewicz, 1990) and also found in blood and milk in various rodents (Denli and Perez, 2010).
Tinospora cordifolia (TC), used as an antioxidant/ antidote in this study, has been recommended from Ayurvedic literature as a compound with variety of active components like alkaloids, steroids, diterpenoid lactones, aliphatics, and glycosides have been isolated from the different parts of the plant body (Upadhyay et al., 2010). As strong antioxidant TC stimulates bile secretion; enlarged spleen; enriches the blood; causes constipation; allays thirst, fever, burning sensation, prevents vomiting; diuretic; cures jaundice; useful in skin diseases; the juice is useful in diabetes, vaginal and urethral discharges (Kirtikar and Basu, 2007; Krishna et al., 2008) general debility, dyspepsia, fever and urinary diseases. Extracts of TC are possible inducers of other antioxidant agents and significantly reduce chemotherapy-induced toxicity of free radicals and lipid peroxidation levels ([a]Rawal et al., 2010). Many outers showed that the extract of TC, with strong antioxidant mechanism, exhibits protective effect in rat hippocampal slices, attenuated the oxidative stress mediated in cells and also has strong free radical scavenging properties against reactive oxygen and nitrogen species, studied by the electron paramagnetic resonance spectroscopy ([b]Rawal et al., 2004). The availability of alkaloids such as choline, tinosporin, isocolumbin, palmatine, tetrahydropalmatine, and magnoflorine, in aqueous and alcoholic extract of TC showed detoxicative effect against OTA and protection of aflatoxin- induced organ-toxicity (Gupta and Sharma, 2011).

MATERIALS AND METHODS

Experimental feed: The contamination of forages with ochratoxin A (OTA) was produced by offungus named *Aspergillus ochraceus* witch produced OTA per kg: 15 ppm solid medium. After that, OTA-contaminated barley flakes were mixed periodically with the feeds for mice, at room temperature 18-23°C.

Collection of the plant material and preparation of TC extract: The stem part of TC for experiment purpose was collected from authenticated Ayurvedic store from a local market at New Delhi, during the month of November, 2013 and was confirmed by expert botanist. Dried stems of TC were cleaned to separate unwanted material and then grinded into coarse powder using mortar and pestle. Fine powder was obtained after grinding in grinder and then passing through sieve. Powdered stems were extracted by Kinetic maceration for 48 hrs using 100% ethanol as solvent. The whole mixture then underwent a coarse filtration by a piece of clean, white muslin cloth which was followed by filtered through whatman no.1 filter paper. The total filtrate was dried till semi-liquid using rotary evaporator (Buchi B-480, India) at 40^0C and was further lyophilized using lyophilizer (IIshin Lab Co. Ltd, USA) to get the crude extract, and was used as a practical approach to supply the mice with described compounds presumed to protect against OTA intoxication in spleen.

Chemicals*:* OTA was purchased and providing from Trakia University, Bulgaria as reference. Dimethyl sulfoxide (DMSO), N-tert-butyl-alpha-phenylnitrone (PBN), 2-(4-carboxyphenyl)-4,4,5,5-tetra-methylimidazoline-1oxyl-3-oxide (Carboxy-PTIO.K) and PBS were purchased from

Sigma Chemical Co, St. Louis, USA. All the chemicals used in this study were with analytical grade.

