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ASSESSMENT OF GENETIC VARIATIONS IN EMS-EXPOSED *PETUNIA* TESTED FOR SALT *IN VITRO* TOLERANCE USING RAPD*

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Key words: *Petunia* × *atkinsiana* D. Don, genetic variability, mutation, salinity, RAPD, DNA.

Abstract

Random Amplified Polymorphic DNA (RAPD) was used to assess the genetic variability within somaclones of *Petunia* cv. Prism Red exposed to 0.5 mM ethyl methane-sulphonate (EMS), and tested for salt tolerance *in vitro*. Twenty RAPD primers were utilized; 8 out of which amplified specific fragments generating a total of 39 alleles, with a mean of 3.9 alleles per locus. Three arbitrary oligonucleotide primers revealed polymorphisms between non-mutated and mutated plants. Whereas, six RAPD primers generated polymorphic products characteristic only for EMS putative mutants or for EMS putative mutants tested for salt tolerance. The extent of polymorphism indicated the existence of variability within DNA in induced mutated somaclones. Cluster analysis using the Nei's similarity coefficient values and UPGMA algorithm detected genetic variation within non-mutated and mutated plants, as they are placed in different clusters/groups far from each other. Similarity matrices mostly ranged from 0.5 to 0.99. Results were indicative that induced mutation bears great potential in improving petunia for salinity resistance which can be considered as potential samples for further breeding programs.

Introduction

One of the main aspects of modern plant breeding is increasing the crop tolerance to environmental stresses, effective use of water potential, resistance to pathogens, as well as the production of high-value bioactive compounds (PENNA and JAIN 2017). One of the major environmental stress

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factors, which limits the growth and development of many important plant species, is salinity. This problem has occurred mainly in the southern countries of Europe (Hungary, Romania, Greece, Italy, and Spain), and reaches from 1 to 3 million ha (DALIAKOPOULOS et al. 2016). In northern Europe, salt is mostly used as a deicing agent for road maintenance and salinization occurs locally. In Poland, saline soil covers approx 5400 ha (DALIAKOPOULOS et al. 2016, KUJAWSKA et al. 2020). Ornamental and floriculture species have high horticultural and esthetic value, therefore it is very important to determine the salt tolerance of popular bedding plants during greenhouse production. The most effective breeding program means that can deal with soil salinity is to grow cultivars that can establish and be productive on such soil (NIU and RODRIGUEZ 2006, BIDABADI et al. 2011, VILLARINO and MATTSON 2011). Salt tolerance of bedding plants is highly variable, depending on the species or even cultivar, climatic conditions, and irrigating methods (NIU and RODRIGUEZ 2006). Development of plants tolerance to salinity in many crops have been reported about a wide range of plant species including sunflower (SADAK et al. 2010), tomato (SHALABY and EL-BANNA 2013, KRUPA-MAŁKIEWICZ and KULPA 2018), Musa (MIRI et al. 2014), petunia (KRUPA-MAŁKIEWICZ and FORNAL 2018).

Belonging to the *Solanaceae* petunia (*Petunia* × *atkinsiana* D. Don) is being one of the most popular bedding flowers worldwide. May serve as a comparative genetic and molecular biology model plant in the exploration of the molecular origin of some of the developmental diversity of important traits. Moreover, petunia has a short lifecycle, easy culture conditions, easy propagation (both sexual and asexual), biochemical analysis, and the development of stable and the plant is amenable to molecular analysis (DARQUI et al. 2017). The progress in the genetic improvement of horticultural and ornamental plants is slow, and hence greater efforts are required to induce mutations (BERENSCHOT et al. 2008, XU et al. 2009, VILLARINO and MATTSON 2011).

Breeding via mutation has been used to obtain genotypes with increased tolerance to environmental stress by BHAGWAT and DUNCAN (1998), JAIN (2000), BAIRU et al. (2011), MIRI et al. (2014), GADAKH et al. (2017), KRUPA-MAŁKIEWICZ et al. (2017), and ABDULHADI et al. (2019). As a result, the obtained putative mutants can have several desirable traits, e.g. disease resistance, high yield, quality, plant architecture, and abiotic stress tolerance (ASLAM et al. 2017, GADAKH et al. 2017, PENNA and JAIN 2017, GERAMI et al. 2019). Interestingly emerging traits in mutated populations can be identified by various biochemical or physiological methods (PENNA and JAIN 2017). However, several putative mutants derived by this method

can have different phenotypic effects, and often a majority of them are discarded possibly due to the lack of appropriate phenotypic (NADEAU 2000, LESTARI 2016, SRIVASTAVA et al. 2018). The use of molecular markers such as randomly amplified polymorphic DNA (RAPD) allow studying the level of diversity and to establish an index of genetic similarity among obtained variants (ABDULHADI et al. 2019). The current study proved that the RAPD technique was efficient in detecting genetic variation and thus can be used in plant breeding programs. Genetic variability using this technique has been studied by BHATTACHARYA et al. (2010) in *Cymbopogon winterianus*; ASLAM et al. (2017) in *Capsicum annuum* L., GADAKH et al. (2017) in sugarcane; and SRIVASTAVA et al. (2018) in orchid.

Therefore, in the present study RAPD technique was used to evaluate the genetic diversity among *Petunia* × *atkinsiana* D. Don cv. Prism Red plants, obtained from callus cultures treated with ethyl methane-sulphonate (EMS) and selected for salt tolerance *in vitro*. This study also helps to identify RAPD markers that would differentiate salinity resistance from susceptible clones.

Materials and Methods

Plant Material and Explant Source

Plants used in this study were obtained by KRUPA-MAŁKIEWICZ et al. (2017) in an experiment aimed to establish a protocol for produce petunia somaclones that are tolerant of salinity stress using *in vitro* EMS mutagenesis and *in vitro* selection method. Details on the protocol of explants, media preparation, culture conditions, and experimental design, as well as regeneration results were described by KRUPA-MAŁKIEWICZ et al. (2017). Clones were obtained from callus culture, initiated from the leaves. Callus was treated by 0.5 mM EMS and screening for salt tolerance using 50, 100, and 150 mM NaCl *in vitro*. Since the present experiment corresponds to the previous one performed by KRUPA-MAŁKIEWICZ et al. (2017), details of the morphological characteristics obtained are shown in Table 1 and Table 2.

Leaves of *Petunia* × *atkinsiana* D. Don cv. Prism Red were plant material. In the genetic analysis using RAPD techniques, to search for differentiating polymorphisms, twenty-five somaclones (Table 2) were selected, including a non-mutated clone as a control (sample No. 1), six clones mutated with 0.5 mM EMS (No. 2 to 7) and eighteen mutated clones tested for salinity (No. 8 to 25). Only DNA fragments that differentiated between the control sample and putative mutants' samples were considered mutation-

-linked markers. Morphological characterization of obtained somaclones was carried out based on six morphological traits (leaf habit, dwarfism, leaf pigmentation, the shape of the leaf, chimerism, and pigmentation of steam) compared to the control (Table 2) (SINK 1984).

Table 1

Somalones of *Petunia* × *atkinsiana* D. Don cv. Prism Red used in the study

Number	Treatment	
	mutagenic treatment 0.5 mm EMS[a]	salinity[b] [mm]
1	0	0
2–7	0.5	0
8–13	0.5	50
14–19	0.5	100
20–25	0.5	150

[a] – clones of petunia treated with 0.5 mM EMS and then screened under salinity
[b] – salt concentration that each clone was able to tolerate during four weeks

Table 2

Morphological characterization of putative mutants of *Petunia* × *atkinsiana* D. Don cv. Prism Red

No.	Trait	Classification types	
		non-mutated	mutated
1	leaf habit	erect	erect (74.2)*, dropping (25.8)
2	dwarfism	normal	normal (49.2), dwarf type (50.8)
3	leaf pigmentation	normal	normal (40.5), chlorosis (59.5)
4	shape of leaf	both sides rounded	both sides rounded (64.3), both sides pointed (35.7)
5	chimerism	absent	absent (68.1), chimerism (31.9)
6	pigmentation of steam	green	green (88.3), light green (7.2), yellow (4.5)

* Percentage of classification is mentioned in parenthesis

DNA Extraction and Quantification

For DNA extraction, approximately 20 mg lyophilized tissue of putative mutants of petunia and their corresponding mother plant, as a control, were isolated separately using the standard protocol of DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. The quality and quantity of DNA samples were measured by the spectrophotometer Epoch (BioTek) by the spectroscopy method. Only samples of high quality were used for RAPD-PCR.

RAPD Amplification

Genetic diversity analysis was performed using RAPD PCR (*Random Amplification of Polymorphic DNA Polymerase Chain Reaction*) technique (WILLIAMS et al. 1990).

PCR amplification was carried out with a set of 20 oligonucleotide primers (Genomed and Biomers), selected for the analysis based on the references on the genetic diversity estimation in petunia (KRUPA-MAŁKIEWICZ and BIENIAS 2018). PCR reaction for RAPD was carried out in a reaction volume of 15 µl containing 10 × PCR buffer with $(NH_4)_2SO_4$, 2.0 mM of dNTPs, 2.0 mM $MgCl_2$, 0.5 µM of primer, 1.0 U *Taq* DNA polymerase (Thermo Scientific), and ~10 ng/µl of template DNA, according to the protocol described by WILLIAMS et al. (1990). Amplifications were carried out in DNA Engine Dyad® Thermalcycler (Biorad), according to the thermal program: initial denaturation step at 94°C for 3 min, 10 cycles – denaturation 94°C 1 min, annealing – 37°C at 30 s, elongation 72°C 30 s, 35 cycles – 94°C at 1 min, annealing 37°C at 30 s, elongation 72°C 1 min with the final extension step at 72°C 5 min.

Electrophoresis

PCR products were mixed with 6 × Loading Dye Solution and were analyzed by electrophoresis on 1.5% agarose gel (Basica LE-Prona) at 8 V/cm for 110 min (in 1 × TBE buffer). Gene Ruler 100 bp DNA Ladder (Fermentas) was used as a size marker. PCR products were stained with ethidium bromide (0.1 mg L^{-1}) under UV light in a Syngene C:Box using GeneSnap Software. Amplification product profiles were scored for the presence (1) or absence (0) of bands, each of which was treated as independent characters regardless of their intensity.

Data Analysis

The profiles generated in *Petunia* genotypes were analyzed to compute polymorphic information content (PIC) values according to ROLDÁN-RUIZ et al. (2000). Data analyses were conducted using the PhylTool software (BUNTJER 2001). The similarity matrix for RAPD primers was constructed using Nei's similarity coefficient values to find genotypic relationships (NEI and LI 1979). The generated matrix of similarities was analyzed by the unweighted pair-group method with arithmetic averages (UPGMA). The strength of the internal branches from the resulting tree was tested with TREECON bootstrap analysis application using 2,000 resamplings (VAN DE PEER and DE WACHTER 1994).

