Evaluation of the Effect of Red Onion Extract Consumption in Mice (*Mus musculus*)

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Red onion (Allium cepa) is commonly used in human diet, representing an important source of flavonoids, with argued therapeutic values in humans. In animals, the tolerance to onion varies according to species and the quantity intake, carnivores being the most sensitive. The experiment aims to identify the negative potential effects in mice after the aqueous red onion extract consumption over 56 days. Similar to some long-term studies performed on other species using aqueous or alcoholic red onion extracts, for the mice of the experimental batch were registered the hepatic and renal disturbances, paraclinically and microscopically registered, which might limit the testing of the effects of the extracts from red onion on mice.

Keywords: red onion, mice, acidity, renal, hepatic, alterations

Red onion (*Allium cepa*) is known as a traditional medicinal plant, being one of many important sources of dietary flavonoids in many countries [1, 2], contributing with a large extent to the overall intake of flavonoids. Simestad et al [3] reported that the quantitative content of anthocyanins in some red onion cultivars was approximately 10% of the total flavonoid content or 39–240 mg·kg^{-1} FW. The anthocyanins are the major pigments responsible for blue and red coloring of some fruits and vegetables such as blueberries, strawberries, red apples, red beetroot, cabbage and lettuce or red onion [4]. Depending on the postharvest conditions of the storage, through cooking and processing were registered a significantly reduced content of anthocyanins or glucosinolates [5, 6]. More studies show that Allium extracts have antioxidant properties [7, 8], antimicrobial [9, 10] and anti-inflammatory effects [11], reduce the blood pressure in hypertensive subjects, the risk of coronary heart disease and stroke [12, 13] or are involved in reducing the hypoglycemia in type 1 and type 2 diabetic patients [14] or other. On the other hand, Halliver et al [15] showed that the antioxidant effects of anthocyanins related to specific disease protection (e.g., cancer) is not strong, the anthocyanins being rapidly absorbed and eliminated, but with a poor efficiency, and mostly as unchanged glycosides [16]. Lately, the replacement of synthetic colorants [17] with natural dyes has begun to increase in both the textile [18] and food industries [19-20], so the use of red onion dyes must be stable and safe in the matter of human and animal toxicity [21]. Humans are the most resistant specie studied, sheep, goats, rats and mice are more resistant to onion toxicosis than carnivores [22-31]. Consumption of as little as 5 g/kg of onions in cats or 15 to 30 g/kg in dogs has resulted in clinically important hematologic changes [32, 33] transient hemoglobinuria and anemia. Daily feeding of onions could have a cumulative effect due to ongoing formation of Heinz bodies in red cells [22-33].

As chemicals, the anthocyanins are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium salts, being composed of an aglycon moiety called anthocyanidin (fig.1) and carbohydrate residues (glucose, rhamnose, xylose, galactose, arabinose, rutinose) [34, 35].

Experimental part

Material and method

The experiment was carried out on albino mice (*Mus musculus*), under conventional conditions growth, using a number of 16 individuals, 3 months aged, which formed two batches of mice, male and female, one control (4 individuals) and one experimental (12 individuals), the last one receiving the red onion extract that was gradually included in the dilution of 1/4 on the first day and after 4 days replacing the drinking water for a total of 56 days.

The mice of the experimental batch were clinically monitored and weighed periodically in the first day, the 14th, the 28th, the 42th and at the final in the 56th days. Blood and organs samples were collected, for this, the experimental batch was divided into 3 groups (G1-G3) who were euthanized according to the legislation provided by the Law no 43/2014 of Romanian Parliament on the protection of animals used for scientific purposes [36] and the Directive 2010/63/EU [37]. During the experiment, mice were fed with balanced nutritional ratio to cover the metabolic requirements. The ethic protocol was followed in accordance to national and institutional guidelines for the protection of animal welfare during experiments. All animals were acclimatized for seven days with laboratory conditions before the experiments began.

Onions were purchased from the local market in Iasi, Romania. There are several methods of obtaining the red onion extract, such as alcoholic extraction or boiling [35, 38-41]. The onions were peeled and dried in the oven at 40^{o}C. In a stainless steel pot of a 3L capacity, 2L of water and 1200 g from about 20 middle sized red onions were added. The mixture was boiled for 30 min at 100^{o}C and cooled to room temperature, after this being filtered and stored in sterile containers. The extract was analyzed in terms of physical and chemical parameters at 4 and 7 days, conducting anthocyanins content determination, *p*H

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values, color stability at both room temperature and refrigeration conditions.