Experimental animals: Specific pathogen-free male Balb/c mice (second line, non- inbred, weighted 25-35 g mice, 5-6 weeks old) were purchased at two-weeks of age, housed in polycarbonate wire floor cages in controlled conditions (12 h light/ dark cycles), temperature of 18–23^{o}C suitable for their age and humidity of 40–60%, with free access to tap water and standard laboratory chow were maintained. Mice were grouped in 3 experimental groups and 1control group (6 animals in each one/ oral pretreatment/ 11 days experiment) and fed respectively: control group - OTA free, standard diet; group I - TC extract, 80 mg/kg, given 3 times for period of 11 days; group II - 15 ppm OTA, daily in diet; group III – 15 ppm OTA and 80 mg/kg extract of TC. All mice were carefully examined and weighed at 3th, 7th and 11th day (**Table 1**), and the consumed feed was measured at the end of experiment. For following up the weight and the changes of spleen and internal organs, organs of all treated groups and controls were compared. Experiments were carried out in accordance with national regulations and the European directive 210/63/EU from 22.09.2010, concerning the protection of animals used for scientific and experimental purposes. After 11th day the mice were dissected and spleen tissues were homogenase in cold PBS solution and studied by direct and spin trapping EPR spectroscopy and spectrophotometric biochemical analyses. Results were analyzed and compared to those of non treated controls.

Electron Paramagnetic Resonance measurements: For all EPR measurements an X-band EMX^{micro}, EPR spectrometer (Bruker, Germany) equipped with standard Resonator was used. Spectral processing was performed using Bruker WIN-EPR and SimFonia software. The levels of the Asc$^{\cdot}$, NO$^{\cdot}$ radicals and ROS production in experimental mice were calculated by double integration of the corresponding EPR spectra registered in the spleen (*arbitrary units*).

***Ex vivo* assay the levels of ROS production in the spleen homogenates of mice by EPR spectroscopy:** The level of ROS productions was studied according to Shi et al., 2005 with some modifications by Zheleva et al., 2011. Briefly, about 0.1 g of spleen samples were homogenized after addition 1.0 ml of 50 mM solution of the spin-trapping agent PBN dissolved in DMSO. EPR settings were as follow: center field 3503 G; sweep width 10.0 G; microwave power 12.83 mW; receiver gain 1 x 10^6; mod. amplitude 5.00 G; time constant 327.68 ms; sweep time 81.92s, 5 scans per sample.

***Ex vivo* assay of the ascorbate radicals levels in the spleen tissue homogenates of mice by EPR spectroscopy:** The Asc$^{\cdot}$ levels in organ homogenates were studied according to Buettner & Jurkiewicz, 1993 with slight modifications. Tissues from spleen were collected in cold saline and processed immediately. Tissue samples were weighed and homogenized in DMSO (10% w/v) and centrifuged at 4000 g, at 4^0C for 10 min. Supernatants were collected and the level of Asc$^{\cdot}$ was evaluated by EPR spectroscopy. EPR settings were as follows: center field 3505 G; sweep width 30 G; microwave power 12.70 mW; receiver gain 1 x 10^4; mod. amplitude 5.00 G; time constant 327.68 ms; sweep time 82.94 s; 1 scans per sample.

***Ex vivo* assay of the nitric oxide levels in the spleen tissue homogenates of mice by EPR spectroscopy**: The levels of •NO radicals were studied according to methods of Yoshioka et al.,1994 and Yokoyama et al.,2004 with some modification. Briefly, to 50μM solution of Carboxy

PTIO.K dissolved in a mixture of 50 mMTris (pH 7.5) and DMSO in a ratio 9:1. To 100µl tissues was added 900µl Tris buffer dissolved in DMSO (9:1) after that the mixture was centrifuged at 4000 rpm for 10 min at 4ºC. 100 µL of sample and 100 µL 50 mM solution of Carboxy PTIO were mixed and EPR spectrum of the spin adduct formed between Carboxy PTIO spin trap and generated •NO radicals was recorded. The EPR settings were as follows: 3505 G centerfield, 6.42mW microwave power, 5G modulation amplitude, 75G sweep widh, 2.5x102 gain, 40.96 ms time constant, 60.42 s sweep time, 1 scan per sample.