Results and Discussion

Genetic variation in mutants can be induced either by physical and chemical mutagens or by specific tissue culture conditions. Many studies (MILER and JĘDRZEJCZYK 2018, ABDULHADI et al. 2019) have reported that the *in vitro* culture alone or combined with mutagenesis can be utilized to generate plants with increased genetic variability and mutants as a potential source of new commercial cultivars (BAIRU 2011, MIRI et al. 2014, LESTARI 2016, ASLAM et al. 2017, GADAKH 2017, MILER and JĘDRZEJCZYK 2018, SRIVASTAVA et al. 2018). Detection of variance is important in the subsequent use of these lines in crop improvement. However, the determination of the range of variability based on the observation of morphological features is difficult. Besides, the basis of the obtained changes may be genetic or epigenetic. Morphological traits are the product of gene and environmental interaction. Therefore obtained phenotypes do not determine the actual level of genetic variation. Among the different molecular techniques, RAPD is widely used to study the variation at DNA level among crops such as sugarcane (YADAV et al. 2006, GADAKH et al. 2017), *Capsicum annuum* L. (ASLAM et al. 2017), *Chrysanthemum morifolium* (MILER and JĘDRZEJCZYK 2018), orchid (SRIVASTAVA et al. 2018). Moreover, it has been indicated in the published studies dealing with the subject that RAPD markers are easy to use, cheap, and require no previous sequence information (SRIVASTAVA et al. 2018).

The genetic variability created in *Petunia* × *atkinsiana* D. Don cv. Prism Red through *in vitro* mutagenesis was efficiently assessed with molecular marker technique (RAPD). Genetic analysis in the characterization of petunia and its somaclones (putative mutants) allows detecting changes that occurred during *in vitro* culture and mutagen treatment. In this study, twenty selected oligonucleotide primers, which amplified stable and reproducible PCR products, were screened. Of these, 12 (pr4, pr176, pr269, pr44, pr66, pr88, pr199, pr107, pr139, pr144, pr920, pr1049) amplified monomorphic products did not confirm the variation within the tested samples. This may indicate the similarity of selected plants to the control plants. This may be due that most morphological variations were caused by a somatic or epigenetic mutation and might be distributed in the non-coding region of the genome.

From the eight RAPD primers (pr815, pr99, pr797, pr 29, pr519, pr447, pr875, pr598), which amplified polymorphic products, 39 alleles were detected, with a mean of 3.9 alleles per locus (Table 3). The size of the polymorphic products varied from 320 bp to 2100 bp. The number of alleles per locus ranged from 1 (pr66) to 8 (pr875). The mean polymorphism percentage was 29.8%, ranging from 20% to 50%. The PIC values for the RAPD loci ranged from 0.07 to 0.92, with an average of 0.66 (Table 3).

Table 3
Polymorphism information of eight primers responded during RAPD analysis of EMS induced mutants of *Petunia* × *atkinsiana* D. Don cv. Prism Red

No.	Primer code	Number of alleles	Polymorphic alleles	Polymorphism [%]	PIC* values
1	pr815	4	1	25	0.92
2	pr99	4	1	25	0.07
3	pr797	4	1	25	0.92
4	pr29	4	1	25	0.48
5	pr519	5	1	20	0.92
6	pr447	4	3	50	0.92
7	pr875	8	2	25	0.28
8	pr598	7	3	43	0.77

* polymorphic information content (PIC)

The polymorphism obtained in electropherograms confirmed the two types of segregation obtained in earlier studies (KRUPA-MAŁKIEWICZ and BIENIAS 2018). The first type of polymorphism, manifested by the absence or presence of a band in control or putative mutants, was observed on electropherograms of three primers pr815, pr99, pr875$_{[500\ bp]}$ (Fig. 1).

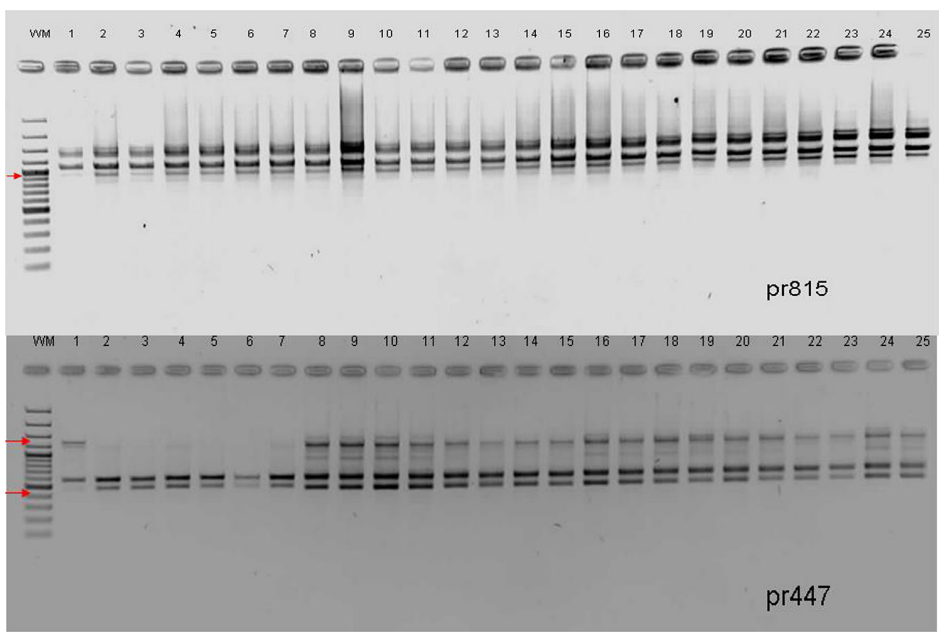


Fig. 1. RAPD profiles generated by primer pr815 generated polymorphic bands characteristic only for EMS putative mutants (2–7) and for EMS putative mutants of *Petunia* tested for salt tolerance (8–25), RAPD profiles generated by primer pr447 representing the absence of a band (490 bp) in control (1) and absence of a band (1250 bp) in putative mutants (2–7) of *Petunia* and arrows show different amplification in each regenerated variant, WM – weight mass DNA Gene Ruler; lines 1–25 see Table 1

The primers pr447, pr29, pr519, pr797, pr875$_{[400\ bp]}$, and pr598 generated polymorphic bands that are characteristic only for EMS putative mutants or for EMS putative mutants tested for salt tolerance (Fig. 1). To ensure that the occurrence of null alleles was not a failure of reaction, the assays were repeated twice. The results showed that the reproducibility was 90–100% under the same amplification conditions.

The results from the present study confirm with the study by YADAV et al. (2006) and GADAKH et al. (2017) among the *in vitro* mutagenized and selected for salt and drought stress sugarcane plants using RAPD marker technique. They screened sixty random decamer primers from which nine and ten gave sufficient intense bands, respectively. The extent of polymorphism, within the mutant clones, indicated the existence of considerable variation at the DNA level. MIRI et al. (2014) tested eleven RAPD primers to assess polymorphism in 22 banana clones. They found that only five amplified products resulted in polymorphic bands. Moreover, the PIC values for the microsatellite loci ranged from 0.25 to 0.85 with an average of 0.56. According to the above-mentioned authors, DNA markers showing an average PIC value of > 0.5 confirms the markers as highly informative.

Determining true genetic similarity between individuals using molecular markers is an important and decisive point for clustering which provides a visual idea about similarity presented in studied genotypes. In the presented study, the RAPD markers produced by eight primers were used to construct a similarity matrix (Table 4). A simple matching coefficient, ranging from 0.50 to 0.98, suggested a rather high similarity between the non-mutated petunia explants and its somaclones. The neighbor-joining method generated a dendrogram showing three main clusters of non-mutated explant and 24 putative mutants (Fig. 2). The first cluster induced only the control (nonmutated explant), while the second one contained the somaclones (2–7) that are only characteristic of EMS putative mutants. The third cluster included putative EMS mutant tested for salt tolerance (8–25). Figure 2 shows that the shortest genetic distances (the highest similarity value) were observed between the nonmutated explant and putative EMS mutants.

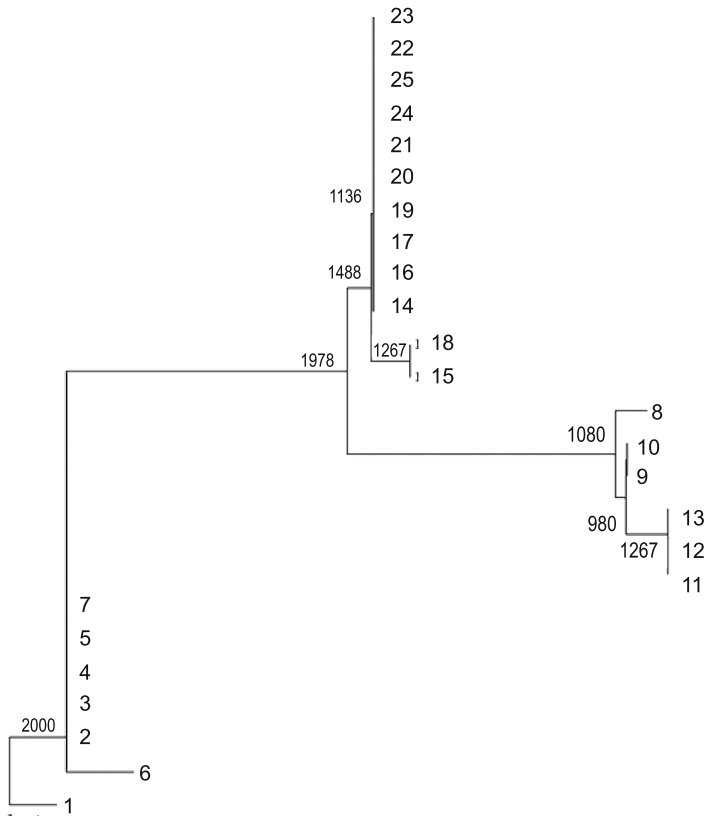
This may suggest that the EMS putative mutants did not accumulate many variations as compared to putative mutants tested for salt tolerance. The results obtained in this study confirm the study of other authors. For example, SHALABY and EL-BANNA (2013) evaluated the genetic similarity, which ranged from 0.82 to 0.99, among putative tomato mutants generated by EMS treatments; using RAPD and simple sequence repeat (SSR) markers. MIRI et al. (2014) reported that banana plants irradiated with gamma rays revealed variations among the clones, using RAPD and SSR markers.

