AZADIRACHTA INDICA OIL AS PROTECTOR OF CHRONIC OCHRATOXIN-INDUCED NEPHROTOXICITY

Iliana Koleva-Korkelia¹, Yanka Karamalakova², Galina Nikolova³

Abstract: Ochratoxin A (OTA) mycotoxin affects protein synthesis, metabolic oxidative pathways and ROS mediation. In the present study, we investigate the antioxidative-therapeutic potential of *Azadirachta Indica Oil* (*A. indica*) against chronic (28 days) OTA-induced nephrotoxicity. Balb/c male mice were exposed to i.e., (1) controls; (2) *A. indica* treated (120 mg/kg b. wt. i.p., given every two days); (3) c) OTA treated (Isolate D2306, 1.25 mg/kg b.wt., i.p., given every two days); d) *A. indica* (120 mg/kg b.wt., i.p.) administered 2h prior to OTA-administration. Till the end of the 28 experimental days of chronic OTA-nephrotoxicity, the mortality rate (± 0) was not observed in mice. Kidney tissue was subjected for the determination of biochemical indexes (the MDA ratio, and antioxidant capability of SOD, and GSH) and EPR- ROS productions. OTA-exposure resulted in twofold significant increases in SOD ($p < 0.004$), GSH ($p < 0.05$) and malondialdehyde (MDA) levels ($p < 0.00$), compared to controls. Further, a nephro-cells experiment was performed to investigate the oxidative stress-protective action of *A. indica*. Our results showed that *A. indica* oil and *A. indica* +OTA combination inhibited OTA-induced nephrotoxicity via a significant reduction of lipid peroxidation ($p < 0.003$), ROS production ($p < 0.005$), and endogenous antioxidant activation. Thus, it can be concluded that *A. indica* treatment neutralized chronic OTA-induced nephrotoxicity, not only by reducing lipid peroxidation but also by improving antioxidant status. Through the present experiments, it was demonstrated that *A. indica* has protective potential in nephron-inflammations.

UDC Classification: 604, **DOI:** <https://doi.org/10.12955/pmp.v2.176>

Keywords: *A. indica*, OTA, ROS, nephron-inflammations

Introduction

Ochratoxins are cyclic pentaketides: dihydroisocoumarin derivatives linked to the L-phenylalanine moiety. Ochratoxin A (OTA) (R) -N - [(5-chloro-3,4-dihydro-8hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl) carbonyl] -L-phenylalanine is a mycotoxin, contaminating cereals, legumes and dried fruits. OTA was found in an isolate of *Aspergillus ochraceus*, and since then several species of *Aspergillus* and *Penicillium* have been described as producers of this mycotoxin (Petrik et al., 2003). Ochratoxin A (OTA) is a ubiquitous nephrotoxic and carcinogenic mycotoxin that is thought to be involved in the etiology of Balkan endemic nephropathy (BEN). Several studies reported that BEN correlates with a very high incidence of otherwise rare urothelial tumors of the renal pelvis and ureters (Atroschi et al., 2000). OTA is found more often and/or in higher concentrations in the food and blood of residents of BEN areas than in other regions. The involvement of OTA in the development of BEN is not yet very well understood. Patients with chronic renal failure treated with dialysis have higher concentrations of OTA in their blood than healthy individuals. Moreover, no decrease in OTA concentration was observed as a result of the treatment (Fuchs & Peraica, 2005).

BEN occurs without an acute phase and is accompanied by anemia of the aplastic or normochromic type and mild proteinuria without hypertension and edema. In blood samples there is a gradual increase in nitrogen and creatinine levels and in the advanced stages of the disease hypertension is reported (Varga et al., 2010). Histopathological changes in the early stages of the disease report degenerative and regenerative processes of the epithelial cells of the proximal tubules in the kidneys without a change in organ size. An increased amount of light eosinophilic, cell-free material is seen throughout the interstitium of the cortex. There are no signs of inflammation and glomerular lesions. In chronic cases, the size of the kidneys is significantly reduced and diffuse cortical fibrosis without inflammatory cells is the most important finding. BEN is more common in women than in men (Fuchs & Peraica, 2005).