***Ex vivo* biochemical analyses of MDA measured specrtophotometrically:** The method estimation of lipid peroxidation of thiobarbituric acid (TBA), which measures Malondialdehyde (MDA)-reactive products, was used (Plaser et al. 1966). In brief, 0.5 mg fresh spleen-tissues, 1 ml physiological solution, and 1 ml 25% trichloroacetic acid were mixed and centrifuged at 7,000 rpm for 20 min. 2 ml protein-free supernatant with 0.5 ml 1% TBA (prepared in 0.025 M NaOH) were added in the reaction mixture. The resultant mixture was then subjected to 95°C for 1 h in a water bath. A pink coloured chromogen complex was formed, readable at 532 nm.

Statistical Analysis: Statistical analysis was performed with Statistica 8.0, StaSoft, Inc. and results were expressed as means ± standard error (SE). A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Ochratoxin-A provokes oxidative stress (Pfohl-Leszkowicz et al., 2007), and causes changes in organs, body weight and food consumption of animals (El-Nekeety et al., 2007; Saad and Abdel-Fattah, 2008). Pretreatment of animals with natural agents such as vitamin A, C and E possessing free radicals potentials and antioxidant- protective properties (Haazele et al., 1993, Stoev et al., 2000; Goel et al., 2002; Subramanianet al., 2002), before OTA administration, significantly reduces the oxidative changes in experimental animals (Reddy et al., 2009; Sangeetha et al., 2011), confirmed by a decrease in the levels of the final products of DNA oxidation and lipid peroxidation and furthermore accelerates the metabolic biotransformation of the toxin.

The main clinical signs (**Table 1**) were observed predominantly in mice from group II: depression and weakness, lurch and tremor and were well expressed after two weeks from the beginning of the experiment. In other groups similar amendments have not been observed. The results of body weight didn't indicate statistically significant decreased of body weight. The present results indicated that OTA- diet group resulted in a significant decrease in food intake and consequently the body weight gain was reduced, in compare with group I, III and controls. Our findings were supported that after *in vitro* exposure to OTA, have shown decreased proliferation of neural progenitor stem cells followed by memory problems, weakness and depression (Sava et al., 2007).

Abdel Fattah and co-worker have reported reduction in the body weight and decreased food consumption in animals, treated with aflatoxins, only. They accept this finding might due to the further protein catabolism, or circuits that regulate homeostasis or decrease the levels of Leptin and re-regulation of energy balance (Abdel- Fattah et al., 2010).

group	3 th day (g)	7 th day (g)	11 th day (g)
	180.2 ± 1.4	212 ± 3.40	255.3 ± 1.25
I – TC (*mean of 6 mice*)	222.6 ± 2.53	230.9 ± 2.21	233 ± 2.14
- OTA (*mean of 6 mice*)	203.2 ± 1.77	219.9 ± 3.73	246.5 ± 1.89
III – TC + OTA (*mean of 6 mice*)	200.8 ± 3.55	216 ± 1.56	257.3 ± 4.36
Controls (*mean of 6 mice*)			
	24	24	23
Number of animals			

Table 1: The mean values of body weight in experiment in 6 mice in groups; ± SE (standard error of the mean); * significant difference towards control group ($p < 0.05$).

In the groups treated with TC and TC + OTA were observed favorable changes in body weight and food intake. Normalization of the body weight and decrease in the oxidative changes and other harmful toxic factors were found after treatment of diabetic rats with TC root extract (Stanely et al., 2000). Stoev et al., 2000 commented that OTA induces an increase in the weight of detoxifying/ eliminating organs (kidney, liver), and also reduce the weight of lymphoid organs. The relative weight of lymphoid organ-spleen was significantly increased in I and III experimental groups and controls opposite from group II on day 11 day from the beginning of the experiment, therefore *Tinospora cordifolia* extract significantly protected spleen and weight changes and stopped direct toxic effect of the toxin on the organs.