Table 4

The simple matching coefficient of similarity matrix for *Petunia* × *atkinsiana* D. Don cv. Prism Red and its putative mutants determined from RAPD analysis by UPGMA cluster analysis

	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	1.00																								
2	0.92	1.00																							
3	0.92	0.99	1.00																						
4	0.93	0.98	0.98	1.00																					
5	0.92	0.98	0.98	0.98	1.00																				
6	0.86	0.94	0.94	0.95	0.94	1.00																			
7	0.92	0.98	0.98	0.98	0.98	0.94	1.00																		
8	0.55	0.63	0.63	0.63	0.63	0.63	0.63	1.00																	
9	0.52	0.60	0.60	0.60	0.60	0.60	0.60	0.98	1.00																
10	0.52	0.60	0.60	0.60	0.60	0.60	0.60	0.98	0.98	1.00															
11	0.50	0.57	0.57	0.57	0.57	0.57	0.57	0.94	0.98	0.98	1.00														
12	0.50	0.57	0.57	0.57	0.57	0.57	0.57	0.94	0.98	0.98	0.98	1.00													
13	0.71	0.57	0.78	0.57	0.57	0.57	0.57	0.94	0.98	0.97	0.98	0.98	1.00												
14	0.68	0.78	0.76	0.78	0.78	0.73	0.78	0.78	0.81	0.81	0.78	0.78	0.78	1.00											
15	0.71	0.76	0.78	0.76	0.76	0.71	0.76	0.76	0.78	0.78	0.81	0.81	0.81	0.97	1.00										
16	0.71	0.78	0.78	0.78	0.78	0.73	0.78	0.78	0.81	0.81	0.78	0.78	0.78	0.98	0.98	1.00									
17	0.68	0.78	0.76	0.78	0.78	0.73	0.78	0.78	0.81	0.81	0.78	0.78	0.78	0.98	0.98	0.98	1.00								
18	0.71	0.76	0.78	0.76	0.76	0.71	0.76	0.76	0.78	0.78	0.81	0.81	0.81	0.97	0.98	0.98	0.98	1.00							
19	0.71	0.78	0.78	0.78	0.78	0.73	0.78	0.78	0.81	0.81	0.78	0.78	0.78	0.98	0.98	0.98	0.99	0.98	1.00						
20	0.71	0.78	0.78	0.78	0.78	0.73	0.78	0.78	0.81	0.81	0.78	0.78	0.78	0.98	0.98	0.98	0.97	0.97	0.98	1.00					
21	0.71	0.78	0.78	0.78	0.78	0.73	0.78	0.78	0.81	0.81	0.78	0.78	0.78	0.98	0.98	0.98	0.96	0.99	0.99	0.98	1.00				
22	0.71	0.78	0.78	0.78	0.78	0.73	0.78	0.78	0.81	0.81	0.78	0.78	0.78	0.98	0.98	0.98	0.97	0.97	0.97	0.97	0.98	1.00			
23	0.71	0.78	0.78	0.78	0.78	0.73	0.78	0.78	0.81	0.81	0.78	0.78	0.78	0.98	0.98	0.98	0.97	0.96	0.98	0.98	0.97	0.99	1.00		
24	0.71	0.78	0.78	0.78	0.78	0.73	0.78	0.78	0.81	0.81	0.78	0.78	0.78	0.98	0.98	0.98	0.99	0.98	0.99	0.97	0.98	0.98	0.99	1.00	
25	0.71	0.78	0.78	0.78	0.78	0.73	0.78	0.78	0.81	0.81	0.78	0.78	0.78	0.98	0.98	0.98	0.98	0.98	0.98	0.96	0.97	0.97	0.98	0.98	1.00

*Abbreviations see Table 1



1–25 see Table 1

Fig. 2. Dendrogram generated by UPGMA cluster analysis representing the genetic relationship among twenty-five accession of *Petunia* × *atkinsiana* D. Don. cv. Prism Red. The number at the branches indicate 2,000 bootstrap replications

They found that genetic distances between the 22 banana clones varied from 0.70 to 1.00. While, GADAKH et al. (2017) evaluated the genetic similarity between controls, salt, and drought-tolerant mutants of sugarcane, which ranged between 0.41 and 0.75.

Conclusions

In conclusion, the genetic variation between non-mutaded and mutated plants of *Petunia* × *atkinsiana* D. Don cv. Prism Red was efficiently assessed with the RAPD-PCR analysis. Chemical mutagenesis, in combination with the tissue culture technique, seems quite appropriate for the improvement of ornamental plants like a petunia, and the variation could be detected at the stage of regeneration, even before hardening in the

greenhouse. This technique has proved to be very sensitive for the characterization of *in vitro*-selected, saline-tolerant, putative mutants of petunia plants.

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COMPUTATIONAL ANALYSIS FOR CHARACTERIZATION AND EVALUATION OF PENTATRICOPEPTIDE REPEAT-CONTAINING PROTEIN (PPR) IN *ARABIDOPSIS THALIANA*

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Abstract

Pentatricopeptide repeat (PPR) proteins are a great group of RNA-binding proteins which critical role plays in different range of biological stages. However, further investigation is necessary for deeper insight to their roles. Here, a total of 41 sequences of *PPR* genes were identified and characterized in *Arabidopsis*. A comprehensive analysis of *PPR* gene was performed containing chromosomal distribution, phylogenetic relationships, conserved motifs, and detection of transcription factor binding sites (TFBs). Analysis of TFBs illustrated that several transcription factors binding sites (TFBs) namely MYB, bZIP, WRKY, Homeodomain, and AP2 act as basic TFBs linked to abiotic stress responses as well as different growth stages. Our findings revealed a positive correlation between PPR promoter regions of genes and other genes. Expression analysis revealed that PCMP-H52 is induced under iron deficiency and shift low to high light stresses. PCMP-H52 was highly up-regulated in senescence stage in *Arabidopsis*. Our results can provide a comprehensive insight into the expression analysis of *PPRs* and their roles in optimizing biological structure and representing varied roles in *PPR* genes.

Introduction

The pentatricopeptide repeat (PPR) proteins are one of the greatest protein families in terrestrial plants. This family has more than 400 members in *Arabidopsis thaliana*, rice, and foxtail millet (Liu et al. 2016). Most researchers have suggested that the PPR proteins are engaged in post

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transcriptional control of gene expression in organelles such as plastid and mitochondria (LURIN et al. 2004). The family members are detected by the arrays tandem of PPR motifs, approximately 35 amino acids, which contain highly degenerate units from 2 to 30 motifs. The PPR family in plants can be divided into the P and PLS subfamilies related to the PPR signature motifs. P class PPR proteins consist of 35 amino acids and lack additional domains whereas, PLS class PPR proteins possess three different types of PPR array repeats of P, short (S), and long (L). Many of PLS subfamily members also encompass C-terminal domains subdivided further into a four subsets: PLS, E, E+ and DYW (RIVALS et al. 2006). Studies have shown that PPR proteins are identified to be localized in the mitochondrial or chloroplast intracellular space, whereas few PPR proteins have been detected to inhabit in other cellular sections such as cytosol and/or nucleus. PPR proteins play a particularly significant role in RNA metabolism such as RNA cleavage, splicing, translation, RNA stability, and RNA editing. Several previous studies have shown that PPR proteins mediate some of the various functions in the plant biological and physiological stages. In addition, PPR proteins have key role in response to plant growth and development as well as biotic and abiotic stresses. For example, PPR40 is one of the PPR proteins that provide a signaling link between mitochondrial electron transport. Mutation of *PPR40* resulted in increased accumulation of reactive oxygen species (ROS) which enhanced toxicity in cell.

In *Arabidopsis*, *LPA66* is encoded in the chloroplast and is necessary for conversion of amino acids and mutation in *LPA66* causing a defect at the RNA transcription level (CAI et al. 2009). HAMMANI et al. (2011) revealed that the Organelle Transcript Processing 87 (*OTP87*) gene encoded a PPR protein which was indicated at the editing of nad7 and atp1 transcripts in *Arabidopsis*. The MLT1 is another pentatricopeptide repeat engaged in the translation of mitochondrial nad7 mRNA in *Arabidopsis*. This protein is a localized membrane-bound mitochondrial protein, indicated its function in nad7 mature mRNA translation. In rice, ASL3 encodes a novel PPR protein with 10 tandem repeats, having an essential role in chloroplast development and seedling growth. Recently, molecular evidences have revealed that PPRs play a vital role in organelle biogenesis and function and, subsequently, on growth, development, and various biotic and abiotic stresses (BARKAN and SMALL 2014). In rice, *WSL5* is important for chloroplast ribosome biogenesis under cold stress. Knock-out of *wsl5* resulted in inability to assemble functional ribosomes due to the abnormal splicing of *rpl2* and *rps12*. Consequently, the absence of RPL2 and RPS12 proteins prevent formation of functional ribosomes (LIU et al. 2018).

Empty pericarp12 (EMP12), a PPR protein, is implicated in the splicing of three nad2 introns and seed development in maize. Mutation in Emp12 severely arrests embryo and endosperm development, leading to embryo lethality in maize (SUN et al. 2019). PPR40 is implicated to increase seed and seedling development of the plants under salt stress, whereas, *ppr40* causes an increased accumulation of ROS, enhanced sensitivity to abiotic stresses, and less intense growth retardation. PPR protein SVR7 is localized in *Arabidopsis* chloroplast and is implicated in RNA processing and plastid gene expression. Further, *svr7* mutants have been demonstrated to aggregate under higher levels of ROS and reveal sensitivity to H_2O_2 with reduced photosynthetic activity (LV et al. 2014). In the present study, comprehensive analysis of *PPR* genes including phylogenetic tree, chromosomal distribution, genes structure, transcription factor binding sites (TFBs), and their gene expression were performed. Our results can provide an understanding on the molecular mechanisms of the *PPR* genes in response to developmental stages and environmental stresses in *Arabidopsis.*

Material and Methods

Phylogenetic Analysis *PPR* Genes of *A. thaliana* and Its Structure Analysis

Gene sequences of 41 PPR proteins of *A. thaliana* was retrieved from the *Arabidopsis* Information Resource (TAIR). All of the gene sequences were confirmed against NCBI and Plant Genome and System Biology (PGSB) databases. Alignment of the sequences of the *PPR* genes was performed using CLUSTALW program with MEGA software version 6. Phylogenetic tree was constructed in the NJ method and diagrams of phylogenetic trees were drawn with MEGA6 software with bootstrap analysis of 1,000 replicates.

Chromosomal Locations and Analysis of TFBS

Chromosome map of *A. thaliana PPR* genes were constructed by Chromosome Map Tools available at TAIR (https://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp). All of *PPR* genes were analyzed to identify their cellular status using CELLO database (http://cello.life.nctu.edu.tw/) (YU et al. 2006). Promoter regions of 41 PPR proteins were analyzed using PlantPAN (http://plantpan2.itps. ncku.edu.tw/) for the detection of transcription factor binding sites (TFBS) in *PPR* gene promoters. Pfam program was used to find out the PPR proteins domain in the predicted sequences.

Analysis of Gene Ontology and Gene Characterization

The list of *PPR* genes were subjected to GO analysis using the Classification Super Viewer web-based tool (http://bar.utoronto.ca/ntools/cgibin/ntools_classification_superviewer.cgi) available from http://bioinfo.cau.edu.cn/. The tool generated an overview of functional classification of a list of AGI IDs based on the GO database. This database was used to identify the biological processes, molecular functions, and cellular component.

