„OVIDIUS“ UNIVERSITY OF CONSTANŢA

DOCTORAL SCHOOL OF APPLIED SCIENCES

RESEARCH FIELD: BIOLOGY

SUMMARY OF THE PhD THESIS

THE EFFECTS OF SOME MYCOTOXINS (PATULIN AND KOJIC ACID) ON ZEBRAFISH (*DANIO RERIO*)

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CONSTANȚA, 2021

CONTENT

Keywords: mycotoxins, patulin, kojic acid, tiger mosquito, *Aedes aegypti,* zebrafish, *Danio rerio,* behaviour, anxiety, enzymes, oxidative stress, hepatotoxicity, nephrotoxicity, brain, pancreas, intestine, myocardium

INTRODUCTION

Microscopic fungi represent a diverse group of organisms (over 100.000 species) (Făgăraș, 2007), a multitude of species causing allergies (An, 2004), mycoses and mycotoxicoses. Fungi are of medical interest due their metabolites, called mycotoxins. These substances are ubiquitous and cause unpleasant symptoms in animals and humans. The main producers of mycotoxins belong to the genera *Aspergillus, Penicillium* and *Fusarium* (An, 2004).

Aspergillus sp. has a global distribution, especially in warm temperate and subtropical areas. Contamination occurs due to improper storage conditions but crops can also be affected. *A. fumigatus, A. flavus, A. niger* produce aflatoxins and are responsible for most human infections: pneumonia, sinusitis, keratomycosis, lung abscess (Howard, Miller, 1996).

Penicillium sp., abundant in temperate areas, occurs due to improper storage conditions. Ochratoxin produced by *P. expansum* is responsible for Balkan endemic nephropathy (CAST, 2003). *P. marneffei* causes infections in immunocompromised patients (Howard, Miller, 1996).

Fusarium sp., a saprophyte with global spread, mainly affects cereals. Some species can cause infestations due to improper storage conditions (CAST, 2003). The produced mycotoxins (Zearalenone, Fumonisin, Deoxynivalenol, T-2 toxin) are very difficult to remove (An, 2004).

Of the more than 300 identified mycotoxins, only a few are commonly found in food and fodder. The main groups of contaminating mycotoxins are represented by Aflatoxins, Trichotecens, Fumonizines, Zearalenone, Ochratoxin and Patulin (An, 2004). The list of economically and medically important mycotoxins also includes: Kojic acid, Penicillic acid, Alternariol, Citrinin, Ergotamine, Gliotoxin, Moniliformin, Roquefortin (CAST, 2003).

Fungi present a global health problem, mycotoxicoses, which are common in underdeveloped countries (Bennett, Klitch, 2003), appear due to the penetration of the mycotoxins into the body through the digestive, respiratory or dermal route. Mycotoxicoses are not transmitted from one individual to another (Carlson, Ensley, 2003). The symptoms depend on the type of mycotoxin, duration of exposure, age, health, sex, genetic factors, nutrition and interaction with other toxins (Bennett, Klitch, 2003).

PART I. CURRENT STATE OF KNOWLEDGE

I.1. Kojic acid – general data

This mycotoxin, produced by *Aspergillus sp.* and *Penicillium sp*., is a food contaminant: sake, soy sauce, cereals, fodder, dairy (Burdock et al., 2001).

Kojic acid has an antibacterial, antidiabetic effect (Rajamanikyam et al., 2017), antifungal (Siddhardha et al., 2010), antiparasitic (Rodrigues et al., 2014), insecticide (Dobias et al., 1977 cited by Mohamad et al., 2010), antioxidant (Chen et al., 1991 cited by Mohamad et al., 2010), skin whitening effect (Prignano et al., 2007).

I.2. Patulin – general data

This mycotoxin is produced by over 60 species of fungi, the most important being of the genus *Penicillium sp., Aspergillus sp.* and *Alternaria alternata* (Afssa, 2006). Patulin contaminates products that are part of human or animal diet: fruits, juices, oilseeds, alcoholic beverages, cereals, dairy products, sausages (Joshi et al., 2013).

The 2006 European Commission Regulation allows a maximum concentration of 50 µg/L. Patulin has insecticidal (Cole and Rolinson, 1972), nematicide (Mayer, 1995 cited by Li et al., 2007), antiparasitic role (Föller et al., 2009) and apoptotic activity (Lupescu et al., 2013).

I.3. The effects of kojic acid and patulin on the body

On **vertebrates**, **kojic acid** has the following effects: decreased heart rate of *Danio rerio* embryos (Choi et al., 2007); anxiogenic action, decreased activity of antioxidant enzymes and increased lipid peroxidation in the brain (Ciornea et al., 2019); pallor of the rodent liver and kidneys (Kynoch, Lloyd, 1977 cited by Burnett et al., 2010); increased liver, kidney mass (Kariya et al., 1979 cited by Burnett et al., 2010), hepatocellular adenoma in rodents (Watanabe et al., 2005 cited by Burnett et al., 2010); cannibalism in rat females (Choudhary et al., 1992 cited by Burnett et al., 2010); thyroid hyperplasia, decreased triiodothyronine and thyroxine levels, increased TSH levels in rats (Fujimoto et al., 1999); lethargy, ataxia, loss of reflexes, tremors, convulsions in rodents (Kynoch, Lloyd, 1977 cited by Burnett et al., 2010).

On **vertebrates**, **patulin** has the following effects: nephrotoxicity in *Danio rerio* embryos (Wu et al., 2012); anxiogenic action, decreased activity of antioxidant enzymes and increased lipid peroxidation in the brain (Ciornea et al., 2019); increased transaminases, hepatocyte vacuolation, pycnosis/caryomegaly, nuclear degeneration in rats (El-Sawi et al., 2012); increased lactate dehydrogenase levels in mice (Gashlan, 2008); increased alkaline phosphatase, hepatocytes degeneration/necrosis, sinusoids dilation, increased urea, uric acid and creatinine levels, renal inflammatory/degenerative processes in mice (Al-Hazmi, 2014); Kupffer cell proliferation, lymphocytic infiltrations, eosinophilia of the rodents jejunal mucosa (Escoula et al., 1977); gastrointestinal inflammatory processes and neutrophilia (McKinley et al., 1982 cited by CAST, 2003); sedation, loss of reflexes, convulsions in rats (De Sarro et al., 1984); increased norepinephrine, dopamine, serotonin levels, locomotor activity and aggression in mice (Al-Hazmi, 2014).

I.4. Oxidative stress induced by mycotoxin exposure

A major effect of mycotoxin exposure is represented by the intracellular accumulation of free radicals, oxidative stress, lipid peroxidation, reduced cell proliferation (Abid-Esefi et al., 2004; Omar, 2013). Under normal physiological conditions, the level of free radicals is kept under control by endogenous antioxidants: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), reduced glutathione (GSH) (Zhang et al., 2015).

I.5. Fish behavior: role and physiology

In the aquatic environment, fish use various cues (visual, olfactory, auditory, water movement, tidal flow, hydrostatic pressure, position of the sun, electromagnetic waves) to determine location and for spatial orientation (Brown et al., 2011).

I.6. Computer method of data analysis "Machine learning"

I.6.1. Used algorithms

Computer algorithms are grouped into three types: supervised learning, unsupervised learning, and reinforcement learning. In supervised learning, used in this experiment, the data is labeled to tell the computer which model to use.

I.6.2. Performance evaluation and data management

Evaluating the model's performance is very important, and the most commonly used performance metric is the classification score (accuracy). Receiver Operating Characteristic (ROC) is a graphical representation. Performance is determined by analyzing the area under the ROC curve (or AUC), the best possible value being 1 and the worst 0.5.

A confusion matrix describes the performance of a classification model on a set of test data. There are cases where an element is missing (corrupt data, incomplete loading/extraction), thus it is necessary to manage the missing values by deleting rows, replacing with average value, predicting missing values, using algorithms that support missing values.

PART II. PERSONAL CONTRIBUTIONS

II.1. Motivation for choosing the research topic

Aedes aegypti females are vectors of Yellow fever, Dengue fever, Chikungunya viruses (Service, 2012). Due to the large spread of these mosquitoes, it is necessary to discover new control measures to which these insects have not acquired resistance.