Different research groups reported strong free radical scavenging properties for *Tinospora cordifolia* extract against *ex vivo* generated superoxide anion ($^{\cdot}O_2^-$), hydroxyl radicals ($^{\cdot}OH$), $Asc^{\cdot}$ radicals, and peroxynitrite anion ($ONOO^-$) ([b]Rawal et al., 2004; Saha and Ghosh, 2012). It was demonstrated TC possessed ability to scavenge free radicals generated during aflatoxicosis and to prevent biological systems from ROS toxicity ([a]Rawal et al., 2004). Certain compounds isolated from TC showed good protection against iron-mediated lipid peroxidation of animal brain homogenates, gamma-ray induced damages (Subramanian et al., 2002) and significantly reduced ROS productions, and the possibility of oxidative stress of treated mice (Gupta and Sharma, 2011).

Results of the present study from the levels of ROS products measured in the spleen homogenates of all treated groups and controls are shown on **Figure 1**. As is seen ROS products in spleen of OTA-treated mice were significantly higher than the controls while in the group I was considerably lower than those of the control mice (mean 0.126±0.09 vs. mean 0.442 ±0.001, $p<0.00$). In group III, when the TC extract were administered along with OTA, was found a decrease in the levels of ROS products comparing to OTA-treated animals (mean 0.5331 ± 0.042 vs. mean 0.789 ± 0.28) and the control group, respectively (mean 0.5331 ± 0.082 vs. mean 0.442 ± 0.013). The study of ROS products confirmed the high oxidative toxicity of OTA. On the other hand statistically insignificant higher level of ROS measured in the group treated with the combination of TC + OTA in comparison with the controls shows that the TC extract contains antioxidant structures which can partially neutralize generated ROS. Moreover, statistically significant lower levels of ROS registered in the TC group in comparison with the other groups shows that the extract contains a sufficient amount of chemical structures having radical trapping capability, which manage to neutralize ROS, generated by the toxin, and the extract.

Our results on the levels of $Asc^{\cdot}$ radicals in the spleen homogenates of control and tested mice are shown on **Figure 2**. Levels of Asc• in the three tested groups were statistically significant lower compared with those of controls. In mice, treated with TC, alone was found almost a fourfold

lower level of Asc• ($p < 0.003$*) compared to the controls (mean 0.058±0.008 vs. mean 0.482 ±0.054). It is well known that in order to prevent other biologically active molecules from being oxidized, water soluble ascorbic acid acts as a chain breaking antioxidant scavenging free radicals such as ROS by donating electrons (Padayatty et al., 2003) and as a result Asc˙ radicals are formed. To explain the lower levels of ascorbate radicals found in spleen homogenates of TC treated mice comparing to the controls we assume that some ingredients of TC extracts act as pro-oxidants generating ROS, that causes partial depletion of the available amount of ascorbic acid confirmed by the registered low levels of Asc•.

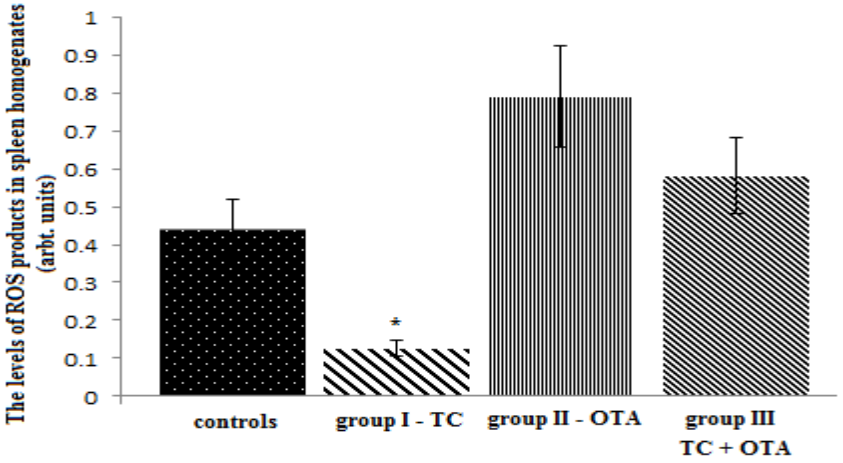

Figure 1. Levels of ROS products expressed in arbitrary units in spleen homogenates of healthy controls and mice treated with TC extract, OTA and combination TC+ OTA, $p < 0.05$; (*).