In recent years, the use of herbal preparations and active ingredients of medicinal plants in healthcare is becoming more common. *Azadirachta indica* (*A. indica*, *Neem*) is one of the most widely used plant extracts. A tonic is prepared from its bark, which is used as a medicine against various skin diseases (Silva et al., 2015). In addition, the bark of *A. indica* exhibits antitumor and interferon activity, and

¹ Trakia University, Medical Faculty, Obstetrics and Gynecology Clinic, Stara Zagora, Bulgaria, iliana_mih@abv.bg

² Trakia University, Medical Faculty, Chemistry and Biochemistry, Stara Zagora, Bulgaria, ykaramalakova@gmail.com

³ Trakia University, Medical Faculty, Chemistry and Biochemistry, Stara Zagora, Bulgaria, gnikkolova@gmail.com

other parts of the plant have antibacterial, antifungal, antimalarial and anticancer effects. Neem oil has anti-fertility activity and stimulates a cell mediated immune response (Ezz-Din et al., 2011).

In Asian countries, *Azadirachta indica* (A. Juss. Neem, Meliaceae) is used as a fertilizer and to control pests in cereals. In fact, in many regions, this fertilizer is a residue from the pressing of Neem seeds in oil extraction, which is used as a botanical insecticide and provides toxicity to maize caterpillars. In most pest control studies, the use of neem seed oil is for cereal protection purposes (Abireh et al., 2020).

In the present study we investigated the antioxidant–therapeutic potential of *Azadirachta Indica Oil* (*A. indica*) in kidney tissue against chronic (28 days) OTA-induced nephrotoxicity. Kidney tissue was subjected to biochemistry analyses with ELISA methods to assay the Malondialdehyde (MDA) ratio, and antioxidant capability of Superoxide dismutase (SOD), and Glutathione (GSH).

All biochemical results were compared to the levels of reactive oxygen species (ROS) productions measured with Electron Paramagnetic Resonance (EPR) spectroscopy.

Materials and Methods

Animals

Experiments were conducted in male Balb/c mice (30–45 g; n = 6 of each), maintained in the animal care facility at the Experimental Laboratory of the Medical Faculty, Trakia University, Stara Zagora, Bulgaria. The animals were fed a normal chow diet and water ad libitum and housed under standard conditions with a controlled temperature (~22 °C) and humidity (~60%) and were exposed to a 12/12-h light/dark cycle. All biological assays were performed in accordance with the guidelines for the care and handling of laboratory animals as recommended by the Ethical Committee for Animals. All protocols were previously approved by the Directive 2010/63/EU/Ethical Committee for Animals of BABH and Trakia University, Stara Zagora, Bulgaria (131/6000-0333/09.12.2016).

Mice were randomly divided into four groups as follows: (1) controls; (2) *A. indica* treated (120 mg/kg b.wt, i.p., given every two days); (3) OTA treated (Isolate D2306, 1.25 mg/kg b.wt, i.p., given every two days); (4) *A. indica* (120 mg/kg b.wt, i.p.) administered 2h prior to OTA-administration. Till the end of 28 experimental days of chronic OTA-nephrotoxicity, the mortality rate (± 0) was not observed in mice.

Biochemistry analyses

At the end of experiment (the 28th day), the mice were anesthetized with Nembutal (50 mg/kg i.p.) and sacrificed. The freshly isolated (un-extravasation with cold 0.9% saline) kidney tissue of all six animals from each group were collected in ice. After homogenization and the addition of solvents, the samples were centrifuged in 4000 rpm at 4°C for 10 min and 300µl of supernatant were stored at -40°C until further assay was done.

Immuno-enzymatic methods

Standard ELISA kits were used to determine the levels of the antioxidant enzymes: Superoxide Dismutase (SOD), and Reduced Glutathione Peroxidase (GSH-Px). The standard ELISA kit was used for Malondialdehyde (MDA) measurements. All enzyme-linked immunosorbent assays were performed according to the procedure described in the respective kit.

Electron paramagnetic resonance (EPR) spectroscopy and ROS evaluation in kidney tissue

EPR spectral processing was performed using Bruker Win-EPR and Sim-fonia Software. The ROS level measurement was according to Shi et al. (2005). The real-time formation of ROS in the serum was investigated by mixing the samples with PBN spin trapping. EPR measurements of all tested samples were conducted at room temperature (18-23°C) on an X-band EMX^{micro}, spectrometer (Bruker, Germany), equipped with a standard Resonator. Quartz capillaries were used as sample tubes.