Zebrafish (*Danio rerio*), native to South and Southeast Asia, inhabit slow-flowing or stagnant waters (Spence et al., 2008). Microbiological analyses of pond water (Saju, 2011), lakes (Bandh et al., 2011), rivers (Kshirsagar and Gunale, 2013), demonstrated the presence patulin and/or kojic acid producing fungi: *Penicillium sp., Aspergillus sp., Alternaria alternata*, many fish species being exposed to these fungi (Kumari and Kumar, 2015; Rao, 2017). The fish in these fungi contaminated waters have an increased level of liver enzymes, which indicates liver damage (Atef et al., 2016).

High doses of kojic acid (204-284 mg/L) led to a 90-95% mortality of exposed *A. aegypti* larvae (Rajamanikyam et al., 2017). Following exposure to 100 mg/L kojic acid, zebrafish embryos showed pericardium edema (Veselinović et al., 2017), which shows the need of testing these concentrations in adults as well.

Based on these studies, I have decided to investigate the effect of these kojic acid doses on zebrafish (*D. rerio*) (adult stage), a species that cohabits and feeds on the larvae of *Aedes aegypti*. Most studies analyze the effects of patulin on *D. rerio* embryos: nephrotoxicity (Wu et al., 2012), hyperactivity (Sidebotham, 2015). An experiment on patulin-injected adults shows changes in locomotor activity (Nguyen, 2014).

I have concluded that these studies should be supplemented with investigations of the effects of patulin on *D. rerio* adults. Although the neurotoxic effect of several mycotoxins has been demonstrated (Zhang et al., 2009, Osuchowski et al., 2005), the behavioral effects induced by patulin and kojic acid on fish are not so in depth studied, which is why I wanted to investigate the effect of these mycotoxins on the anxiety of *D. rerio*.

The tested patulin dose, 70 μg/L, significantly exceeds the limit of 50 μg/L imposed by the European Commission and represents: 0.38 % of the LD50 of *D. rerio* larvae and 0.41 % of the LD50 of adult mice (https: // pubchem. ncbi.nlm.nih.gov).

II.2. Goals

The main objectives of the thesis are to determine the effects of kojic acid and patulin on anxiety, oxidative stress, internal organs and the existence of correlations between the administered toxin and fish behavior.

II.3. MATERIALS AND METHODS

II.3.1. Biological material

II.3.1.1. Animals

We used zebrafish (*Danio rerio*, freshwater, omnivorous species), purchased from local producers (Iasi, Romania) with a body weight ranging between 0.48 - 0.74 g.

The breeding and use of laboratory animals were in accordance with the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA).

II.3.1.2. Captivity conditions

The fish were kept in 70 L aquariums, the water temperature was constant (24°C), the day-night cycle: 12 h light, 12 h dark. The fish were ded twice a day (at 8:00 and 20:00). The water was changed every 3 days (1/3 of the water volume). The fish acclimatization period lasted 7 days.

II.3.1.3. Mycotoxins and dosage

Kojic acid was purchased from Carl Roth GmbH + Co KG (Karlsruhe, Germany). Patulin was purchased from Romer Labs Diagnostic GmbH (Tulln, Austria).

The animals were divided into 5 groups of 20 individuals: control (nothing was administered for 7 days); patulin (70 µg/L patulin was administered daily, 7 days); kojic acid I (100 mg/L kojic acid was administered daily, 7 days); kojic acid II (204 mg/L kojic acid was administered daily, 7 days); kojic acid III (284 mg/L kojic acid was administered daily for 7 days).

For the administration of the desired amount of toxin we used an AND HR-202 (4 decimals accuracy) analytical balance. The administration of patulin and kojic acid was done by immersion.

II.3.2. Determination of Patulin and Kojic Acid effects on zebrafish behavior (*Danio rerio*)

The Novel Tank Test (Fig. 1) was performed on the 8th day after the start of the experiment.

Fig. 1 - Aquarium used for Novel Tank behavioral test

II.3.2.1. Novel Tank (NTT) anxiety assessment test

The Novel Tank Test assesses the fish anxiety.

Each of the 60 fish involved in the test was placed in a rectangular aquarium and allowed to move freely. The location of the fish in the lower/upper area of the aquarium is an anxiety indicator (Bencan et al., 2009).

The observation was 6 minutes, using a Logitech HD webcam.

Using ANY-maze®software, for each individual we recorded: number of entries in the upper zone of the aquarium, traveled distance (m), immobility time (s), mobility time (s), latency to enter the top zone of the aquarium (s), time in the bottom zone of the aquarium (s), time in the top zone of the aquarium (s).

II.3.2.2. Statistical analysis

The statistical analysis was performed using ANOVA variation analysis (two-way ANOVA). All the results are expressed as the mean ± standard error of the mean. The values of the general index F for which $p < 0.05$ were considered statistically significant.

II.3.3. Application of statistical analysis and computer methods to discover the existence of correlations between the state of intoxication and fish behavior

II.3.3.1. Statistical analysis

I have used the 5 groups of zebrafish (Control, Patulin 70 µg/L, Kojic acid 100 mg/L, 204 mg/L and 284 mg/L) subjected to the NTT behavioral test. I have obtained 60 valid records from 60 individuals and the variables recorded by ANY-maze®software were monitored.

I have started with a statistical analysis for the NTT test (using the One Way ANOVA test) and the Principal Component Analysis (PCA) technique to reduce the system's dimensionality (using the AnalyzeIt software).

ANOVA test - is a test for comparing the means, the hypotheses for this test being:

- H_0: Averages of the populations from which the lots come from are equal
- H_1: At least one of the averages of the populations from which the lots come from differs from the others

The variable p of the test has the same interpretation as in the case of the other statistical tests:

- $p > 0.05$ H_0 is not rejected, the difference is insignificant at the 95% significance threshold
- $p < 0.05$ rejects H_0 (significance threshold of 95%). At least 2 averages differ significantly
- $p < 0.01$ rejects H_0 (significance threshold of 99%). At least 2 averages differ significantly
- $p < 0.001$ rejects H_0 (significance threshold 99.9%). At least 2 averages differ significantly

We want to know if the NTT test contains sets of variables that indicate the existence of correlations between the administered toxin and fish behavior.

II.3.3.2. Factor analysis

Factor analysis (principal component analysis - PCA) aims to reduce data complexity, highlighting the correlations between variables. The data are represented in an n-dimensional space, and the factor analysis identifies a k-dimensional subspace of the data ($k < n$) (https://profs.info.uaic.ro/~val/statistica/StatWork_11.pdf).

The variance of each main component show how much of the initial data set variation is represented by the main component (https://analyse-it.com).

A two-dimensional monoplot represents the information contained in 2 of the main components, being an approximation of the original multidimensional space (https://analyse-it.com).

To evaluate whether a variable is well represented in the graph, we will analyze the length of the vector and its proximity to the circle's radius: a vector of large length indicates that the variable is well represented; the short length of the vectors indicates a poor representation; it is not recommended to draw conclusions about the relationships between/with these poorly represented variables (https://analyse-it.com).

The angle between the vectors represents an approximation of the correlation between the variables: an angle below 90˚ indicates a positive correlation; a 90˚ angle indicates a lack of correlation; an angle of approx. 180˚ indicates a negative correlation (https://analyse-it.com)

II.3.3.3. Algorithms and data management

I have used supervised machine learning algorithms (Support Vector Machine (SVM), KNN, XGBoost, Decision tree, Random Forest) in the Python programming language and accessing the Google Colaboratory environment that provides access to Tesla K80 GPUs. The .ipynb created files will be saved in Google Drive account.

I have created a program to test machine learning algorithms on classification problems in the case of NTT behavioral test. It started with data scanning, basic imports and then it covered the classification algorithms: it calculated model accuracy for the test data set and for the training one (to discover a possible over-fitting), it calculated the Confusion Matrix and Area Under the Curve (AUC). I used the notebook [https://www.kaggle.com/mgabrielkerr/visualizing-knn-svm-and-xgboost-on-iris-dataset].

The same analysis was repeated for the situation in which the fish groups treatment was reduced to 2 components: control - 0 and intoxicated -1.

The dataset has 8 dimensions. I used PCA to reduce it to 2 dimensions, for a better data browsing and understanding. I used this notebook [https://towardsdatascience.com/pca-using-python-scikit-learn-e653f8989e60].

The same analysis was repeated for the situation in which the fish groups treatment was reduced to 2 components (binary analysis): control - 0 and intoxicated - 1.

The unbalanced classes situation appears in the data sets characterized by a disproportionate observations ratio for each class. The accuracy is no longer reliable in measuring the performance.