Expression Study of *PPR* Genes

To evaluation the *PPR* gene expression, microarray expression data were taken (ZIMMERMANN et al. 2008) from *Arabidopsis thaliana* database using Affymatrix *Arabidopsis* ATH1 Genome Array. In addition, the genes up and/or down regulated by 1.5 folds were considered as differentially expressed genes (DEG) and these DEG find out using "Perturbations" tool under biotic and abiotic stresses. Differentially expressed genes (DEG) were utilized to generate gene expression heatmap using compendium--wide analysis in genevestigator program. The "red" and "green" colors reflect up and down-regulation of genes, respectively. 'Development' tool was used to identify *PPR* gene expression study using microarray AT-AFFY-ATH1-0 dataset in 10 developmental stage (seed germination, seedling young rosette, developed rosette, bolting, young flower, developed flower, flowers and siliques, senescence).

PPR Proteins Sequence Identification and Domain Analysis

Peptide length, molecular weight, and PI were calculated using the ProParam tool (https://web.expasy.org/protparam/). Characterization of *PPR* genes were performed from PGSB PlantsDB. Sub-cellular localization was predicated using Plant-Mploc server (http://csbio.sjtu. edu.cn/bioinf/plant multi) (HALL 2002). MEME program (http://alternate.meme--suite.org/tools/ meme) and the Pfam tool were utilized to detect the conserved motifs and domains of PPR proteins, respectively. Motifs functions were determined using the hmmscan (https:// www.ebi.ac.uk/Tools /hmmer /search/hmmscan) tool. Then, detected PPR sequences were aligned using Muscle, and identity residue was calculated. GSDS program was utilized to analyze the exon-intron structures of *PPR* genes.

Results and Discussion

Characteristics of 41 *PPR* genes of *A. thaliana* taken from TAIR database are illustrated in table 1 and their locations on each chromosome have also been represented. In this analysis, the names of the PPR proteins, their corresponded protein ID, and the 5'-upstream promoters of each *PPR* gene were surveyed and chromosomal location of the related *PPR* genes have been depicted (Table 1).

Chromosomal Organization of *PPR* Genes and Phylogenetic Analysis

To characterize the genomic distribution of the *PPR* genes on the *A. thaliana* genome, we extracted their chromosomal locations from the PGSB database and found their positions. Total of 41 *PPR* genes were mapped irregularly to the five chromosomes. While chromosome 3 and 5 each were found to possess 10 *PPR* genes, five *PPR* genes were observed to be located in chromosome 4 (Fig. 1). Chromosome 2 was detected to possess the least number of *PPR* genes (4) while the highest number of *PPR* genes (11) were found on chromosome 1. Gene clusters are orders of functionally related genes on a chromosome and clustering of some *PPR* genes were evident on all of the chromosomes, indicating valuable information about their evolution (XING et al. 2018). Accordingly, based on the Holub's criterion, we found 11 *PPR* gene clusters containing a total of eight genes. The clustering patterns of these sequences were compared with their placement on each chromosome, confirming the presence of the sequences of the same chromosomal origin in the same phylogeny (LIU et al. 2016). Only one gene cluster was found on each of the chromosomes 2 and no cluster was found on chromosome 4. Chromosome 5 has the highest number (5) of gene clusters, whereas the least number (only 1) of gene cluster was identified on chromosome 2. The phylogenetic tree was constructed on the nucleotide sequences of 41 *Arabidopsis* PPR proteins using the NJ method. The tree was classified into two distinct subfamilies (P and PLS subfamily) – Figure 2. However, some of PPR members of the PLS subfamily were clustered with the P subfamily, which is consistent with the results from the *Arabidopsis* phylogenetic analysis in which some of the PPR proteins possessed the PLS structure, but were clustered into the P subfamily. Our results agreed with XING et al. (2018) where some of the PPR proteins were classified in PLS subfamily and were clustered with the P subfamily (XING et al. 2018).

Table 1

Description of *Arabidopsis PPR* genes and their cell position

No.	Specification	Gene name	Promoter regions	Localization	Gene size	Chromosome number	Protein length (aa)	Strand	Type of domain
1	*AT1G09410*	PCMP-H18	3035400-3037687	mitochondrial	2118	1	705	+strand	PLS-type
2	*AT3G11460*	PCMP-H21	3608250-3610121	mitochondrial	1872	3	623	+ strand	PLS-type
3	*AT5G52630*	PCMP-E49	21350375-21352333	chloroplast	1767	5	588	+ strand	P-type
4	*AT1G20230*	PCMP-H69	7009568-7012107	mitochondrial	2539	1	759	+ strand	PLS-type
5	*AT2G37310*	PCMP-E5	15664800-15667115	mitochondrial	2280	2	759	-strand	P-type
6	*AT3G12770*	PCMP-E51	4056953-4059284	chloroplast, mitochondrial	2203	3	733	-strand	PLS-type
7	*AT1G03100*	PCMP-E55	743885-746701	mitochondrial	2595	1	864	-strand	PLS-type
8	*AT3G02010*	PCMP-E56	337906-340442	mitochondrial	2478	3	825	-strand	PLS-type
9	*AT1G56690*	PCMP-E57	21253686-21256048	mitochondrial	2115	1	704	+ strand	P-type
10	*AT5G61400*	PCMP-E35	24681550-24683514	mitochondrial	1965	5	654	+ strand	P-type
11	*AT5G37570*	PCMP-E103	14923911-14926333	chloroplast	1653	5	550	-strand	PLS-type
12	*AT1G77010*	PCMP-H13	28942710-28944827	chloroplast, mitochondrial	2088	1	695	+ strand	PLS-type
13	*AT3G15930*	PCMP-H3	5387444-5389690	chloroplast	2247	3	748	+ strand	PLS-type
14	*AT1G26900*	PCMP-H38	9319643-9321512	mitochondrial	1719	1	572	-strand	PLS-type
15	*AT1G31430*	PCMP-E105	11253912-11255745	chloroplast	1802	1	599	-strand	P-type
16	*AT1G32415*	PCMP-E25	11695596-11697964	mitochondrial	2286	1	761	+ strand	P-type
17	*AT1G33350*	PCMP-E101	12089249-12091743	mitochondrial	2285	1	760	-strand	P-type
18	*AT5G52850*	PCMP-E29	21414935-21417616	chloroplast, mitochondrial	2682	5	893	-strand	PLS-type
19	*AT3G25970*	PCMP-H56.1	9500016-9502253	chloroplast	2106	3	701	+ strand	PLS-type
20	***AT2G17140***	**PCMP-H52**	**7462809-7466898**	mitochondrial	3816	2	1271	+ strand	PLS-type
21	*AT5G43790*	PCMP-H43	17591929-17593666	chloroplast	1475	5	490	-strand	PLS-type
22	*AT3G14730*	PCMP-H36	4949178-4951346	mitochondrial	1962	3	653	-strand	PLS-type

23	*AT2G39620*	PCMP-H84	16518890-16521544	mitochondrial	2511	2	836	-strand	PLS-type
24	*AT4G20770*	PCMP-H22	11130762-11133086	mitochondrial	2325	4	774	-strand	PLS-type
25	*AT2G46050*	PCMP-H5	18939262-18941034	mitochondrial	1773	2	590	+ strand	PLS-type
26	*AT3G08820*	PCMP-H52.1	2676990-2679265	chloroplast	2062	3	686	-strand	PLS-type
27	*AT3G02330*	PCMP-H58	473774-476662	mitochondrial	2712	3	903	-strand	PLS-type
28	*AT5G47460*	PCMP-E37	19252463-19254336	chloroplast	1731	5	576	-strand	P-type
29	*AT1G68930*	PCMP-E54	25918066-25921034	mitochondrial	2232	1	743	+ strand	P-type
30	*AT4G14050*	PCMP-H31	8103521-8105637	mitochondrial	1877	4	624	-strand	PLS-type
31	*AT4G37170*	PCMP-E46	17498411-17500655	mitochondrial	2076	4	691	-strand	P-type
32	*AT3G22690*	PCMP-E30	8021229-8024594	chloroplast, mitochondrial	3306	3	1101	-strand	P-type
33	*AT5G50390*	PCMP-E31	20520600-20523212	chloroplast, mitochondrial	2192	5	729	-strand	P-type
34	*AT4G14820*	PCMP-E39	8507268-8510113	chloroplast	2245	4	747	-strand	P-type
35	*AT5G48910*	PCMP-E45	19832785-19835148	chloroplast	2063	5	686	-strand	PLS-type
36	*AT5G08305*	*AT2G17140*	2669944-2671782	chloroplast	1632	5	543	-strand	PLS-type
37	*AT4G38010*	At1g03100	17859338-17861407	mitochondrial	1680	4	559	-strand	PLS-type
38	*AT5G16420*	At5g61400	5367971-5370242	mitochondrial	1608	5	535	+ strand	P-type
39	*AT1G09220*	**PCMP-E33**	2977792-2979466	mitochondrial	1675	1	557	-strand	P-type
40	*AT4G18840*	PCMP-E90	10336566-10340378	mitochondrial	1638	4	545	-strand	P-type
41	*AT3G21470*	At5g16420	7563503-7565160	chloroplast	1572	3	523	+ strand	P-type