For the total anthocyanins (fig. 1) content determination, the following reagents were used :

a. *p*H = 1.0 buffer (potassium chloride, 0.025M) - weigh 1.86 g KCl into a beaker and add distilled water to approximately 980 mL. Measure the *p*H and adjust to 1.0 (±0.05) with HCl (approx. 6.3 mL). Transfer to a 1 L volumetric flask, and dilute to volume with distilled water.

b. *p*H = 4.5 buffer (sodium acetate, 0.4M) - weigh 54.43 g $CH_3COONa \cdot 3H_2O$ in a beaker and add distilled water to approximately 960 mL. Measure the *p*H and adjust to 4.5 (±0.05) with HCl (approx. 20 mL). Transfer to a 1 L volumetric flask and dilute to volume with distilled water.

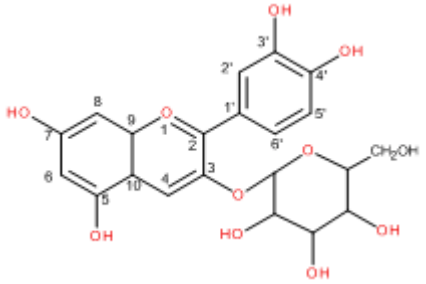

Fig.1. Structure of cyanidin-3-glucoside (CYD-3-GLU) [39]

Procedure: two dilutions of the test sample, one with *p*H 1.0 buffer and the other with *p*H 4.5 buffer were prepared from 1 mL extract diluted to 25 mL with the corresponding buffer and left to equilibrate for 15 min. Absorbencies of test portions diluted with *p*H = 1.0 buffer and *p*H = 4.5 buffer were determined at both 510 nm and 700 nm. The diluted test portions were read versus a blank cell filled with distilled water within 20-50 min of preparation.

Calculations: The anthocyanin pigment concentration, in mg/L, is calculated as follows:

$$\text{Anthocyanin pigment} = A \times MW \times DF \times 10^3/(\varepsilon \times l),$$

where: $A = (A_{510nm} - A_{700nm})_{pH=1.0} - (A_{510nm} - A_{700nm})_{pH=4.5}$; MW (molecular weight) = 449.2g/mole for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor = 25; l = path length in cm; ε = 26 900 molar extinction coefficient, expressed in $L \cdot mol^{-1} \cdot cm^{-1}$, for cyd-3-glu; 10^3 = factor for conversion from g to mg [39].

For the color stability study, the extract of anthocyanins was stored in a dark place at room temperature for 7 days and also in the refrigerator. The absorbance was determined at days 1, 4 and 7 in buffer diluted samples prepared with the same dilution factor as the initial ones [34, 35].

pH determination: The extract's *p*H values were determined in days 1, 4 and 7 during storage at room temperature compared to same extract maintained in the refrigerator, at 4 degree Celsius, with a Hanna Instruments pH-meter, model HI 98103 equipped with a plastic body, combined *p*H electrode model HI 1230B [34].

Total acidity determination: Both extracts were tested in days 1, 4 and 7 for the total acidity value by potentiometric titration with a 0.1 N NaOH solution, using also the Hanna Instrument pH-meter described above [34, 35-41].

Calculations: $A\% = V \times 0.1 \times 63 \times F \times 10^3/50$ mg oxalic acid/100 mL extract,

where: V = mL of NaOH solution consumed for 5 mL extract analyzed; F = correction factor for the sodium hydroxide solution (NaOH 0.1N) = 1.0046.

Blood samples were taken from each mouse by cardiac puncture after anesthesia, obtaining about 1.3 mL of blood which was transferred to the vacutainer with EDTA to assess hematological parameters and to tubes without anticoagulant that were centrifuged to obtain the serum component for serum liver enzyme assay. Hematologic parameters were determined using the Sysmex kx-21n Analyzer, while serum alanine aminotransferases were assessed photometrical using the Reflotron Plus automated analyzer.

Results and discussions

Regarding the anthocyanins values, both extracts had a slight increase after 4 days and decreased after 7 days, but the differences were in favor of the refrigerated extract, being included into table 1 and figure 2.

For the room temperature extract, *p*H values dropped, as expected, with small differences after 4 days and more after 7 days, when the extract developed also a mould pellicle. The registered values were represented in table 1 and figure 3.