The case of reducing the fish treatment to 2 components (control - 0 and intoxicated – 1) belongs to the unbalanced classes situation. For the unbalanced classes encountered in the NTT analysis, I have explored 5 ways to manage the situation: over-sampling of the minority class, under-sampling the majority class, changing the performance evaluation, using penalty algorithms, using decision trees - based algorithms. I used the notebook [https://elitedatascience.com/imbalanced-classes].

NTT databases contain missing values. For simplicity, all the missing values were replaced with the average value of the corresponding column.

II.3.4. Determination of some biochemical parameters that measure the oxidative stress induced by Patulin and Kojic Acid

After performing the behavioral test, 4 fish from each group were anesthetized and decapitated. The brain was washed with saline, then homogenized in buffer and centrifuged (Maheswari et al., 2014; Dumitru et al., 2018). The obtained supernatant was used to determine the catalase, glutathione peroxidase activity and malondialdehyde concentration.

II.3.4.1. Determination of catalase level (CAT)

CAT activity was determined by the Sinha (1972) spectrophotometric method adapted according to Maheswari et al. (2014), Dumitru et al. (2018) and read at 570 nm using a spectrophotometer. Enzymatic activity is measured in U/mg protein.

II.3.4.2. Determination of glutathione peroxidase level (GPX)

GPX activity was determined by the Fukuzawa and Tokumura spectrophotometric dosing method, adapted according to Dumitru et al. (2018).

One unit of glutathione peroxidase is the amount of enzyme required to oxidize 1 nmol GSH/min. Enzymatic activity is measured in U/mg protein.

II.3.4.3. Determination of malondialdehyde level (MDA)

The MDA concentration was determined spectrophotometrically using the thiobarbituric acid method, adapted according to Dobrian et al. (2001), Dumitru et al. (2018). The samples were read at 532 nm using a spectrophotometer. The MDA concentration is measured in nmol/mg protein.

II.3.4.4. Statistical analysis

For the biochemical analysis, brain tissue samples were taken from 4 individuals in each group. Then, the statistical calculations were performed by using standard statistical packages (Microsoft Excel). Results were expressed as mean ± standard error.

II.3.5. Determination of histopathological effects induced by Patulin and Kojic Acid

II.3.5.1. Histological preparation and staining

After euthanasia, 8 fish from each group were fixed for 48 h in 10% formaldehyde, then immersed for 24 h in Bouin solution. The preparation of histological samples was done by paraffin inclusion, longitudinal sectioning (5 μm thickness), trichrome Masson staining (Hematoxylin-eosin-methylene blue) presented by Diaconita et al. (1953), Șincai (2000).

Histological examinations were performed with a AmScope B120 microscope, the images were captured with a 5 mp MicroQ Pro digital camera.

I have compared the histological changes (of the liver, pancreas, intestine, kidney, myocardium, brain) in all the patulin, kojic acid treated groups and the control group.

II.3.6. Experimental models

The effects of patulin have been studied mainly on zebrafish embryos/larvae (Wu et al., 2012; Yuan et al., 2014; Sidebotham, 2015; Muthulakshmi et al., 2018). The exposure duration varied between 24-90 h, observing the appearance of malformations, cardiovascular, histological, behavioral effects, the level of antioxidants. Only a student's experiment (Nguyen, 2014) tests the toxin’s effects on locomotor activity.

As a starting point in choosing the duration of fish exposure, I have chosen Gashlan's (2008) study, in which one of the mice groups was exposed to patulin for a week in order to determine the histopathological changes in the liver and antioxidants levels.

The effect of kojic acid on zebrafish has been studied mainly on the embryonic stage: Chen et al. (2015), Hsu et al. (2016), Veselinovic´ et al. (2017), Lajis et al. (2017), Thach et al. (2017). The exposure duration was 48-72 h, observing the mortality rate, the appearance of cardiovascular malformations, changes in heart rate, the effect on neutrophils.

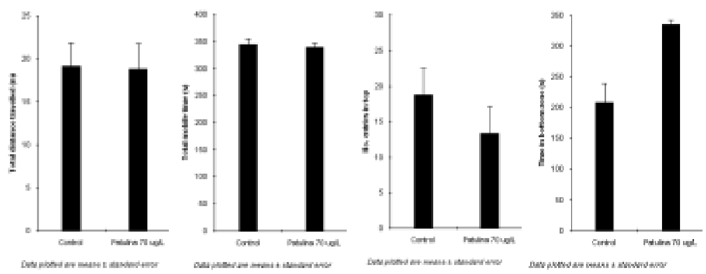
II.4. RESULTS AND DISCUSSIONS

II.4.1. PATULIN AND KOJIC ACID EFFECTS OF ON ANXIETY. APPLYING SOME COMPUTER METHODS OF DATA ANALYSIS TO IDENTIFY THE EXISTENCE OF CORRELATIONS BETWEEN POISONING STATUS AND BEHAVIOR

II.4.1.1. Patulin

II.4.1.1.1. Effects on anxiety

The locomotor activity of the patulin-exposed fish took place mainly in the lower part of the aquarium (Fig. 2), unlike those in the control group which swam uniformly in the aquarium.
Compared to the control group, the distance traveled is slightly shorter for the patulin-exposed fish. This is due to a shorter mobile time and a significantly reduced number (by 27%) of entries in the upper area of the aquarium (Fig. 2 a, b, c).



a **b** **c** **d**

Fig. 2 - Patulin effects on behavioral parameters (Novel Tank Test): a – travelled distance (m); b – mobility time (s); c – no. of entries in top zone; d – time in bottom zone (s)

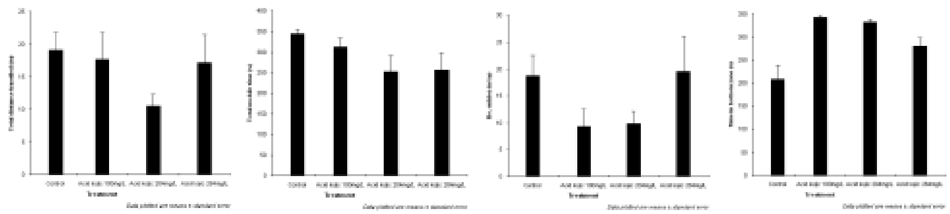
The decreased number of entries in the upper area of the aquarium shows that the intoxicated fish swam mainly in the lower area of the aquarium. The time spent in the lower part of the aquarium is much increased (by about 61%) in patulin treated individuals (Fig. 2 d).
The values of all these parameters demonstrate a state of anxiety due to the toxic effects of patulin.

The anxiogenic effect induced by **patulin** decreases the fish ability to explore the environment, to find food, partners and spawning grounds, all these being essential for the survival of the individuals and species.

II.4.1.2. Kojic acid

II.4.1.2.1. Effects on anxiety

The locomotor activity of the kojic acid-exposed fish took place mainly in the lower part of the aquarium (Fig. 3), unlike those in the control group which swam uniformly in the aquarium.
The traveled distance is reduced in kojic acid-exposed fish (Fig. 3 a). This is due to a shorter mobile period (by 25-27% in the case of fish exposed to 204 mg/L and 284 mg/L kojic acid) and a significantly reduced number of entries in the upper area of the aquarium (by approx. 50% in in the case of fish exposed to 100 mg/L and 204 mg/L kojic acid) (Fig. 3 b, c).



a **b** **c** **d**

Fig. 3 - Kojic acid effects on behavioral parameters (Novel Tank test): a – travelled distance (m); b – mobility time (s); c – no. of entries in top zone; d – time in bottom zone (s)

The reduced traveled distance of the individuals exposed to 204 mg/L kojic acid (45% lower than the control group) is correlated with the shortest mobility time (27% shorter) and a significantly reduced number of entries in upper area of the aquarium (48% lower). The reduced locomotor activity of the fish and the increased time spent in the bottom area of the aquarium show that the 204 mg/L dose exhibits strong anxiogenic effects.

At 100 mg/L kojic acid, individuals record the minimum number of entries in the upper area of the aquarium. The locomotor activity carried out mainly in the bottom zone of the aquarium, the traveled distance and the duration of mobility show an anxiogenic effect.

At 284 mg/L kojic acid, the number of entries in the upper area is slightly increased, and the traveled distance is close to that recorded in non-intoxicated individuals. The anxiety is exhibited by the reduced duration of mobility (by 25%). These values demonstrate an intense locomotor activity, meaning that the fish swam rapidly trying to leave the toxic environment.