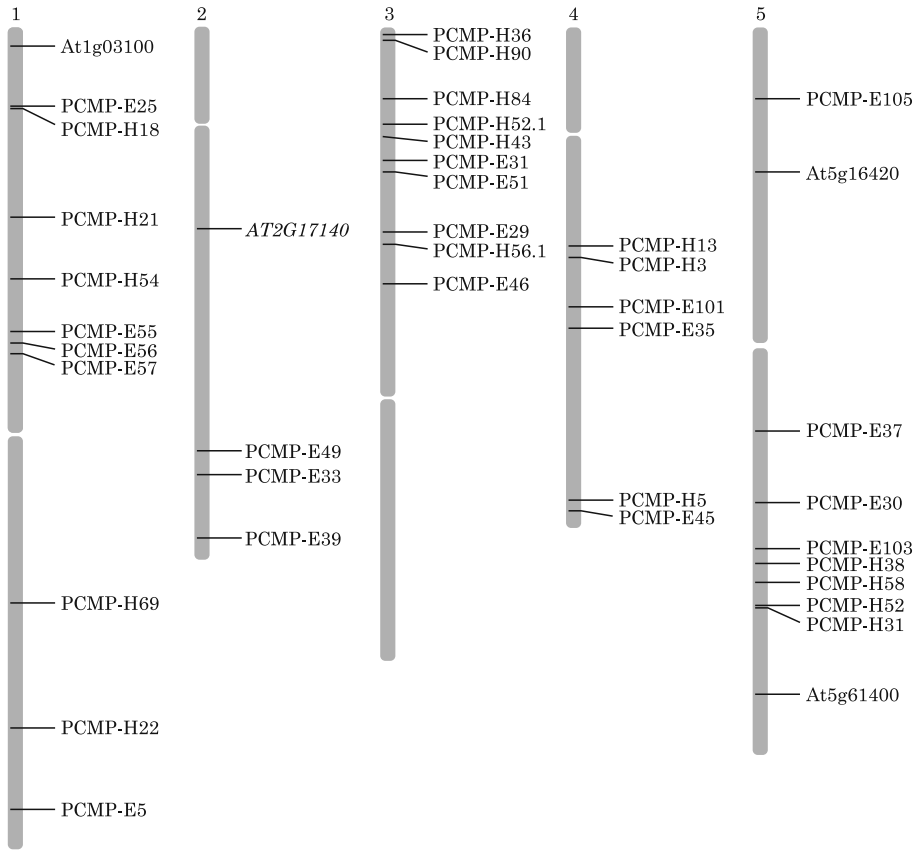

Fig. 1. Chromosomal distribution of 41 different *Arabidopsis PPR* genes

Phylogenetically, 41 *PPR* genes were divided into two different clusters with bootstrap values of 0 to 100 (Fig. 2). Cluster 1 (PLS subfamily) contained nine sequences from chromosome 1, while cluster 2 (P subfamily) had two sequences belonging to the chromosome 1. Cluster 1 contained four sequences (PCMP-H5, PCMP-H52, PCMP-E5, and PCMP-H84) from chromosome 2 while cluster 2 was formed of only one sequence (*AT2G17140*) from chromosome 2. Cluster 2, as the smallest clade, included 15 sequences; of which one (PCMP-E56) was from chromosomes 3, four sequences (3100, E39, E46, E90) from chromosome 4, and seven sequences (PCMP-E29, *AT5G61400*, PCMP-E103, PCMP-E35, PCMP-E31, and PCMP-E49*)* from chromosome 5. Cluster 1, as the largest clade, consisted three nucleotide sequences from chromosome 5 and the remaining nine sequences belonged to chromosome 1, two sequences were belonged to chromosome 4, eight sequences belonged to chromosome 3, and four sequences belonged to chromosome 2.

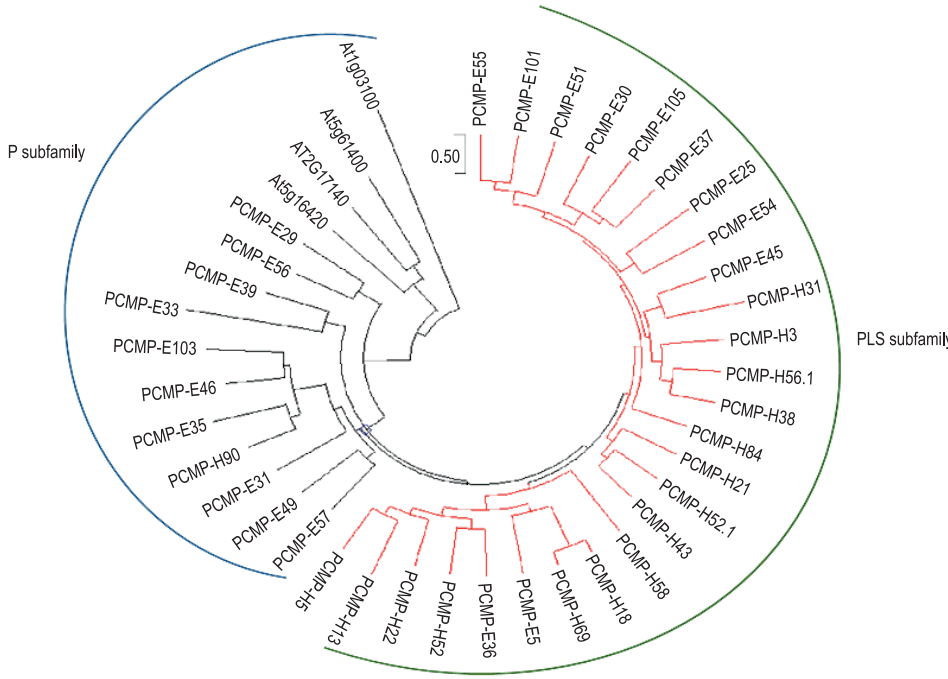

Fig. 2. Molecular Phylogenetic analysis of *PPR* genes from *A. thaliana* by Maximum Likelihood method. The green demonstrated the P class and the black demonstrated the PLS class. The tree was produced in MEGA7.0 with the NJ method and 1000 bootstrap replicates

Analysis of the TFBs

TF families (MBY, bZIP, WRKY, Homeodomain, and AP2) have been identified on the promoter regions of both strands, and were mostly located in the upstream region of 1000 bp. Description of the first five most frequently occurring TFBs of the total detected elements is provided in Table 2. In the following section the importance of surveyed TFBs in the *PPR* genes upstream promoter regions have been investigated, carefully considering their correlation with regulatory patterns and their roles.

Mby. MYB transcription factors play a key role in controlling various processes like metabolism, development, differentiation, defense and responses to biotic and abiotic stresses (AMBAWAT et al. 2013, SAIDI et al. 2020a). Microarray expression study showed at *MYB60* gene is up-regulated upon exposure pathogen and abiotic stress (RASHEED et al. 2016). Five MYB members, *MYB15*, *MYB20, MYB44, MYB52,* and *MYB96* genes are implicated in drought, ABA, salt or/and cold responses. In another study *OsMYB48-1* has been over-expressed in transgenic rice which resulted in lower rate of water loss and improved drought tolerance in comparison to non-transgenic line under drought stress (XIONG et al. 2014).

Table 2

Summary of the transcription factor binding sites (TFBS) detected in the promoter regions of *PPR* genes in *A. thaliana*

TF/ Motifs related to	Specification	AT1G03100	AT1G09220.1	AT1G09410.1	AT1G20230.1	AT1G31430.1	AT1G32415.1	AT1G33350.1	AT1G56690.1	AT1G68930	AT1G77010.1	AT2G17140	AT2G37310.1	AT2G39620.1	AT2G46050	AT3G02010.1	AT3G02330.1	AT3G08820.1	AT3G11460.1	AT3G12770	AT3G14730.1	AT3G15930.1	AT3G21470	AT3G22690	AT3G25970.1	AT4G14050.1	AT4G14820.1	AT4G18840	AT4G20770.1	AT4G37170.1	AT4G38010.1	AT5G08305	AT5G16420.1	AT5G37570.1	AT5G43790	AT5G47460	AT5G48910.1	AT5G50390.1	AT5G52630	AT5G52850.1	AT5G61400
Hormone response	EIN3; EIL	2	4	2	6	3	5	6	4	2	6	3	2	5	2	6	4	3	2	6	3	5	5	6	1	0	1	5	6	5	6	0	3	3	2	1	3	1	0	1	4
	AP2	4	16	15	15	13	16	32	20	13	30	28	49	30	25	34	25	21	22	25	30	25	6	24	31	33	58	30	37	18	13	21	15	17	6	26	40	26	14	17	14
	BES1	0	0	0	0	0	0	3	0	0	0	2	2	2	2	3	0	2	2	0	0	2	3	2	0	0	1	2	0	2	3	0	2	0	0	0	1	0	0	0	0
Tissue-specific	TCR	3	1	2	5	1	2	5	3	4	3	4	1	1	5	1	4	3	2	4	5	4	2	3	6	3	5	4	3	3	3	0	5	3	6	0	0	2	4	2	3
	SBP	17	3	3	4	21	6	20	3	4	20	22	22	6	3	18	19	21	23	3	20	4	21	23	3	18	22	3	3	3	35	3	2	12	3	19	14	4	15	4	2
	WOX	1	3	4	2	0	2	1	1	1	1	1	3	3	3	0	3	3	3	9	9	10	5	4	5	2	4	3	4	1	4	2	5	4	3	3	4	4	3	0	3
	AT-Hook	12	7	11	8	6	6	8	8	13	4	23	5	12	6	5	6	5	4	9	9	9	11	6	6	12	12	10	11	6	8	3	4	9	17	9	14	14	6	15	7
Light response	GATA	13	4	16	13	17	16	18	16	14	9	15	5	17	11	13	12	18	10	17	16	11	9	18	17	12	17	14	14	8	14	17	15	15	14	17	11	12	14	12	17
Cell cycle	E2F	2	2	0	2	1	2	2	4	0	1	1	2	0	0	1	2	1	1	1	2	0	1	1	1	1	2	1	1	1	1	2	1	0	1	1	1	1	1	1	1
Basic transcription	NF-YB	2	3	2	2	2	1	1	2	1	1	1	2	3	2	2	2	2	1	3	2	1	2	2	1	1	2	1	1	1	1	1	2	1	1	1	1	1	2	1	1
	TBP	4	5	2	9	1	0	8	3	8	7	5	7	5	2	3	3	1	4	1	6	4	4	5	7	7	8	7	2	5	5	8	5	3	4	8	6	7	2	3	7

Other binding	TCP	2	9	3	5	3	3	4	3	4	2	4	4	3	0	9	4	6	3	15	4	3	2	8	4	5	9	11	9	2	2	3	5	4	4	4	6	3	9	4	6
	WRKY	26	26	6	14	37	20	28	3	32	24	35	31	29	20	10	16	31	23	19	15	31	19	19	3	13	34	26	27	19	2	34	23	12	13	26	24	12	2	16	24
	MYB	22	22	55	39	19	15	14	16	57	41	55	39	38	47	39	39	12	40	44	51	46	37	46	41	35	41	47	50	41	19	26	43	58	31	36	45	50	47	43	40
	ZF-HD	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	GRAS	0	0	0	6	2	1	2	1	1	2	1	1	0	0	0	0	2	2	0	0	0	1	1	0	1	1	0	0	0	1	0	0	0	0	2	0	0	0	0	0
	bZIP	8	19	30	17	24	20	35	20	28	34	42	41	40	35	42	25	34	33	39	23	38	32	47	27	14	46	32	27	23	18	25	30	9	36	28	10	21	31	29	31
	Dof	10	16	12	6	15	16	14	10	8	10	15	10	9	12	15	15	12	10	6	14	7	12	11	8	14	15	15	12	15	10	10	12	10	5	3	9	15	13	14	12
	bHLH	8	12	6	8	7	9	40	10	17	12	13	39	40	35	40	10	16	40	15	9	10	38	12	8	7	10	14	8	34	14	10	10	10	10	12	10	9	8	16	11
	NAC	9	9	18	17	10	12	18	15	7	6	10	5	4	9	17	10	9	7	7	6	7	7	12	12	9	10	8	9	5	6	9	11	8	4	5	8	7	7	8	6
Stress response	CDS	0	0	0	1	2	2	2	1	1	1	1	2	1	1	2	1	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	HSF	3	2	1	1	2	2	2	2	2	1	1	0	0	0	0	0	0	3	0	0	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	C2H2	7	7	8	8	9	7	7	9	9	9	8	9	7	5	10	7	5	5	17	8	4	7	14	8	16	13	5	4	3	6	8	7	6	21	5	11	16	1	9	6
	Homeo-domain	12	18	16	26	11	14	13	17	20	16	22	14	16	14	21	20	16	19	20	29	28	13	22	25	11	26	25	15	14	16	17	14	17	15	20	28	12	23	13	15

bZIP. In plants, bZIPs are master regulators of several processes including seed formation, pathogen defense, light signaling, abiotic and biotic stress responses (RAHAIE et al. 2011, SAIDI et al. 2020b). Some bZIP are activated by salt stress, and act as salt stress sensor in *Arabidopsis*. In addition, stress-inducible expression through participating transfer from the endoplasmic reticulum to the nucleus and subsequently up-regulation of salt stress genes. Beside abiotic stress control, nine of bZIP TFs increased expression during the course of *Ustilago maydis* infection in maize where same expression profile was observed by *Colletotrichum graminicola* infection (WEI et al. 2012).