Table 1
VARIATIONS OF THE ANTHOCYANINS VALUES AND pH OF ONION ESTRACTS AT ROOM AND REFRIGERATOR TEMPERATURE

During storage	Anthocyanins values (mg/mL)		pH		Acidity (mg oxalic acid/100mL extract)	
	Room (t = 20°C)	Refrigerator (t = 4°C)	Room (t = 20°C)	Refrigerator (t = 4°C)	Room (t = 20°C)	Refrigerator (t = 4°C)
Initial	23.38	23.38	6.26	6.26	75.95	75.95
4 days	28.39	37.16	6.24	6.31	82.28	88.61
7 days	26.30	32.98	4.99	6.3	202.53	94.93

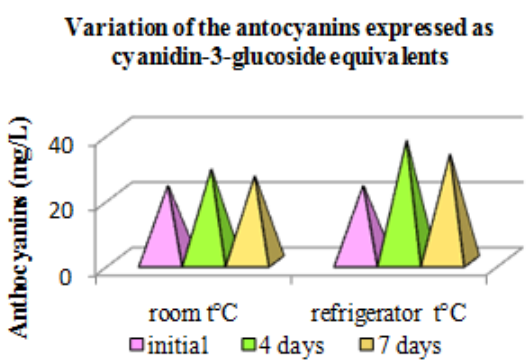

Fig. 2. Variations of the Anthocyanins Values at Room/Refrigerator Temperature

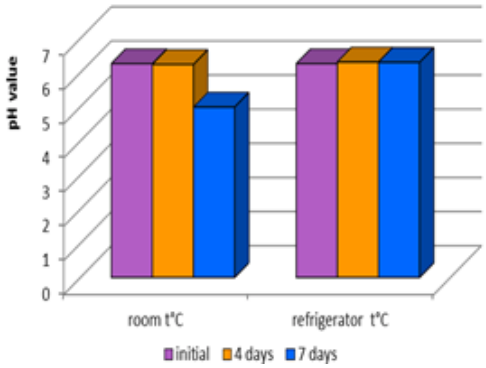

Fig. 3. Variation of the *p*H Values of The Onion Extract at Room/Refrigerator Temperature

For the room temperature extract, acidity values increased after 4 days and became more than double after 7 days storage (table 1, fig. 4).

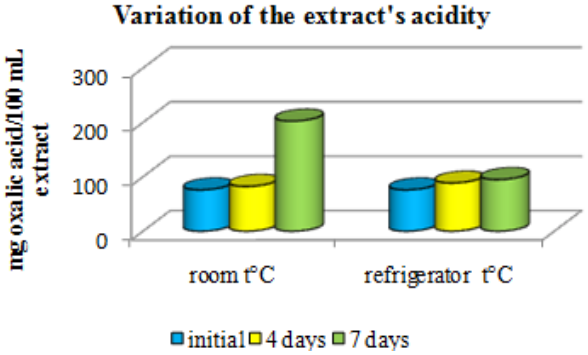

Fig. 4. Acidity Values of Onion Extract at Room/Refrigerator Temperature

From the clinical point of view, no apparent health disturbances were noted in the experimental batch, the mice were energetic, consuming both food and red onion extract in the age-specific quantities. On the other hand, it was registered an obvious increase in body weight of mice in the experimental batch, as seen in table 2, of 2-3 g at each weighing compared to the control group of only 0.5-1.3 g, and at the end of the experiment, mice increase their weight by almost half of it.

The determination of hematological parameters at 28, 42 and 56 days of experiment, as registered in table 3, shows that the values of RBC, PCV, HGB, TWBC and PLT were increased, while VEM, HEM and CHEM recorded insignificant changes at 28 and 42 days of experiment, with an increase in MCHC and MCH in the 56th day of the experiment. It is known that these indicators are very important in anemia identification in humans and animals [23-26]. The variation in the values of some blood components can be based on the action of onion extract. Increased MRC can occur due to the antioxidant effect of anthocyanins by stimulation of erythropoiesis. At the same time, there was noticed an increase in TWBC that shows an organism's defense against onion extract [42, 43] stimulating the immune system. Samson et al. [43] argued that substances from onion extract seem to act as oxygen consumers in vivo, so they may compete with hemoglobin for oxygen, leading to tissue hypoxia, directly stimulating the kidney by releasing erythropoietin. Consequently, the increase of the hemoglobin levels occurs by the indirect effect on erythropoietin [43, 44].