The time spent in the bottom zone is much increased in kojic acid-exposed fish (Fig. 3 d).

At 100 mg/L and 204 mg/L kojic acid, the time spent in the lower part of the aquarium is more than 60% longer than the unintoxicated fish. These 2 groups register the lowest number of entries in the upper area of the aquarium, proving an accentuated anxiety.

At 284 mg/L kojic acid there was a 34% increase in time spent in the lower part of the aquarium. This variable is maximum in the case of the lowest kojic acid dose. Higher doses increase mobility in the whole aquarium, by inducing restlessness in the intoxicated fish.

The increased time spent in the aquarium's bottom zone and the reduced duration of mobility prove the fish anxiety.

The anxiogenic effect induced by kojic acid decreases the fish ability to explore the environment, to find food, partners and spawning grounds, all these being essential for the individuals and species survival.

II.4.1.3. Determining the correlations between poisoning status and zebrafish behavior by using statistical and computer methods

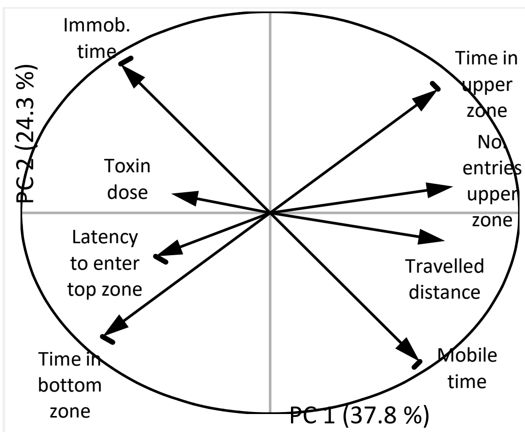
For most groups of variables, "H_0 is not rejected, the difference is insignificant at the significance threshold of 95%". Exceptions were the variables "Time spent in the upper zone" and "Time spent in the lower zone" for which "H_0 is rejected with the significance threshold of 99.9% - At least 2 averages differ significantly".

Thus, we found that the NTT test contains variables that vary depending on the toxin/dose administered, which indicates a correlation between the behavior of zebrafish and the mycotoxin/dose used.

The first 2 main components represent 62.1% of the variance of the original 8 variables set (Fig. 4 b) and provide an useful approximation of the relationships between the variables. The relationships between the variables are easier to visualize in the monoplot (Fig. 4 a):

- negative correlation between time in the upper zone and time in the bottom zone (represented by vectors that form an angle of approx. 180˚), negative correlation between duration of immobility and duration of mobility (represented by vectors that form an angle of approx. 180˚)

The number of entries in the top zone, the traveled distance, the latency to enter the top zone and the toxin dose are not well represented (vectors length is reduced); it is not recommended to draw conclusions about the relationships between/with these variables.



a

Principal components

Component	Variance	Proportion	Cummulative proportion
1	3.027	0.378	0.378
2	1.944	0.243	0.621
3	1.476	0.184	0.806
4	0.778	0.097	0.903
5	0.676	0.085	0.988
6	0.099	0.012	1.000
7	0.000	0.000	1.000
8	0.000	0.000	1.000

b

Fig. 4 - Testul NTT: a - 2-dimensional correlation Monoplot of the coefficients of the first 2 principal components (62.1%); b – variance table

The lowest over-fitting (difference between training data accuracy and test data accuracy) was recorded in the case of the KNN algorithm (20%) and the highest accuracy values were recorded on the training data (approx. 100%) for XGBoost, Decision Tree and Random Forest, but the over-fitting values are high (over 70%) (Table 1).

Table 1 NTT. Accuracy of machine learning algorithms

Algorithm	Accuracy of training data	Accuracy of test data
SVM	0.62	0.11
KNN	0.48	0.28
XGBoost	1.00	0.17
Decision Tree	1.00	0.22
Random Forest	0.98	0.28

On the reduced data set there was a higher accuracy of the classification algorithms (Table 2). SVM, KNN exhibit the lowest overfitting (1%), a high accuracy (90%) and the AUC value is 0.75. Random Forest, Decision Tree exhibit good results: 100% accuracy, 30% overfitting and 0.6 AUC. XGBoost obtained a high accuracy (95%), low overfitting (6%) and a high AUC value (0.75). The confusion matrix has the most errors in the case of the minority class, but much reduced.

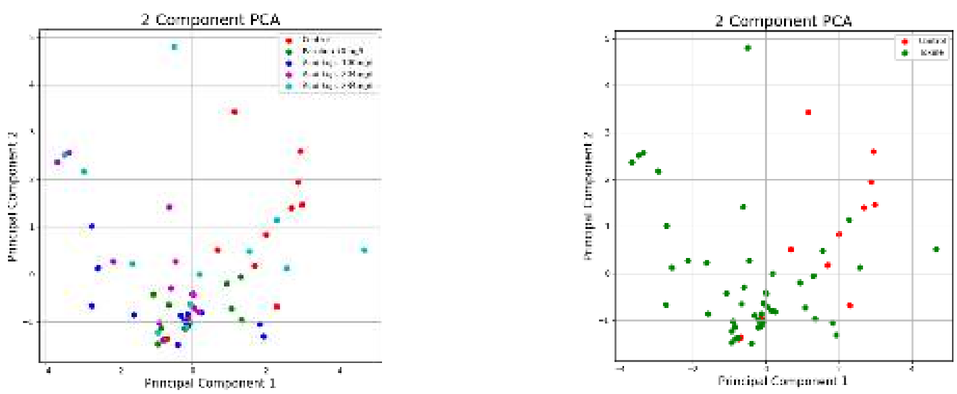
Table 2 NTT. Accuracy of machine learning algorithms on reduced data

Algorithm	Accuracy of training data	Accuracy of test data	Confusion matrix	Area Under Curve
SVM	0.90	0.89	[0 1]	0.75
KNN	0.88	0.89	[0 1]	0.75
XGBoost	0.95	0.89	[0 1]	0.75
Decision Tree	1.00	0.67	[0 1]	0.6071428
Random Forest	1.00	0.78	[0 1]	0.5892857

The high accuracy, high AUC value and lowest overfitting show that SVM and XGBoost are the best performing classification algorithms for our model in low data.

The Python code projects the original data (8 dimensions) into 2 dimensions. The new components are just the 2 main dimensions of the variant. While converting 8-dimensional space into 2-dimensional space, some variance (information) is lost. The first main component contains 43.44% of the variance and the second component contains 26.79% (70.23% together) : array ([0.43440277, 0.26793531])

In the original data set, most classes are not well separated from each other, only the "Control" class being slightly separated from the others (Fig. 5 a). In the case of the reduced data set we observe a clearer separation of the "Control" class from the "Toxins" class (Fig. 5 b). Thus, the existence of a correlation between the state of intoxication and the behavior of zebrafish is demonstrated.



a **b**

Fig. 5 - PCA applied to NTT test: a – on the original data set; b – on the reduced data set

The variance value (70%) is close to the value calculated using PCA analysis using statistical algorithms.

Following the data reduction by assigning 2 values to the fish treatment (0- non-intoxicated, 1- intoxicated), the accuracy of our model (obtained by launching the P3 program in Annex 4) is approximately 88 % : [0 1] 0.883333333 **(accuracy)**

The model predicts both classes (minority and majority).

By exploring several ways to manage the situation of unbalanced classes we obtained:

1. over-sampling of the minority class: [0 1] 0.854166666 (**accuracy**)

The model predicts both classes and the accuracy is high.

2. under-sampling of the majority class: [0 1] 0.791666666 **(accuracy)**

The model predicts both classes and the resulting accuracy seems lower (but not by much).

3. changing the performance evaluation:

The model trained on the under-sampled data set has 0.909722222 (AUC)

The original model trained on the unbalanced data set has 0.909722222 (AUC)

The original model trained on the unbalanced data set had an accuracy of 88%, much higher than that of 79% of the model trained on the under-sampled data set.

However, the latter has an AUC value of 91%, the same as the original model.

4. using penalty algorithms:

[0 1] 0.883333333 **(accuracy)** 0.918402777 **(AUC)**

5. using decision trees - based algorithms:

[0 1] 1.0 **(accuracy)** 1.0 **(AUC)**

Amazing results, 100% accuracy and 100% AUC.