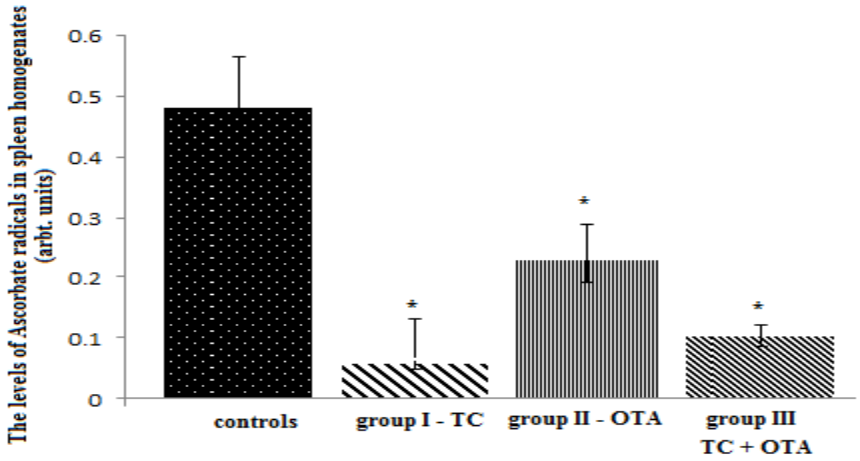

Figure 2. Levels of Asc• radicals expressed in arbitrary units in spleen homogenates of healthy controls and mice treated with with TC extract, OTA and combination TC+ OTA, $p < 0.05$; (*).

Goel and co-workers report that chronically increased oxidative stress from elevated levels of aflatoxins in the organs may increase levels of lipid peroxidation by decreasing cellular oxygen radical scavenging capacity (Goel et al., 2002), which results in modification in membrane

phospholipids and disorganization of cell function. By the present study has been demonstrated that combination of TC + OTA (group III) causes considerable decrease in the MDA levels in spleen (**Figure 3),** in comparison with the group treated with the toxin, only (mean 4.03 ± 0.29 vs. mean 5.73 ± 0.88) and were close to the controls (mean 4.03 ± 0.29 vs. mean 3.16 ± 0.35). As is seen in the group treated with TC was registered the lowest quantity of MDA reactive substances which additionally confirms scavenging abilities of TC extract against ROS generated in the spleen during lipid peroxidation processes induced by OTA. This our result was in accordance with a number of studies on the protective effect of TC extracts against oxidative toxicity induced in animals by various toxins (Singh et al., 2006; Prince et al., 2004; [a,b]Sharma V and Pandey, 2011; Gupta and Sharma, 2011)

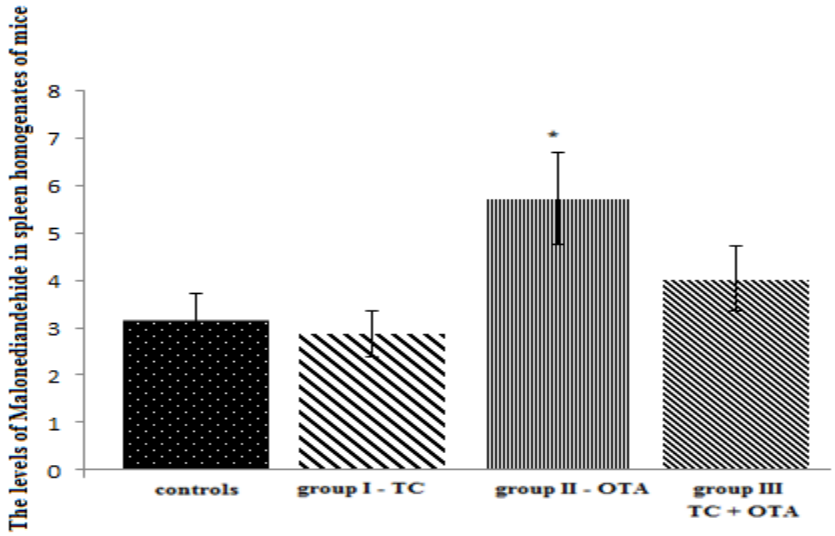

Figure 3. Levels of MDA in spleen homogenates of healthy controls and mice treated with TC extract, OTA and combination TC+ OTA, $p < 0.05$.