WRKY. Members of this family of WRKY contain at least a conserved class of DNA-binding region for abiotic and biotic stress management in rice (ROSS et al. 2007). The upregulation of some members of WRKY TFs have been reported to be positive regulators of drought tolerance. Also, under salt and pathogen infection, most WRKY were significantly upregulated (JIANG et al. 2009, SAIDI and HAJIBARAT 2019).

Homeodomain. Homeodomain encoded by homeobox genes contain a specific DNA sequence that provides instruction for making a string of 60 protein building blocks. This TFBs was increased against pathogen infection (COEGO et al. 2005)

AP2. AP2 has a role in controlling seed mass, seed development, and development of the ovule and seed coat (OHTO et al. 2005). AP2, as a novel role, is incorporated in the floral homeotic gene APETALA2 during *Arabidopsis* fruit development (RIPOLl et al. 2011).

In the present study, five TFBs were identified among 41 *PPR* gene promoters with the greatest number of TFBs detected in MYB and least number of TFBs identified in AP2 (Table 2). Also, the maximum and minimum number of TFBs was observed on PCMP-H52 and PCMP-E55, respectively. Although the correlation between TFBs and genes response under stress conditions need more experimental and systematic analysis of most of PPR proteins, these results only showed the stress-responsive nature of *PPR* genes (CHEN et al. 2018). In silico analysis of transcription factor binding site has demonstrated that the availability of a TFBs bZIP in PCMP-H52 is far higher as compared to PCMP-E55 promoter region. Pervious study is shown that TFBs "bZIP" has been identified to be implicated in drought, senescence, and pathogen defense response (JAKOBY et al. 2002, CHEN et al. 2018) and it could be expected that PCMP-H52 might be highly responsive to drought stress. Further experimentation is required with the high and low TFBs possessing PPR promoters to unravel their biological significance (DAS et al. 2019).

Gene Expression of *PPR* Genes in Developmental Stages

To investigate the expression profile of the PPR proteins in *Arabidopsis* development, we analyzed microarray dataset in genevestigator (Fig. 3).

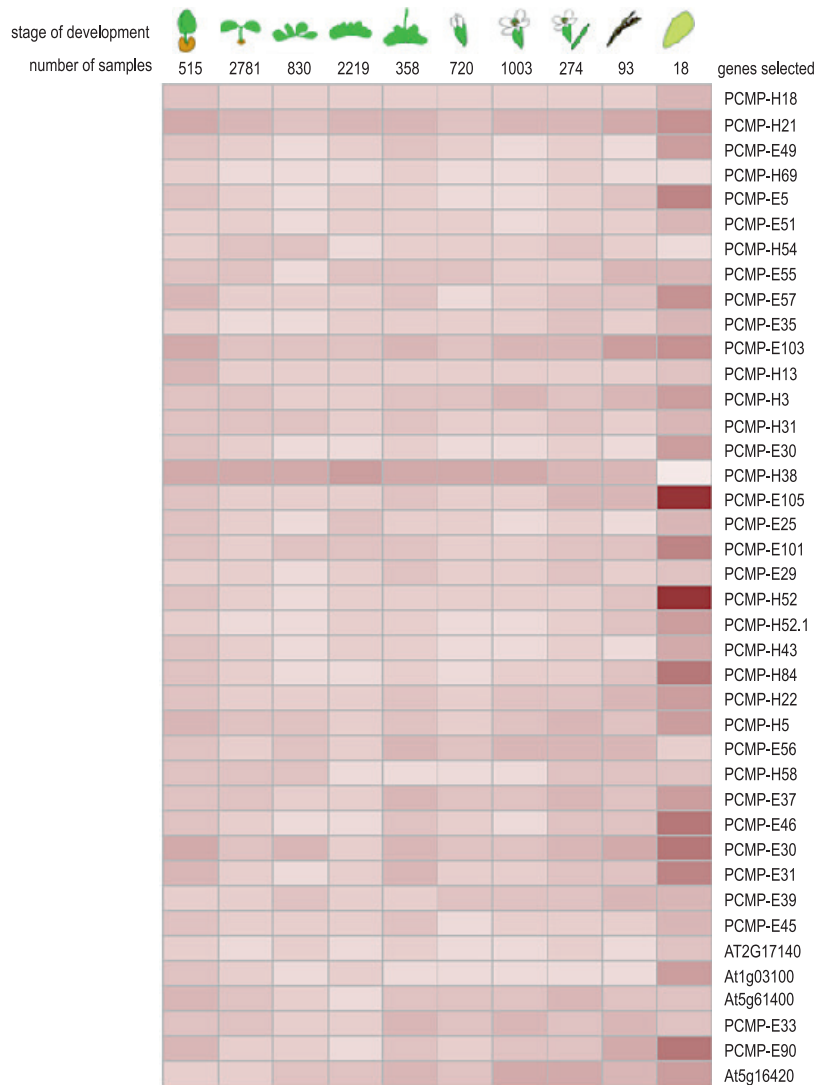

Fig. 3. Expression profiles of *PPR* genes at different developmental stages of *A. thaliana*

PCMP-H38 was a highly expressed in all stages of development except senescence, where, PCMP-E105 and PCMP-H52 were highly expressed in senescence stage. PCMP-H21 was slightly up-regulated in all developmental stages in *Arabidopsis* (Fig. 3). In the course of seed germination stage, all

genes showed almost similar level of expression. Expression of all genes were maximal during senescence and seed germination stage whereas, expression of these genes was minimal during the young rosette and flower development stages.

Gene Expression of *PPR* Genes in Abiotic and Biotic Stresses

Expression analysis of *A. thaliana PPR* genes in response to abiotic stress was surveyed by genevestigator (Fig. 4). Based on our results, a positive correlation was obtained between PPR up-stream promoter regions of genes and presence of TFBs. Based on the available microarray data, it has been observed that PCMP-H52 and PCMP-E105 were highly up-regulated in response to iron deficiency and shift low to high light stresses. Whereas, these genes were down-regulated in RNA labeling and EMS mutation (Fig. 5). Additionally, the gene expression data showed that the gene expression of most *PPR* genes were up-regulated in response to a shift low to high light stress. But, majority of *PPR* genes were down-regulated in response to RNA labeling and EMS mutation. Furthermore, some *PPR* genes were up-or down-regulated in response to cordycepin stress whereas, others showed no response to cordycepin stress.

Plants utilize complex signaling pathways containing stress-related TFBs and regulate their compatibility to changing stresses. To identify linked TFBs to abiotic stresses, the 5' up-stream sequences of *PPR* genes of *A. thaliana* were surveyed using PlantPAN. In this study, PCMP-H52 and PCMP-E105 were expressed under iron deficiency and shift low to high light stresses perhaps due to the presence of both MYB and bZIP. MYB has been shown to possess regulatory effects on cell fate, hormonal action, response to environmental factors, as well as in the control of Fe transport, and tissue partitioning under iron deficiency (WANG et al. 2018).

Microarray data also showed up-regulation of most genes during iron deficiency which can be related to the presence of bZIP. These results are in agreement with the finding that bZIP possesses indirect roles in Fe-response in *Arabidopsis* (SINCLAIR et al. 2018). Cordycepin, a transcription inhibitor, causes premature termination of protein synthesis. PCMP-H58 and PCMP-E33 were up-regulated after cordycepin; this can be due to the presence of MYB and bZIP in their promoter regions. Whereas, other genes were down-regulated under cordycepin stress. BZIP present in PCMP-E35, PCMP-E5, and PCMP-E51 play an important role in glucose-ABA interaction network, regulating mRNA decay in cordycepin stress (MATIOLLI et al. 2011). Most of *PPR* genes are highly expressed under a shift low to high light stress condition which can be due to presence of MYB TFBs, which act as a clock-controlled element (YANG et al. 2018).

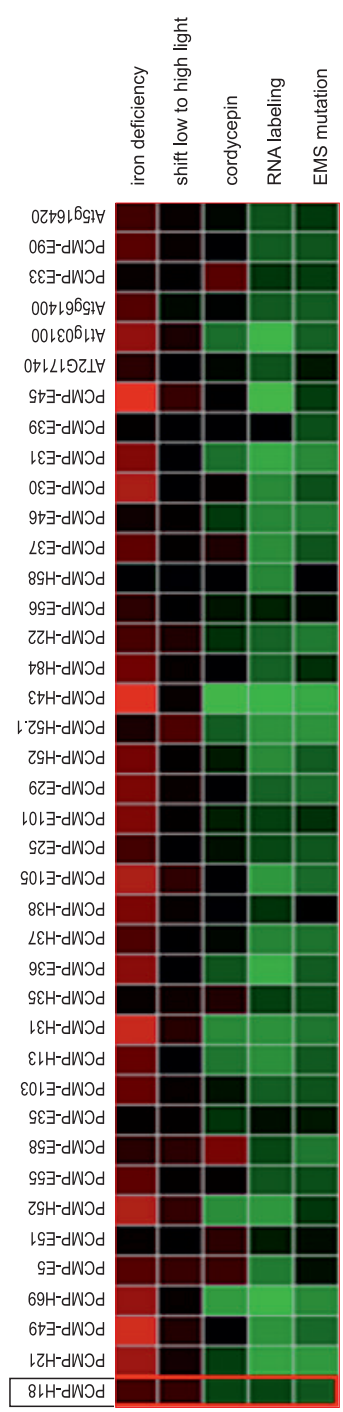

Fig. 4. Heatmap representation of expression analysis in response to biotic stresses of *A. thaliana PPR* genes based on microarray data present at Genevestigator

Structure and Characteristic Analysis of *PPR* Genes

The characteristics of the *PPR* genes were analyzed in detail. The length of protein sequences of *PPR* genes ranged from 490 (PCMP-H43) to 1271 (PCMP-H52) amino acids and their gene size ranged from 1475 (PCMP-H43) to 3816 (PCMP-H52) KDa (Table 1).

To examine the structural diversity of *PPR* genes, exon-intron distribution and conserved motifs were analyzed according to maize and Brachypodium (SAIDI and HAJIBARAT 2018, SUN et al. 2019). Gene structure analysis revealed that the number of introns in the *PPR* genes of the three clusters ranged from 1 to 2. Most of the PPR members were classified in the same subfamily. Both PCMP-H43 and PCMP-E35 genes each contained two exons. The PCMP-H43 and PCMP-E35 possess 1 intron (Fig. 5).