Also, red onion extract also acts on thrombopoiesis under the effect of thrombopoietin which is produced by the liver, kidneys and bone marrow, stimulating platelet

Experimental Batch	Weight on the first day of experiment X(g)	Weight on the 14th day of experiment X(g)	Weight on the 28th day of experiment X(g)	Weight on the 42nd day of experiment X(g)	Weight on the 56th day of experiment X(g)
G1 group	25.00	28.50	31.40	-	-
	25.00	29.00	32.00	-	-
	26.00	29.00	31.90	-	-
	25.50	29.50	32.50	-	-
G2 group	26.50	29.80	31.00	33.00	-
	25.00	27.50	30.00	33.70	-
	24.80	28.00	31.00	33.00	-
	25.50	28.80	31.50	34.20	-
G3 group	25.50	28.33	31.66	34.50	36.20
	25.00	28.90	31.80	34.00	37.00
	26.00	28.00	30.50	34.70	37.40
	25.00	28.50	31.10	33.80	37.15
Control batch	25,50	26.0	27.3	-	-
	25,80	26.4	27.8	-	-
	26,10	26.8	28.2	28.9	30.00
	25,00	26.3	27.8	29.00	30.50

Table 2
VARIATION OF MICE WEIGHT ALONG THE 56 DAYS OF EXPERIMENT

Parameters	Control group	G 1 mice (28th day of experiment)	G 2 mice (42nd day of experiment)	G 3 mice (56th day of experiment)
RBC (X10^6/mm^3)	9.89	9.98	10.01	10.09
HT(%)	48.30	47.80	43.40	48.20
HGB (g/dL)	12.30	14.60	12.90	15.30
MCV (fl)	48.90	47.90	47.50	47.70
MCH (Pg)	14.50	14.70	14.10	15.10
MCHC (g/dL)	29.60	30.60	29.70	31.70
TWBC (X10^3/mm^3)	2.33	5.95	1.64	6.26
NEUT# (X10^3/mm^3)	0.34	3.38	0.59	3.45
LYM# (X10^3/mm^3)	1.83	2.26	0.89	4.16
PLT (X10^3/mm^3)	831	1051	1001	835
MON(X10^3/mm^3)	0.05	0.27	0.07	0.55
EOS (X10^3/mm^3)	0.06	0.02	0.07	0.08
BAS (X10^3/mm^3)	0.05	0.01	0.02	0.02
Uric acide (mg/dL)	1.20	1.80	1.50	1.50
Seric Magnesium (mg/dL)	2.95	2.69	3.35	2.10

Table 3
HAEMATOLOGICAL PARAMETERS OF MICE FROM CONTROL AND EXPERIMENTAL GROUP

RBC - red blood cell count, HGB - hemoglobin concentration, MCV - mean cell volume, MCH - mean cell hemoglobin, MCHC - mean cell hemoglobin concentration, TWBC - total white blood cell count, NEUT# - absolute neutrophil count, LYM# - absolute lymphocyte count, absolute count of the summation of monocyte (MON), eosinophil (EOS) and basophil (BAS), PLT - platelet count.

Table 4
HEPATOTOXICITY PARAMETERS IN CONTROL AND EXPERIMENTAL MICE GROUPS

Indicators	Control batch	G 1 mice (28th day of experiment)	G 2 mice (42nd day of experiment)	G 3 mice (56th day of experiment)
ALAT (TGP) U/L	50	33	24	30
ASAT (TGO) U/L	139	326	202	219
LW g	1.76	0.9	1.3	1.47

ALAT - Alanine-Amino-Transferase, ASAT - Aspartate-Amino-Transferase, LW - Liver Weight

production at 28 and 42 days, 56 days to normal, Adebolu et al [45] and Ugwu & Omale [46] observed significant decreased of PCV values after a long consumption.

The indicator parameters routinely implemented during nonclinical assessments for hepatotoxicity include such as ALAT, ASAT, LW, albumin, urea, nitrogen, electrolytes, total CO_2, glucose, triglyceride, cholesterol etc. Significant variation in these parameters indicates a hepatic disorder [47, 48]. It was registered an increase of ASAT and a decrease in ALAT values, leading to the idea that the red onion extract might produce toxic effects on the liver, which could be identified by histological analysis of it (table 4).

Macroscopically, it was noticed the decrease of liver volume, aspect associated with LW value which might show both toxicity and atrophy of the hepatocytes as in the case of garlic and onion high concentration extract consumption for a period of 90 days as Samson et al [43] registered in 2012.