Using Electron Pparamagnetic Resonance (EPR) spectroscopy Rawal and co-workers have demonstrated that TC exhibited free radical scavenging properties against ROS and nitrogen species. The same authors suggest that the ability of TC to scavenge free radicals probably due to the availability of choline, tinosporin, isocolumbin, palmatine, tetrahydropalmatine, and magnoflorine in TC herb ([a]Rawal et al., 2004). Jagetia and Baliga, 2004 assume that extracts of different medicinal plants, including TC might be potent therapeutic agents for scavenging of NO and peroxynitrite, for regulation of pathological conditions caused by chronicle toxicity. As is seen on Figure 4 our results on scavenging abilities of TC extract against nitric oxide are in support of the above mentioned studies. Administration of OTA at a dose of 15 ppm caused significant increase (5.992 ± 1.01, $p<0.03$) in the levels of nitric oxide in spleen tissues as compared to the respective values of the control mice (3.441 ±0.56).

The levels of •NO radicals decreased insignificantly when administration of the toxin was combined with oral supplementation of ethanolic extract of TC at a dose of 80 mg/kg (mean 4.009 ± 0.193), but the values were close to the controls (mean 4.009 ± 0.193 vs. mean 3.441 ±0.56, $p<0.03$), respectively.

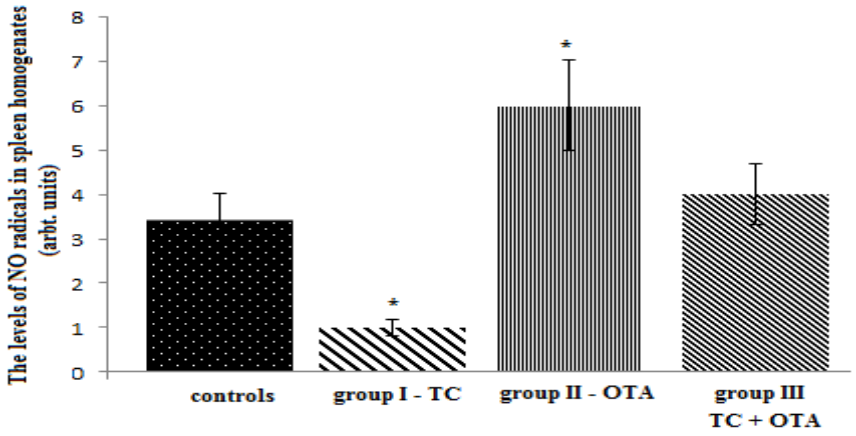

Figure 4. Levels of •NO radicals (in arbitrary units) in organ homogenates of healthy controls and mice treated with TC extract, OTA and combination TC+ OTA, $p < 0.05$; (*).

Moreover, group treated with the TC, alone showed the lowest levels of •NO radicals in the spleen tissues confirming once again the radical scavenging capabilities of TC constituents not only against ROS but RNS, as well.

CONCLUSION

In conclusion, using EPR spectroscopy we have demonstrated increased levels of some "real time" biomarkers of oxidative stress such as $Asc^{\cdot}$, $NO^{\cdot}$ radicals and ROS products in the spleen of mice after treatment by OTA. Moreover combination of OTA with oral administration of TC extract led to significant improvement in the levels of oxidative stress biomarkers in mice spleen.
In the light of these results, TC extract behaves as a good scavenger of ROS and RNS and might find application in further studies in order to find application in the pharmaceutical and food industry as a protector against various mycoses

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