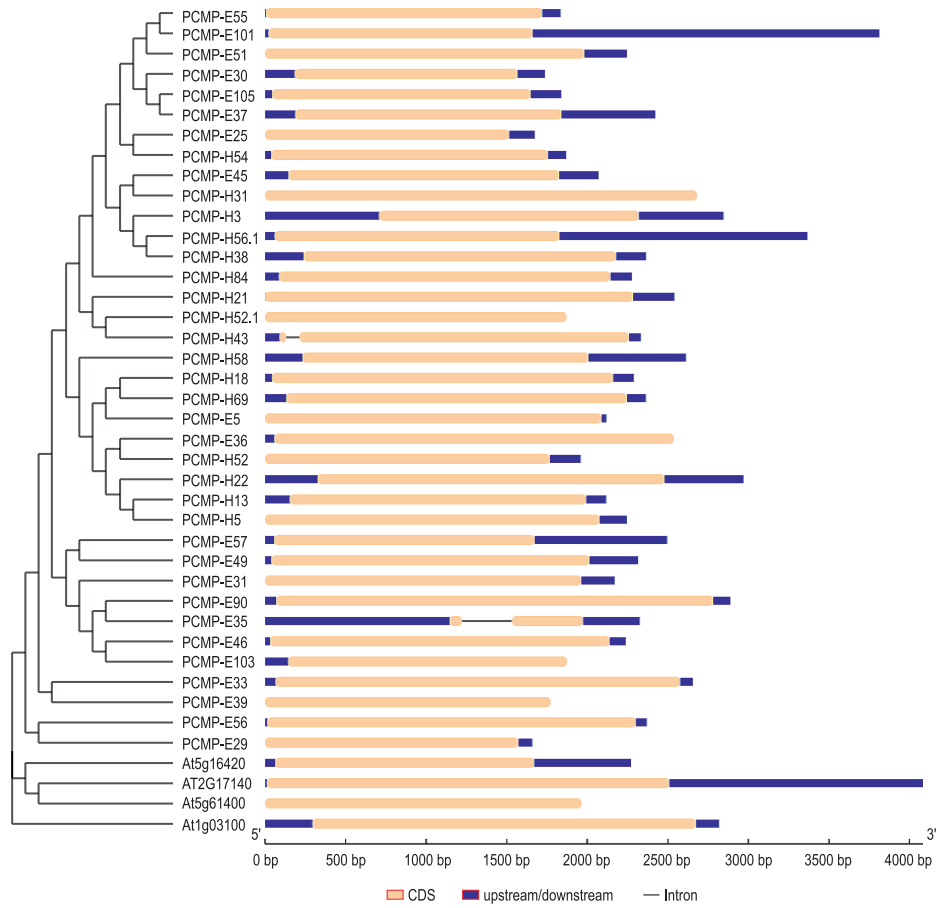

Fig 5. Distributions of the conserved motifs detected by GSDS and displayed in different colored boxes

Multiple Sequence Alignment, Conserved Motifs, and Domain Architectures in PPR Proteins

To obtain more understanding into the structure characteristics of the PPR proteins and conserved motif analysis, their amino acid sequences were submitted to MEME program. As shown in Figure 6, three motifs were identified in the PPR family members which were explored to encode functional domains when subjected to Pfam. Motif 3 was annotated as DYW family of nucleic acid deaminases while, motifs 1 and 2 were assigned by the Pfam as PPR domain. Highly similarity motifs are expected to have similar functions. DYW family belonged to PLS group having three motifs sequences namely, motif 1, 2, and 3. PPR proteins relevant to P group contained two motifs (1 and 2) (Fig. 5, Fig. 7).

The sequence alignment was performed among the reduced amino acid sequences of the 41 templates by Muscle, and identity residue was calculated (Fig. 6). The three general domain such as E, E^+, and DYW were identified as the dedicated motifs in the domain PLS-type proteins. According to other findings, the PLS subfamily have four subclasses: 1) proteins that do not possess none of the three motifs, 2) proteins have only E motif, 3) proteins with both E and E^+ motifs, and 4) proteins with the E, E^+, and DYW motifs (Fig. 8). Whereas, P-type proteins lack E, E^+ and DYW. Table 1 and Table 2 supplies details of the numbers of *PPR* genes in each subgroup and characterization of type motifs. The largest difference is the number of *PPR* genes in the PLS/P subgroups, with 24 *PPR* genes in PLS-type but only 17 in P-type (Table 1, Fig. 1). Previous studies have shown that these three motifs were located in C-terminal ends of *PPR* proteins and which were only present in PPR proteins and PCMPs and not in any other proteins of *Arabidopsis* (LURIN et al. 2004). AUBOURG et al. (2000) concluded that both E and E^+ motifs were extremely degenerate, but DYW motif was highly conserved in the amino acid sequence (Fig. 6).

As described earlier, the DYW domain is a typical feature of the PLS subfamilies. Hence, the domain architectures in PLS subfamilies were analyzed. Results showed that most of the PPR proteins included the DYW domain. However, some members of the PLS groups do not possess a DYW domain, required for site-specific editing factors in chloroplast. The DYW domain, one of the PLS family, is the candidate domain for cytidine deaminase, a highly evolutionarily domain correlated with RNA editing. While, P-type is a type of classical PPR proteins with p-motifs, having a vital role in RNA-interacting with other protein and RNAs ae well as the role played by combinatorial motifs (Fig. 6).

PLS-subfamily
E
E+
DYW

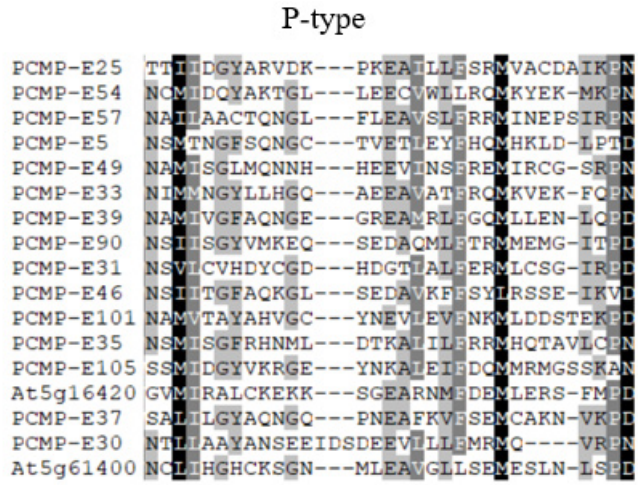

Fig. 6. Sequence alignment of the PPR motifs in *A. thaliana*. The amino acid sequences of the three domains are shown in boxes

Gene Ontology Annotation and Subcellular Localization Prediction

GO analysis was performed the using BAR database, suggesting the putative participation of PPR in various biological processes, molecular functions and cellular components (Fig. 8). All 41 PPR proteins were grouped into seven separate categories of biological processes. Our finding suggested that a most of PPR were probably associated to metabolic and cellular, followed by cell organization and biogenesis processes within mitochondria and chloroplasts including RNA editing, RNA splicing, RNA stability and translation, response to abiotic or biotic stimulus, and response to stress. PPR proteins were indicated to participate in molecular function such as hydrolase activity and other binding. The second most frequently annotated molecular function was nucleic acid and protein binding function, which is in agreement with the role of PPR10 protein in interaction with protein/RNA using two binding sites (BARKAN et al. 2012). Cellular component prediction suggested that PPR proteins were localized in mitochondria (60%), chloroplasts (30%), ribosome (6%), and cytosol (4%). It has been reported that PPR proteins are targeted approximately 54% in mitochondria and 28% in chloroplast (CHEN et al. 2018). The GO analysis results predicted that the PPR proteins incorporated in different biological and molecular processes, seed development, and fertility and can provide useful information for further gene functions studies in *Arabidopsis*. Our results have revealed that great PPR proteins are localized in mitochondria and a few in chloroplast. It has been reported that the majority of PPR proteins are indicated to target both mitochondria and chloroplast (SMALL and PEETERS 2000).

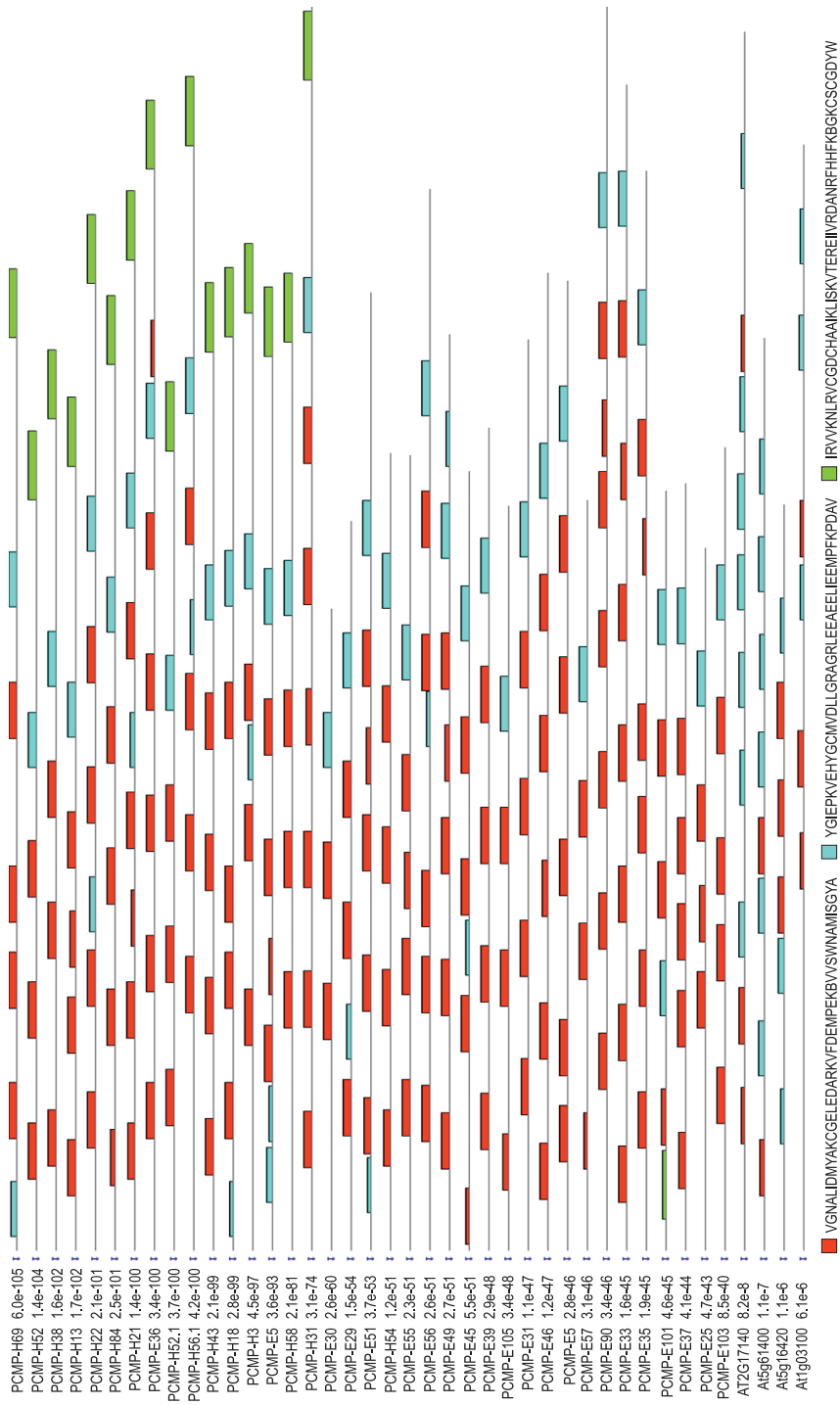

Fig. 7. Conserved motifs detected by MEME and displayed in different colored boxes