For our model, the most efficient ways to manage the unbalanced classes situation proved to be the usage of decision trees algorithms, their hierarchical structure allowing them to learn signals from both classes (majority and minority).

II.4.1.5. PARTIAL CONCLUSIONS

1. The locomotor activity of the **patulin**-exposed individuals took place predominantly in the lower part of the aquarium, unlike the non-intoxicated individuals which swam uniformly.

2. The low number of entries in the upper area of the aquarium, correlated with the values of the traveled distance and the duration of fish mobility, show that individuals exposed to **patulin** exhibited an increased state of anxiety due to mycotoxin toxicity.

3. The fish from the **patulin**-exposed group exhibit a beta (subordinate) behavior.

4. The anxiogenic effect induced by **patulin** decreases the fish ability to explore the environment, to find food, partners and spawning grounds, all these being essential for the survival of the individuals and species.

5. The locomotor activity of **kojic acid**-exposed individuals took place predominantly in the lower part of the aquarium, unlike the non-intoxicated individuals which swam uniformly.

6. The prolonged time spent by the fish in the lower part of the aquarium and the reduction of locomotor activity show that the **204 mg/L kojic acid** dose induces strong anxiogenic effects.

7. In the case of the 100 mg/L **kojic acid** dose, the fish anxiety is shown by the minimum number of entries in the upper area of the aquarium and by the locomotor activity carried out mainly in the lower area of the aquarium.

8. At the maximum dose of **kojic acid** (284 mg / L), an incresed locomotor activity is recorded, meaning that the fish swam rapidly, trying to leave the toxic environment. The state of anxiety is shown by the reduced mobility time.

9. All **kojic acid-**exposed groups exhibit a state of anxiety, the increased mobility of the fish exposed to the maximum dose is caused by a restlessness due to the mycotoxin's toxicity.

10. **Kojic acid**-exposed fish exhibit an increased state of anxiety and a beta (subordinate) behavior.

11. The anxiogenic effect induced by **kojic acid** decreases the fish ability to explore the environment, to find food, partners and spawning grounds, all these being essential for the survival of the individuals and species.

12. Both **patulin** or **kojic acid** show significant effects on zebrafish brain by inducing an increased state of anxiety and a subordinate behavior.

13. Following the ANOVA test we found out that the Novel Tank test contains variables that correlate with the administered toxin.

14. The Novel Tank test proved to be conclusive in the behavioral analysis of the fish, proving that the intoxicated fish had anxiety problems.

15. We detected a decision tree algorithm, which, by analyzing the variables measured in the Novel Tank test, can be trained to classify the behavior of fish that were exposed to one of the toxins used in the experiment.

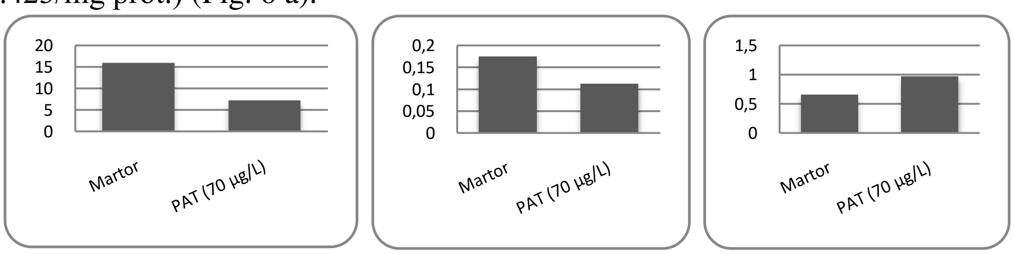
16. We trained and saved a Support Vector Machine (SVM) model for subsequent assessments of an animal's state of intoxication based on the 8 variables analyzed in the Novel Tank test.

II.4.2. DETERMINATION OF OXIDATIVE STRESS INDUCED BY PATULIN AND KOJIC ACID

II.4.2.1. Patulin

II.4.2.1.1. Effects on catalase

In the case of the patulin-treated group, CAT activity decreased by more than 50% (7.226±0.423/mg prot.) (Fig. 6 a).



a **b** **c**

Fig. 6 - Enzyme level of the patulin-exposed group: a – catalase concentration (CAT), b – glutathione peroxidase concentration (GPX), c - malondialdehyde concentration (MDA)

II.4.2.1.2. Effects on glutathione peroxidase

In the case of the patulin-treated group, GPX activity decreased by 35% (0.113±0.009 U/mg prot.) (Fig. 6 b).

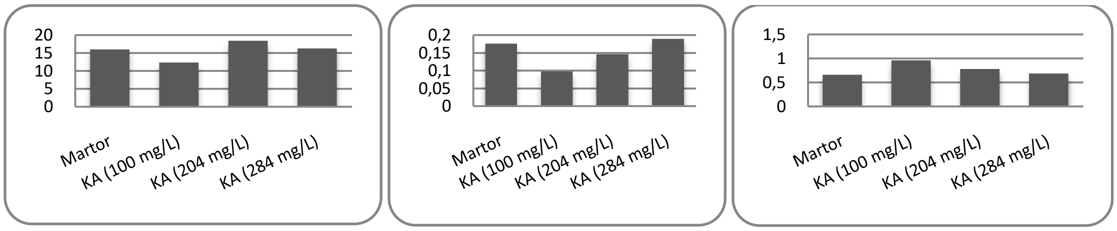
II.4.2.1.3. Effects on malondialdehyde

MDA values (Fig. 6 c) were minimal in the control group. Patulin increased these values by approx. 48% (0.973±0.044 nmol/mg prot).

II.4.2.2. Kojic acid

II.4.2.2.1. Effects on catalase

At 100 mg/L kojic acid, CAT activity (Fig. 7 a) recorded a value of 12.258 ± 0.658 U/mg prot. (76% of the value of the control lot). At 204 mg /L and 284 mg/L there were increases in CAT activity by 15% (18.357 ± 0.981 U/mg prot.), respectively 2% (16.258 ± 1.002 U/mg prot.).



a **b** **c**

Fig. 7 - Enzyme level of the kojic acid-exposed group: a – catalase concentration (CAT), b – glutathione peroxidase concentration (GPX), c - malondialdehyde concentration (MDA)

In the case of high kojic acid doses, the increase in CAT activity is explained by the need to remove the high level of hydrogen peroxide and free radicals synthesized on it.

II.4.2.2.2. Effects on glutathione peroxidase

At 100 mg/L and 204 mg/L kojic acid there were decreases in GPX activity (Fig. 7 b), the value reached being 55% (0.097 ± 0.0084 U/mg prot.), respectively 83% (0.146 ± 0.0076 U/mg prot.) of the control group value. At 284 mg/L kojic acid, GPX had the highest values (0.189 ± 0.0081 U/mg prot.), surpassing those of non-intoxicated fish.

Comparing the values of the 3 intoxicated groups, we find that GPX activity increases as the toxin concentration increases. In this way, the enzyme contributes to the protection of the tissue against free radicals, the level of which is increased due to the toxic action of kojic acid.

II.4.2.2.3. Effects on malondialdehyde

MDA values (Fig. 7 c) were minimal in the control group (0.6598 ± 0.023 nmol/mg prot.). At 100 mg/L kojic acid, the MDA concentration (0.956 ± 0.042 nmol/mg prot.) increased by 45%. At 204 mg/L and 284 mg/L kojic acid, the MDA concentration increased by 18% and 4%, respectively (0.778 ± 0.029 nmol/mg prot., respectively 0.684 ± 0.038 nmol/mg prot.).

The increase in MDA concentration is a consequence of the increased concentration of free radicals (against the background of intoxication with mycotoxin doses), these increases correlating with the increase in the activity of the two enzymes involved in the detoxification process (CAT and GPX).

II.4.2.3. PARTIAL CONCLUSIONS

1. Patulin exposure causes a decrease in CAT activity. This decreases the ability of zebrafish brain tissue to defend against the action of oxygen free radicals and favors the conditions of oxidative stress.

2. Following kojic acid exposure, CAT activity did not change significantly compared to the control group. Thus, we can say that the administered kojic acid doses (100 mg/L, 204 mg/L and 284 mg/L) do not affect CAT, so this enzyme can perform its function of defending the brain tissue from the toxic action of oxygen free radicals. By analyzing the effect of the two mycotoxins on CAT, we can state that patulin is more toxic than kojic acid to brain tissue.