NO· evaluation in kidney tissue

Based on the methods of Yoshioka et al, (1996) and Yokoyama et al. (2004) we developed and adapted the EPR method for the evaluation the NO radical (NO·) levels. The EPR spectrum of the spin adducts formed between the spin trap Carboxy. PTIO and generated NO· was recorded. NO· levels were calculated as double integrated plots of EPR spectra and the results were expressed in arbitrary units. The EPR settings were: 3505 G centerfield, 6.42 mW microwave power, 5 G modulation

amplitude, 75 G sweep width, 2.5×10^2 gain, 40.96 ms time constant, 60.42 s sweep time, 1 scan per sample.

Asc $\cdot$ evaluation in kidney tissue

Ascorbate radical (Asc $\cdot$) levels were studied according to Bailey (2004), with some modification. EPR settings were as follows: 3505.00 G center field, 20.00 mW microwave power, 1.00 G modulation amplitude, 15 G sweep width, 1×10^5 gain, 40.96 ms time constant, 60.42 s sweep time, 10 scans per sample.

Statistical Analysis

Statistical analysis was performed using Statistic 8.0 (Stasoft, Inc.), one-way ANOVA Student- T-tests to determine significant difference among data groups. The results were expressed as means $\pm$ standard error (SE). A value of $p < 0.05$ was considered statistically significant.

Results

In the present study we investigated the antioxidant–therapeutic potential of *Azadirachta Indica* Oil (*A. indica*) in kidney tissue against chronic (28 days) OTA-induced nephrotoxicity.

The results were compared with mice treated with OTA alone and with combination *A. indica* + OTA. Based on literature data (Rodrigues et al., 2019), we selected the 120 mg/kg body weight dose as the "protector" dose for *Azadirachta Indica* Oil (*A. indica*).

ROS levels in kidney tissue

Figure 1 shows the level of ROS, Asc radicals and NO radicals in healthy untreated mice and mice treated with OTA, *A. indica* and combinations of *A. indica* and OTA.

The ROS level (Figure 1) in kidney tissue of mice treated with OTA were statistically significantly higher compare to the healthy controls (mean 3.38 ± 0.4 vs mean 1.36 ± 0.1 , t-test, $p=0.000$) and the group treated with *A. indica* (mean 3.38 ± 0.4 vs mean 1.49 ± 0.08 , t-test, $p=0.000$). The combination of *A. indica* + OTA showed a statistically significant decrease compared to the OTA group (mean 2.00 ± 0.07 vs mean 3.38 ± 0.4 , t-test, $p=0.1$). The results from the group treated with combination *A. indica* + OTA was higher, but not statistically compare to the controls (mean 2.00 ± 0.07 vs mean 1.36 ± 0.1 , t-test, $p=0.000$), and *A. indica* group (mean 2.00 ± 0.07 vs mean 1.49 ± 0.08 , t-test, $p=0.000$).

NO radical levels in kidney tissue

Figure 1 presents a statistically significant increase in the NO levels in kidney homogenate from mice treated with OTA compared to control (< 0.001). The groups treated with combination OTA + *A. indica* show a statistically insignificant reduction compared to the OTA group, and a statistically significant increase in the NO levels compared to mice treated only with *A. indica* and control.

Asc $\cdot$ levels in kidney tissue

The levels of Asc radicals in the group treated with OTA are statistically significant higher compared to the control group (mean 1.92 ± 0.01 vs mean 0.59 ± 0.003 , t-test, $p=0.000$) and the *A. indica* group

(mean 1.92 ± 0.01 vs mean 0.84 ± 0.02 , t-test, $p=0.000$). The group treated with combination OTA + *A. indica* show statistically significant lower levels relative to samples treated only with OTA, and statistically insignificant higher levels compare to the control ($p=0.1$) and *A. indica* ($p=0.3$).

Lipid Peroxidation levels

Products of lipid peroxidation measured as the level of MDA (Table 1) in the group treated with OTA was statistically significantly higher to controls ($p<0.00$, t –test). The statistically significant increase compared to controls was observed in the group treated with combination OTA + *A. indica*. The MDA levels were statistically insignificantly lower in the *A. indica* treated group compared to controls ($p = 0.3$) and the combination OTA + *A. indica* group ($p=0.1$).