The histological examination revealed in control group a normal hepatic morphology (fig. 5a), but also phenomena of hyperemia, the venules from the portobiliary space being enlarged in experimental group of mice (fig. 5b). Hepatic tissue destructions are also observed, along with the appearance and multiplication of oval cells in portobiliary space which is characteristic for reparative phenomena in liver (fig. 5c).

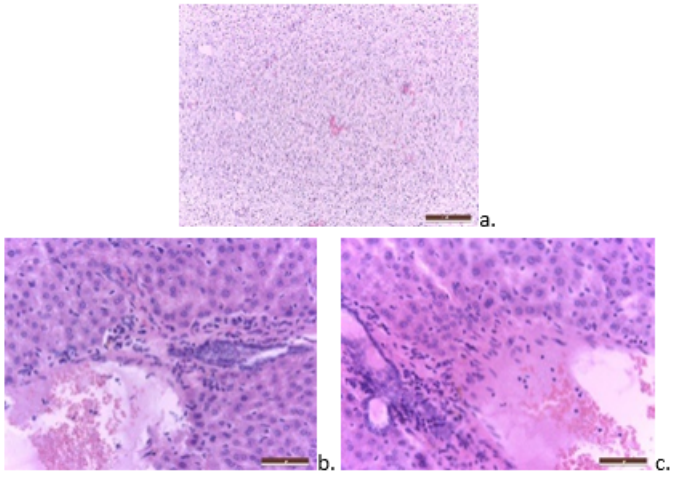

Fig. 5. Mouse Liver: a. Control group-normal morphology, HE staining, 100×; b. Experimental group, 42nd day of experiment, Reactive hyperemia, HE staining, 200×; c. Experimental group, 56th day of experiment, Distended porto-billiary space, HE staining, 200×.

Concerning the mice kidneys at 42 days of experiment, the normal renal aspect of control group (fig. 6a) differs from experimental group, where the Malpighi corpuscles show a series of changes represented by the presence of a rich basophilic protein in the capsular space (fig. 6b). In the cortical area there are frequent spaces filled with blood cells. At 56 days of experiment, the number of Malpighi corpuscles is lower, many are degenerate and there are blemishes in the area of the small calyx, many areas with

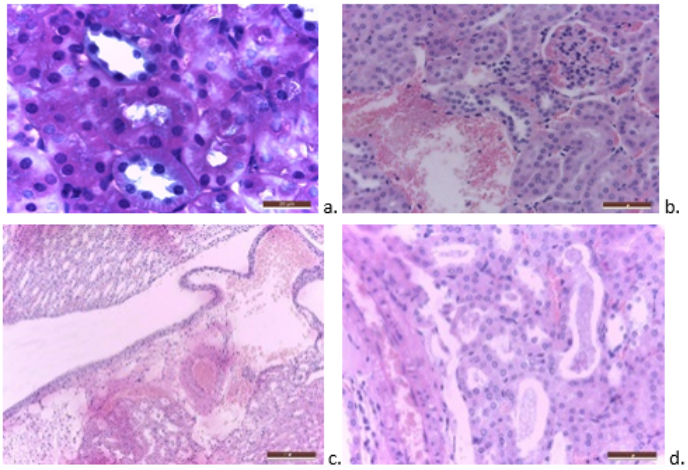

Fig. 6. Mouse Kidney: a. Control group. Normal renal morphology. HE staining 400×, b. Experimental group, 42nd day of experiment-glomerular alterations, HE staining 200×, c. Experimental group, 56th day of experiment, Irregular, pale areas in minor calyx, HE staining, 100×; d. Experimental group, 56th day of experiment, Plasma deposition in cortical areas, HE staining, 200×

blood plasma formations appear in the corticum in one of the mice (fig. 6c,d).

Conclusions

The renal injuries found at experimental group of mice are similar with what happened in cats intoxication with Ibuprofen, represented by coagulative necrosis and epithelial vacuolization [48]. Renal hypoxia may produce the interstitial inflammation and glomerulosclerosis. Onions contain toxic components that may damage red blood cells and determine hemolytic anemia accompanied by the formation of Heinz bodies in erythrocytes of animals such as cattle, water buffaloes, sheep, horses, dogs, and cats [21-30] but not in the case of mice involved in experiment. The hepatic and renal alterations detected in our experimental study although do not reflect a high degree of consistency, suggesting a low receptivity in mice for the components of onion extract.

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