3. Following patulin exposure, the GPx activity in the zebrafish brain decreases as compared to the control group. We can say that, by affecting two essential enzymes of the brain tissue antioxidant system (CAT and GPx), patulin favors the installation of oxidative stress. It is possible that the oxidative stress from the zebrafish brain tissue correlates with the anxiety exhibited by these fish during the behavioral test.

4. Following kojic acid exposure, the GPx activity changes proportionally with the administered dose (100 mg/L, 204 mg/L or 284 mg/L), but the changes are insignificant compared to the control group. Based on our experimental data, we can say that the activity of CAT and GPx, remains within normal physiological limits, so that the antioxidant system of the zebrafish brain tissue remains functional.
5. In the case of patulin-exposed group, the MDA level in the zebrafish brain increases, compared to the control, indicating that this tissue is affected by oxidative stress. MDA is an indicator of the lipid peroxidation process induced by the toxic activity of oxygen free radicals.
6. In the case of kojic acid exposure, for all three doses (100 mg/L, 204 mg/L and 284 mg/L), the MDA level falls within normal physiological limits. We can say that the oxidative stress has not established in the zebrafish brain tissue, because the two antioxidant enzymes, CAT and GPx, remain functional.

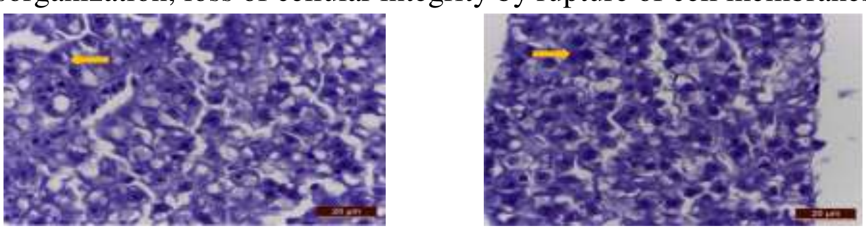
II.4.3. PATULIN AND KOJIC ACID EFFECTS ON THE INTERNAL ORGANS

II.4.3.1. Patulin

II.4.3.1.1. Effects on the liver

After the examination of the the control group individuals, no histological changes were observed in the liver, kidneys, intestines, pancreas or myocardium.

The hepatocytes of the patulin-exposed fish have suffered a severe alteration: swelling, cytoplasm vacuolation, triglyceride loading (Fig. 8 a), nuclear hyperhydration (Fig. 8 b), cytoplasmic disorganization, loss of cellular integrity by rupture of cell membranes.



a **b**

Fig. 8 - Hepatocytes alteration: a - triglyceride loading (→); b – nuclear hyperhydration

II.4.3.1.2. Effects on the renal tissue

The presence of large, glassy lumps with a homogeneous, acidophilic structure is observed in the nephrocytes (Fig. 9). The rupture of the apical pole of the cells is followed by the formation of hyaline cylinders in the tubes lumen. The increased incidence of hyaline bodies may result in renal blockage. Hyalinosis does not reflect the lesion of the nephrocyte, but the fact that the physiological mechanism is saturated.

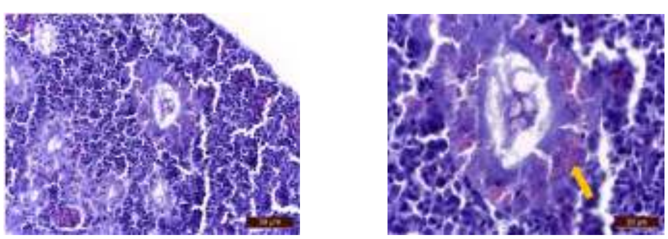


Fig. 9 - Tubular epithelium hyalinosis, hyaline bulges in the cytoplasm of nephrocytes (→).

On the kidneys, patulin induces moderate-severe degeneration of the urinary epithelium, nephrocytes swelling, cytoplasm vacuolation, nuclear hyperhydration (Fig. 9), loss of cellular integrity, interstitial capillary congestion, monocyte infiltration of the urinary epithelium.

II.4.3.1.3. Effects on the intestine

Enterocyte hyalinosis is exhibited by the presence of glassy lumps in the cytoplasm (Fig. 10). This shows a nutrients transport deficiency from the intestinal contents and can cause malabsorption.

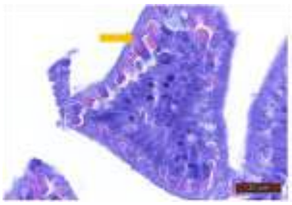


Fig. 10 - Enterocytes hyalinosis (→)

II.4.3.1.4. Effects on the pancreas

In the endocrine pancreas, patulin induces partial coagulative necrosis of the Langerhans Islets (Fig. 11). This effect will affect the production of endocrine pancreatic hormones and alterate the metabolism.

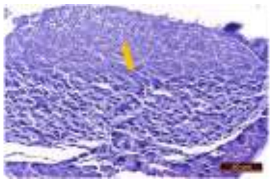


Fig. 11 - Endocrine pancreas necrosis (→)

II.4.3.1.5. Effects on the heart

Myocardial edema is noted by the appearance of interfibrillary spaces (Fig. 12), swollen and hyperhydrated myocardocytes. These effects can cause a heart rate reduction and a decrease in the amplitude of heart contractions.

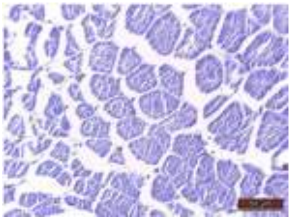


Fig. 12 - Myocardial edema

II.4.3.2. Kojic acid

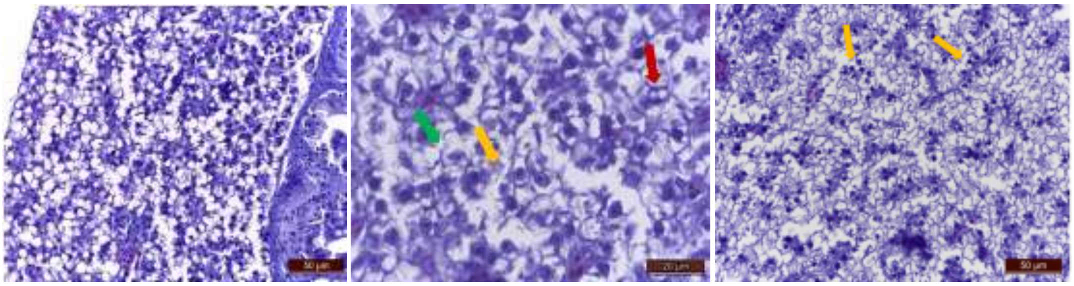
II.4.3.2.1. Effects on the brain

The fish from the 100 mg/L kojic acid-exposed group exhibited a slight meningeal congestion.

II.4.3.2.2. Effects on the liver

The hepatic degeneration in groups exposed to 100 mg/L and 204 mg/L kojic acid, is characterized by hepatocytes hyperhydration or vacuolation and loss of cell integrity (Fig. 13 a, b), some hepatocytes are empty (without nuclei and cytoplasm).

Severe and uniform liver damage, swelling, hepatocyte disintegration, cell membrane fragmentation, nuclear karyolysis/pycnosis occur at 284 mg/L (Fig. 13 c).
The inflammatory component is absent.

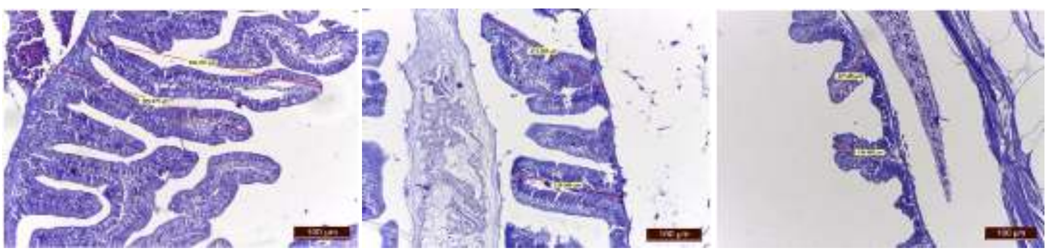


a **b** **c**

Fig. 13 - Kojic acid effects on the liver: a – 100 mg/L; b – 204 mg/L; c – 284 mg/L
Vacuolation (→), fragmentation of cell membranes (→), nuclear caryolysis/pycnosis (→).