The table 1 present results from Enzyme linked immunoassays

Table 1:				
	group	MDA	SOD	GSH-Px
	control	2.545 ± 0.07	12.881 ± 0.4	62.486 ± 0.6
	OTA	4.248 ± 0.2 ($p<0.05$) vs control	3.271 ± 0.1 ($p<0.05$) vs control	23.483 ± 1.1 ($p<0.05$) vs control
	<i>A. indica</i>	2.406 ± 0.1	12.931 ± 0.6 vs OTA group	61.451 ± 1.6
	<i>A. indica</i> + OTA	3.311 ± 0.12 ($p<0.05$) vs control	10.588 ± 0.1 ($p<0.05$) vs control	50.943 ± 0.79 ($p<0.05$) vs control
Source: Author				

Antioxidan enzyme -SOD levels

SOD levels (table 1) were statistically significantly lower in the OTA treated group compared to the control ($p<0.00$), the *A. indica* group ($p<0.00$), and the combination *A. indica* + OTA group ($p<0.00$). A statistically significant increase was observed in the group treated with combination *A. indica* + OTA compared to control, as well as the group treated with *A. indica* alone. The levels of antioxidant enzyme SOD were statistically significantly higher in the group treated with combination *A. indica* + OTA compared to the group treated only with OTA ($p<0.00$).

Reduced Glutathione Peroxidase (GSH-Px)

The same statistically significant decrease is shown in the group treated with OTA compared to control, the *A. indica* group and the combination *A. indica* + OTA group. Also, statistically significantly lower was the result of the combination *A. indica* + OTA group compared to control ($p<0.00$) and the *A. indica* group ($p<0.00$), which were statistically significantly higher to the OTA group ($p<0.00$).

Discussion

OTA has been shown to be nephrotoxic to animals and to cause kidney nephrotoxicity in mice and rats Hoehler et al. (1997). OTA is accumulated in human food and animal feed, due to favorable weather conditions and climate, and/or to inappropriate food storage (Koszegi and Poor, 2016). According to Atroshi et al. (2000) OTA-induced ROS/RNS are elective but not mandatory apoptosis regulators. Moreover, ROS and NO have a major role in the cell damage mediation. Organism defense mechanisms attempt to minimize free radical production and harmful oxidation action; defense mechanisms include antioxidant enzymes -SOD, and GSH-Px, as well as natural antioxidants. Petrik et al. (2003) induced apoptosis with OTA in the kidneys of rats and reported that apoptosis was accompanied by oxidative stress, which manifested itself in elevated MDA levels, increased ROS, and statistically significantly reduced SOD activity. ROS and RNS levels may be further elevated in OTA-treated cells. Moreover, OTA promotes the expression of both the enzyme responsible for inducing nitric oxide synthase (iNOS) and the activity of dimethylarginine dimethylaminohydrolase (DDAH). According to Sorrenti et al. (2013) elevated levels of iNOS and DDAH further increase NO $\cdot$ synthesis and nitrite / nitrate concentration. Therefore, high NO $\cdot$ levels cause nitrosative stress due to its reaction with $\cdot O_2^-$, which forms peroxynitrite (ONOO $^-$) and increases the level of nitrogen dioxide (NO $_2$) and $\cdot OH$.

Elevated levels of NO $\cdot$, NO $_2$ and $\cdot OH$ lead to a decrease in the levels of antioxidant enzymes SOD and GSH-Px. Mycotoxin-induced free radical damage is likely to be increased by lipid peroxidation chain

reactions and expressed with elevated MDA levels. In addition, ROS is involved in the regulation of cell death by inducing apoptosis and increasing $\cdot\text{O}_2^-$ formation. Our results (Table 1) show that combining OTA with an antioxidant (*A. indica*) prevents cell damage and statistically significantly increases the levels of the antioxidant enzymes -SOD and GSH-Px, while also statistically significantly decreases the levels of ROS, $\text{NO}\cdot$, $\text{Asc}\cdot$ and MDA.

These results coincide with a study by Atroshi et al. (2000) which found that after 14 days, mice treated with OTA, showed a decrease in GSH activity. The authors treated the mice with combined antioxidants, which improved the liver's antioxidant/detoxification system, expressed in elevated glutathione levels. Abireh et al. (2020) investigated the therapeutic effect of aqueous leaf extract of *Azadirachta indica* on ibuprofen-induced nephrotoxicity in Wistar rats. The authors concluded that the administration of *A. indica* leaf extract led to the resolution of ibuprofen-induced kidney damage. Thus, it may serve as an option for the treatment of renal impairment resulting from the ingestion of ibuprofen, once the molecule responsible for this effect has been identified. Like most natural antioxidants, the extract of *Azadirachta* leaves and *A. indica* oil has a high content of polyphenols. A number of studies have reported the antioxidant effects of polyphenols associated with increased inhibition of lipid peroxidation and free radical scavenging.