II.4.3.2.3. Effects on the intestine

At 100 mg/L kojic acid, there were no significant changes in the size of the intestinal villi, but at 204 mg/L and 284 mg/L, the intestinal villi are undersized, with a reduction of 38% and 68%, respectively (Fig. 14 b, c). The atrophy of the intestinal villi can lead to malabsorption. No fusion of intestinal villi was found.



a **b** **c**

Fig. 14 - Kojic acid effects on the intestine: a – 100 mg/L; b – 204 mg/L; c – 284 mg/L

II.4.3.2.4. Effects on the heart

The cardiac muscle fibers are degenerated, with slightly vacuolated sarcoplasm (Fig. 15). There is a moderate-severe myocardial congestion, capillaries ectasia and erythrocyte overloading. These effects can cause a heart rate reduction and a decrease in the amplitude of heart contractions.

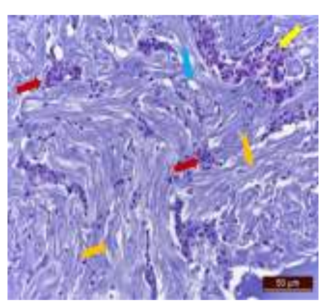


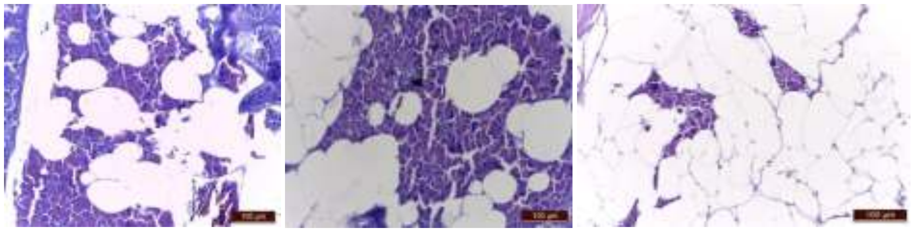
Fig. 15 Degenerated cardiomyocytes (→), vacuolation (→). Congestion (→), ectasia, capillary overload (→).

II.4.3.2.5. Effects on the pancreas

The interstitial lipid infiltration of the exocrine pancreas and the compression of the acini are proportional to the administered kojic acid dose.

At 100 mg/L kojic acid, the lipid infiltration of the pancreas led to the disorganization of its architecture, the exocrine pancreas acini being compressed by lipid deposits (Fig. 16 a).

At 204 mg/kg and 284 mg/L kojic acid, the severe lipid infiltration isolates and compresses the exocrine acini (Fig. 16 b, c), causing the atrophy of the exocrine pancreas.



a **b** **c**

Fig. 16 - Kojic acid effects on the pancreas: a – 100 mg/L; b – 204 mg/L; c – 284 mg/L

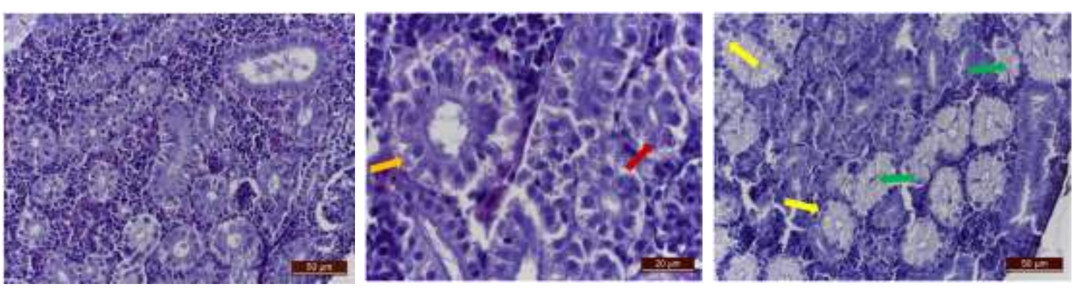
Lipid infiltration and acini atrophy will negatively affect the production of pancreatic digestive enzymes. Nutrient digestion will be deficient, contributing to malabsorption.

II.4.3.2.6. Effects on the renal tissue

At 100 mg/L kojic acid, the moderate degeneration of the urinary tract is shown by a moderate alteration of the epithelium (Fig. 17 a): nephrocytes swelling, granular cytoplasm, nuclear hyperhydration, reduction of tubular lumens.

At 204 mg/L kojic acid, the presence of hyaline spherules is noticed in the proximal tubules (Fig. 17 b). Hyalin denotes an altered glomerular filtration, resulting in massive proteinuria.

At 284 mg/L kojic acid, there is swelling of the tubular epithelium and the tubular lumens dissapearance (Fig. 17 c). Nephrocytes are swollen, with abundant cytoplasm, hypertrophied nuclei and prominent nucleoli. The presence of a renal cyst was noted in the case of a single individual.



a **b** **c**

Fig. 17 - Kojic acid effects on the kidneys: a – 100 mg/L; b – 204 mg/L; c – 284 mg/L
Nephrocyte degeneration: granular cytoplasm (→), hyaline spheres (→), swelling of the tubular epithelium, with cancellation of the tubular lumens (→), prominent nucleoli (→).

II.4.3.3. PARTIAL CONCLUSIONS

1. The **patulin-**exposed group exhibits histological changes in the liver, kidneys, intestines, pancreas and myocardial tissue.

2. On the **liver**, patulin causes severe alteration of hepatocytes: swelling, vacuolation of the cytoplasm, nuclear hyperhydration, triglycerides loading, loss of cellular integrity; liver damage will alter the metabolism, acid-base balance, thermoregulation, antitoxic function, bile and enzymes synthesis.

3. On the **kidneys**, patulin induces a moderate to severe degeneration of the urinary epithelium (swelling, vacuolation, nuclear hyperhydration, loss of cellular integrity, interstitial capillary congestion, monocytic infiltration) and hyalinosis (oversaturation of the physiological mechanism); kidney damage has negative effects on excretion, on maintaining the body's acid-base balance, reabsorption/secretion in the urinary tract, renin and erythropoietin formation.

4. In the case of the **intestine**, patulin induces enterocytes hyalinosis, which can cause malabsorption and, consequently, decreased energy reserves, affecting cell structure and function and the synthesis of hormones, enzymes and nucleic acids.

5. In the **endocrine pancreas**, patulin induces the partial coagulative necrosis of the Langerhans Islands. This effect will have repercussions on the production of endocrine pancreatic hormones and, implicitly, on metabolism.

6. On the **heart**, patulin induces myocardial edema, shown by interfibrillary spaces, myocardiocytes swelling and hyperhydration. These effects can decrease the heart rate and the amplitude of heart contractions.

7. Following exposure to kojic acid, the tissue damage is progressive, corresponding to the increase of the toxin's dose.

8. In the case of the **nervous system** of the kojic acid-exposed fish, the mild histological lesions are correlated with the insignificant changes of the oxidative stress enzymes levels.

9. In the **liver**, the toxic degeneration is gradual but severe from the minimum dose of kojic acid (100 mg/L) (hyperhydration, loss of hepatocyte integrity), high doses (204 mg/L and 284 mg/L) of kojic acid, leading to hepatocytes vacuolation, cell membranes fragmentation, karyolysis/nuclear pycnosis, the liver having a uniform degenerative appearance, but the inflammatory component is absent; by damaging the liver, all kojic acid doses will alter the metabolism, acid-base balance, thermoregulation, antitoxic function, bile and enzymes synthesis.

10. At the **intestine,** there is a shortening of the villi as the dose of kojic acid increases: at 100 mg/L, there were no changes of the intestinal villi size; at 204 mg/L kojic acid, the intestinal villi appear shortened, exhibiting a size decrease of approx. 38%; at 284 mg/L kojic acid, we have registered the atrophy of intestinal villi and a size decrease of approx. 68%, but not their fusion. In the case of high kojic acid doses, the shortening of the villi can lead to malabsorption, decreased energy reserves, altered cell structure and function and an impaired synthesis of hormones, enzymes and nucleic acids.

11. The **exocrine pancreas** is severely affected, lipid infiltrations being more and more pronounced, as the kojic acid dose increases: at 100 mg/L, the lipid infiltration led to the disorganization of the pancreas structure, the acini being compressed by lipid deposits, and at high doses (204 mg/L, 284 mg/L), severe lipid infiltration led to isolation, compression and atrophy of the pancreatic acini. Lipid storage in the pancreas reflects a lipid metabolism disorder;

lipid infiltration and acini atrophy will negatively affect the synthesis of pancreatic digestive enzymes, thus, contributing to malabsorption.

12. In the **heart**, kojic acid causes myocardial congestion, ectasia and capillary overload, myocardiocytes vacuolation and degeneration. These effects can decrease the heart rate and the amplitude of heart contractions.

13. In the case of the **kidneys**, the degeneration of the urinary tract epithelium increases progressively as the kojic acid dose increases: at 100 mg/L, there is a slight nephrocytes swelling, granular cytoplasm and nuclear hyperhydration, the tubular lumens being slightly reduced; at 204 mg/L, we have noted the presence of hyaline spheres, which denote an altered glomerular filtration; at 284 mg/L, the swelling of the tubular epithelium (nephrocytes have abundant cytoplasm, hypertrophied nuclei and prominent nucleoli) leads to the disappearance of the tubular lumens; kidney damage has negative effects on excretion, on maintaining the body's acid-base balance, reabsorption/secretion in the urinary tract, renin and erythropoietin formation.

II.5. FINAL CONCLUSIONS

1. In the case of **patulin** or **kojic acid-**exposed fish, the decrease of the locomotor activity and its conducting in the lower part of the aquarium, proves that both patulin and kojic acid induce a pronounced state of anxiety. The anxiety, present at all doses of kojic acid, increases as the kojic acid dose increases.

2. The high mobility of the individuals exposed to the maximum **kojic acid** dose (284 mg/L) is due to the restlessness induced by this mycotoxin, the fish trying to leave the toxic environment by moving rapidly in the upper area of the aquarium. The reduced mobility time demonstrates the presence of anxiety.

3. By intensifying the anxious behavior, both **patulin** and **kojic acid** have demonstrated significant effects on the nervous system; the anxiogenic effect induced by these mycotoxins will decrease the survival chances of the individuals and the species.

4. In the case of the Novel Tank behavioral test, 'Time spent in the upper area' and 'Time spent in the lower area' correlate with the administered toxin and dose.

5. In the case of **patulin**-exposed group, there is a decrease of the brain catalase and glutathione peroxidase activity, which decreases the ability of this tissue to defend against the action of oxygen free radicals. By affecting these two essential antioxidant enzymes, patulin intoxication favors the installation of oxidative stress in the brain tissue; oxidative stress may correlate with the anxiety exhibited by these fish during the behavioral test.

6. In the case of **kojic acid**-exposed groups, the brain catalase and glutathione peroxidase activity varies depending on the used mycotoxin dose (100 mg/L, 204 mg/L or 284 mg/L), but the changes are insignificant compared to the control group. Thus, the activity of these enzymes remains within normal physiological limits, and the antioxidant system of the zebrafish brain tissue remains functional.

7. Comparing the effect of the two mycotoxins on catalase activity, we can say that, in the zebrafish brain tissue, patulin is more toxic than kojic acid.

8. In the case of the **patulin**-exposed group, the malondialdehyde level in the zebrafish's brain increases compared to the control group, indicating that, due to the toxic action of oxygen free radicals, the establishing of the oxidative stress in this tissue.

9. In the case of the 3 **kojic acid**-exposed groups, the level of malondialdehyde in the cerebral tissue falls within normal physiological limits; due to the fact that the two antioxidant enzymes (CAT and GPx) remain functional, we can say that, in the brain of kojic acid-exposed zebrafish, oxidative stress was not established.

10. Individuals in the control group did not show histological changes in the brain, liver, kidneys, intestines, pancreas or myocardium.

11. **Patulin** or **kojic acid**-exposed fish exhibit histological changes in the liver, kidneys, intestines, pancreas and myocardium.

12. In the case of **kojic acid**-exposed groups, the tissue alterations are progressive, corresponding to an increase in the administered dose.

13. In the case of the nervous system, the mild histological lesions induced by **kojic acid** are correlated with the maintenance of oxidative stress enzymes (CAT, GPx, MDA) within normal physiological limits.

14. In the **liver**, patulin induced a severe alteration and **kojic acid** induces a gradual but severe hepatic degeneration starting at the minimum dose, at the maximum dose the liver having an uniform degenerative appearance. Both mycotoxins cause swelling and loss of hepatocyte integrity, but in the case of kojic acid there are also alterations of the nuclear structure; the inflammatory component is absent in the case of both used mycotoxins.

15. In the **kidneys**, both patulin and the maximum kojic acid dose cause hyalinosis of the tubular epithelium, which can lead to kidney failure. Patulin induces a moderate-severe degeneration, congestion of the interstitial capillaries, an inflammatory component being recorded: monocytic infiltration of the urinary epithelium. Kojic acid induces the degeneration of the urinary tract epithelium, the tubular lumens are progressively reduced until they disappear, as the kojic acid dose increases. Unlike the patulin-exposed group, the inflammatory component is absent, but nuclear structure alterations have been recorded.

16. In the **intestine**, the effects of the 2 mycotoxins are different, but both can lead to malabsorption: patulin causes enterocytes hyalinosis, and kojic acid causes a decrease of the of intestinal villi size. The minimum kojic acid dose (100 mg/L) did not cause significant changes of the intestinal villi size, but in the case of high doses (204 mg/L and 284 mg/L) there is a significant decrease of the intestinal villi size as the toxin dose increases; no fusion of intestinal villi was found in any of the intoxicated individuals.

17. In the case of **pancreas** there is a delimitation of the action of the 2 mycotoxins: patulin alters the endocrine pancreas (induces partial necrosis of the Langerhans Islands, which will alter the production of endocrine pancreatic hormones and metabolism) and kojic acid alters the exocrine pancreas: lipid infiltrations are increasing as the toxin dose increases, leading to the compression, isolation and atrophy of the pancreatic acini; lipid storage in the pancreas reflects a lipid metabolism disorder, thus contributing to the occurrence of malabsorption.

18. On the **heart**, we have recorded different myocardiocytes alteration depending on the used toxin: patulin leads to myocardiocytes swelling and myocardial edema, and kojic acid causes myocardial congestion, interstitial capillary ectasia and myocardiocyte degeneration; through their effects, both patulin and kojic acid can alter the heart rate.

19. The behavioral and physiological effects on *Danio rerio* adults, prove the high toxicity of the patulin (even at a dose of only 0.38% of the zebrafish larvae LD50), and the 3 kojic acid doses exhibit increased toxicity to zebrafish, altering both the behavior and physiology.

20. *Danio rerio* species is severely affected following exposure to both patulin or kojic acid, the toxic effects exhibiting behavioral (anxiety), biochemical (oxidative stress) and histological alterations (on liver, kidney, heart, brain, pancreas, intestine). Both patulin and kojic acid impact the zebrafish life quality, decreasing the survival chances of individuals and the species.

National and international scientific contributions

1. We have evaluated the effects of 2 mycotoxins, patulin and kojic acid, on adult zebrafish (*Danio rerio*), the effects of these mycotoxins being studied mainly on rodents.

2. We have tested the effects of a patulin dose (70 μg/L) that has not been previously applied in toxicological studies involving zebrafish.

3. We have tested, for the first time, the effects of 3 kojic acid doses on adult zebrafish. The 100 mg/L dose was only tested on *Danio rerio* embryos, and the effects of the 204 mg/L and 284 mg/L doses have not been previously studied.

4. We have studied the effects of these mycotoxins on zebrafish behavior, the novelty consisting in the analysis of anxiety, which has an extremely important role in the survival of individuals and the species.

5. We have investigated, for the first time, the oxidative stress induced by these mycotoxins on the zebrafish brain, determining the activity of 2 antioxidant enzymes (catalase, glutathione-peroxidase) and establishing the tissue level of malondialdehyde, the lipid peroxidation marker.

6. We have studied the effects of these mycotoxins on the zebrafish internal organs, recording the histopathological changes in the brain, heart, liver, kidneys, pancreas and intestine. The histopathological study of the pancreas and intestine is a new element in the analysis of the mycotoxins effects on zebrafish.

7. By studying the effects of the kojic acid doses, we have assessed the ecological impact on fish (survival chances of individuals and species). Our results proved that kojic acid induces negative effects on the ichthyofauna and, implicitly, on the habitat balance.

8. We have detected, for the first time, an algorithm (Decision Tree), which can be trained to classify the behavior of patulin or kojic acid-exposed fish, only by analyzing the variables of the Novel Tank test.

9. We have trained and saved a Support Vector Machine algorithm model in our local working directory, for further estimates of an animal's intoxication status based on the characteristics of some variables from the Novel Tank test.

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