Conclusion

In conclusion, the use of *A. indica* oil as a protector in OTA toxicity is a promising renal agent and this protective activity of *A. indica* oil may be due to its antioxidant activity, which normalizes impaired renal function.

References

- Abireh, I. E., Ozioko, O. M., Ozor, I. I., Finbarrs-Bello, E., Ozioko, U. S., & Egbo, F. (2020). *Azadirachta indica* (Neem) Leaf Extract Effect as an Option of Treatment of Ibuprofen-induced Nephrotoxicity. *Journal of Advances in Medicine and Medical Research*, 56-62.
- Atroshi, F., Biese, I., Saloniemi, H., Ali-Vehmas, T., Saari, S., Rizzo, A., & Veijalainen, P. (2000). Significance of apoptosis and its relationship to antioxidants after ochratoxin A administration in mice. *J. Pharm. Pharm. Sci*, 3(281), e291.
- Bailey, D.M., (2004) Ascorbate, blood-brain barrier function and acute mountain sickness: A radical hypothesis. *Wilderness & environmental medicine*.15:231–233.
- Ezz-Din, D., Gabry, M. S., Farrag, A. R. H., & Moneim, A. E. A. (2011). Physiological and histological impact of *Azadirachta indica* (neem) leaves extract in a rat model of cisplatin-induced hepato and nephrotoxicity. *Journal of Medicinal Plants Research*, 5(23), 5499-5506.
- Fuchs, R., & Peraica, M. (2005). Ochratoxin A in human kidney diseases. *Food additives and contaminants*, 22(s1), 53-57.
- Hoehler, D., Marquardt, R. R., McIntosh, A. R., & Hatch, G. M. (1997). Induction of free radicals in hepatocytes, mitochondria and microsomes of rats by ochratoxin A and its analogs. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1357(2), 225-233.
- Kőszegi, T., & Poór, M. (2016). Ochratoxin A: molecular interactions, mechanisms of toxicity and prevention at the molecular level. *Toxins*, 8(4), 111.
- Petrik, J. T. Zanic-Grubisic, K. Barisic, S. Pepelnjak, B. Radic, Z. Ferencic, I. (2003) Cepelak Apoptosis and oxidative stress induced by ochratoxin A in rat kidney *Arch. Toxicol.*, 77, 685
- Rodrigues, M. P., Astoreca, A. L., Oliveira, Á. A. D., Salvato, L. A., Biscoto, G. L., Keller, L. A. M., ... & Keller, K. M. (2019). In vitro activity of neem (*Azadirachta indica*) oil on growth and ochratoxin A production by *Aspergillus carbonarius* isolates. *Toxins*, 11(10), 579.
- Shi H, Sui Y, Wang X, Luo Yi, Ji L. (2005) Hydroxyl radical production and oxidative damage induced by cadmium and naphthalene in liver of *Carassius auratus*. *Comp Biochem Physiol Part C Toxicol Pharmacol.*;140:115–121.
- Silva, M. S., Broglio, S. M. F., Trindade, R. C. P., Ferreira, E. S., Gomes, I. B., & Micheletti, L. B. (2015). Toxicity and application of neem in fall armyworm. *Comunicata Scientiae*, 6(3), 359-364.
- Sorrenti, V., Di Giacomo, C., Acquaviva, R., Barbagallo, I., Bognanno, M., & Galvano, F. (2013). Toxicity of ochratoxin A and its modulation by antioxidants: a review. *Toxins*, 5(10), 1742-1766.
- Varga, J., Kocsabé, S., Péteri, Z., Vágvölgyi, C., & Tóth, B. (2010). Chemical, physical and biological approaches to prevent ochratoxin induced toxicoses in humans and animals. *Toxins*, 2(7), 1718-1750.
- Yokoyama, K., Hashiba, K., Wakabayashi, H., Hashimoto, K., Saton, K., Kurihara, T., Motohashi, N., Sakagami, H., (2004). Inhibition of LPS-stimulated NO production in mouse macrophage-like cells by Tropolones. *Anticancer Res*.
- Yoshioka, T., Iwamoto, N., Lto, K., (1996). An application of Electron Paramagnetic Resonance to evaluate nitric oxide and its quenchers. *J. Am. Soc. Nephrol.* 7, 961-965.