Biological process			
2.54	*0.289*	**7.254e-08**	unknown biological processes (Input set freq.: 0.65; 0.25)
1.85	*1.301*	0.318	electron transport or energy pathways (Input set freq.: 0.02; 0.01)
0.53	*0.163*	**5.105e-03**	other metabolic processes (Input set freq.: 0.21; 0.41)
0.51	*0.157*	**3.078e-03**	other cellular processes (Input set freq.: 0.21; 0.42)
0.27	*0.196*	0.092	cell organization and biogenesis (Input set freq.: 0.02; 0.08)
0.21	*0.152*	**0.041**	response to abiotic or biotic stimulus (Input set freq.: 0.02; 0.11)
0.19	*0.136*	**0.023**	response to stress (Input set freq.: 0.02; 0.12)
Molecular function			
1.6	*0.291*	**0.012**	other binding (Input set freq.: 0.41; 0.25)
1.48	*0.55*	0.095	hydrolase activity (Input set freq.: 0.17; 0.11)
1.14	*0.427*	0.152	DNA or RNA binding (Input set freq.: 0.17; 0.14)
1.04	*0.276*	0.137	unknown molecular functions (Input set freq.: 0.26; 0.25)
0.51	*0.256*	0.090	protein binding (Input set freq.: 0.07; 0.14)
Cellular component			
6.9	*0.437*	**2.930e-26**	mitochondria (Input set freq.: 0.85; 0.12)
1.37	*0.954*	0.356	ribosome (Input set freq.: 0.02; 0.01)
0.99	*0.398*	0.174	chloroplast (Input set freq.: 0.14; 0.14)
0.65	*0.402*	0.224	unknown cellular components (Input set freq.: 0.04; 0.07)
0.63	*0.232*	0.069	other intracellular components (Input set freq.: 0.14; 0.23)
0.33	*0.236*	0.148	cytosol (Input set freq.: 0.02; 0.07)
0.17	*0.099*	**1.771e-04**	other cytoplasmic components (Input set freq.: 0.04; 0.27)
0.1	*0.073*	**1.506e-04**	other membranes (Input set freq.: 0.02; 0.24)

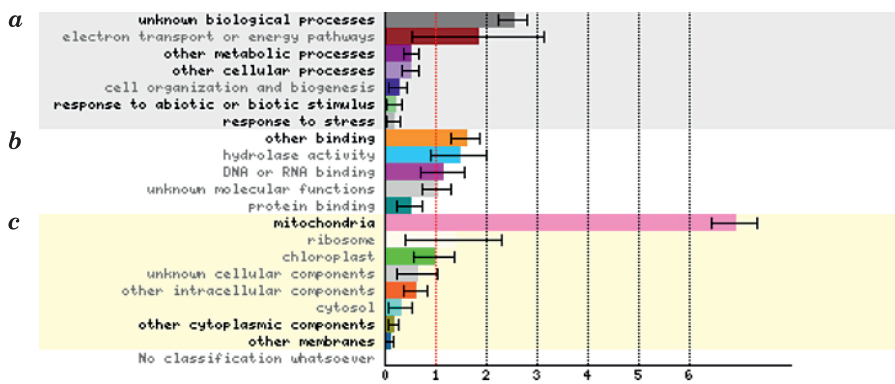

Fig. 8. GO classification of the PPR proteins. The BAR database defined the GO under three main categories: (*a*) biological processes; (*b*) molecular functions and (*c*) cellular component

Conclusion

In the current study, computational analysis of 41 PPR proteins of *Arabidopsis* revealed the presence of various types of TFBs. Consequently, analysis of PPR proteins on phylogeny, transcription factor binding sites (TFBs), chromosomal location, stress associated TFBs, expression profiles in different tissues and biotic stresses, and analysis of conserved motifs were performed based on bioinformatics. Our findings identified five

important TFBs such as MYB, bZIP, WRKY, Homeodomain, and AP2 which were involved in abiotic and biotic stress. According to analysis of TFBs, PCMP-H52 was found to be different from other *PPR* genes. The PCMP-H52 revealed a significant role in different abiotic stresses, possibly due to having multiple TFBs in its promoter region. Microarray data indicated that PCMP-H52 gene was up-regulated during senescence stage and iron deficiency and a shift low to high light stresses. Also, analysis of PCMP-H52 gene expression revealed that this gene can be considered for resistance to abiotic stresses in plant breeding programs.

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Supplement 1

Table Appx. 1

The sequences and the Pfam annotations of conserved motifs in PPR proteins

No. motifs	Specification	–
1	VGNALIDMYAKCGELEDARKVFDEMPEKBVVSWNAMISGYA	PPR
2	YGIEPKVEHYGCMVDLLGRAGRLEEAEELIEEMPFKPDAV	PPR_1
3	IRVVKNLRVCGDCHAAIKLISKVTEREIIVRDANRFHHFKBGKCSCGDYW	DYW family of nucleic acid deaminases

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TARTRAZINE INCREASES OXIDATIVE STRESS AND MODULATES THE EXPRESSION OF APOPTOTIC-REGULATORY GENES IN *DROSOPHILA MELANOGASTER*

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Key words: Tartrazine, *Drosophila melanogaster*, food additive, oxidative stress, apoptosis.

Abstract

In this study, the effect of exposure to a food colorant tartrazine was investigated on biomarkers of oxidative stress and apoptosis in *Drosophila melanogaster*. *D. melanogaster* eggs were grown on cornmeal medium containing tartrazine at different concentrations (0, 250, 500, 1000, 2000 mg/kg). Third instar larvae of *D. melanogaster* were harvested. Oxidative stress markers were assayed spectrophotometrically while the level of expression of some apoptosis-regulatory genes were quantified using reverse polymerase chain reaction technique. Tartrazine significantly ($p < 0.05$) increased the levels of hydroperoxides and malondialdehyde in a dose-dependent manner. Interestingly, glutathione level increased among the groups, while the activities of glutathione transferase were reduced across the treatment groups. Tartrazine exposure resulted in misexpression of apoptosis and stress genes with a significant reduction in ANCE, HSP 27 genes. This study suggests that tartrazine induced oxidative stress and could induce apoptosis in *D. melanogaster* larvae, thus a risk factor in developmental toxicity.

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Introduction

Tartrazine (E102) is a synthetic lemon-yellow azo dye commonly used as food, drug and cosmetic colourant (El-Keredy 2017, Khayyat et al. 2017, Tripathy et al. 1989). It is also employed as a dye for wool and silk (Sarikaya et al. 2012, Tripathy et al. 1989). Due to its low cost and stability, tartrazine is widely used in numerous consumables, including food products (Amin et al. 2010, Khayyat et al. 2017, Walton et al. 1999). Some non-food consumables containing tartrazine include cosmetics, food supplements and some specific prescription drugs (Amin et al. 2010). Allowed Dietary Intake (ADI) of tartrazine is 7.5 mg/kg/day (Amin et al. 2010, Walton et al. 1999).

Most producers involved in food industry use food additives to achieve the desired organoleptic characteristics of each product. Conversely, these additives may have adverse health effects as demonstrated in several studies (Bateman et al. 2004, Raposa et al. 2016). As the consumption of these substances increased, the incidence of diseases, notably, eczema, headache, allergic asthma, diarrhoea, hyperactivity and hypersensitiveness also increased (Uysal et al. 2015). The ingestion of tartrazine has also been associated with some behavioural changes in children such as irritability, restlessness, and sleep disturbance (Bateman et al. 2004, Rowe 1988).

Tartrazine toxicity may result directly or indirectly from the biotransformation of its azo linkage (Chequer et al. 2011). Metabolic reduction of tartrazine in the intestine of the animal by the intestinal microflora results in the formation of two metabolites – sulfanilic acid and aminopyrazolone (Chung et al. 1992, Russ et al. 2000). These metabolites of tartrazine can generate reactive oxygen species (ROS), causing oxidative stress, and affect hepatic and renal architectures and biochemical profiles (Himri et al. 2011, Khayyat et al. 2017). Oxidative stress has been implicated in several diseases (Caro and Cederbaum 2004) and the process has also been established as a key pathomechanism of inflammatory, apoptotic and ageing processes (Askari et al. 2018, Hamishehkar et al. 2014, Raposa et al. 2016, Selvakumar et al. 2018).

There are contradictory reports of the toxicity of food colorants in literature. Azo dyes have been reported to be genotoxic, inducing DNA damage in treated animals (Tsuda et al. 2001). Conversely, another research showed no significant increase in the number of micronuclei found even at high dose of tartrazine while investigating for DNA damage in mice. However, tartrazine and azorubine increased the mRNA level of CYP1A1, which are pivotal in the metabolic activation of certain procarcinogenic

substances in the liver of mice (POUL et al. 2009). Also, altered kidney and liver function and oxidative stress biomarkers were observed in male rats after tartrazine and azorubine intake (AMIN et al. 2010). In the same study, alanine aminotransferase, aspartate aminotransferase, total protein, and albumin levels were significantly increased in the treated groups compared with the control. Furthermore, tartrazine exposure reportedly disrupted hepatic oxidative balance in the animals (AMIN et al. 2010). Increased tissue concentrations of enzymes involved in the oxidative mechanisms indicate that these food colorants may intervene in the multi-step process of inflammation and carcinogenesis (RAPOSA et al. 2016). Another comparative *in vivo* toxicity study administered different doses of tartrazine to guinea pigs showed no cytotoxic changes in tissues and organs and reported absence of the development of neoplastic alteration in the experimental animals (RUS et al. 2010).

The common fruit fly, *Drosophila melanogaster*, is a well-studied and highly tractable genetic model organism for understanding the molecular mechanism of human diseases (PANDEY and NICHOLS 2011). The extensive knowledge of the genetics of *Drosophila melanogaster* and the long experimental experience with this organism has made it of unique usefulness in mutation research and genetic toxicology (SARIKAYA et al. 2012). Many fundamental biological, physiological, and neurological properties are conserved between mammals and *D. melanogaster*. Nearly 75% of human disease-causing genes have a recognisable match in the genome of *Drosophila* and 50% of the fly protein sequences have mammalian homologs (PANDEY and NICHOLS 2011, SARIKAYA et al. 2012). Furthermore, *Drosophila* is a widely accepted model organism used in *in vivo* genotoxicity and mutagenicity studies (SARIKAYA et al. 2012, WANG et al. 2018).

It is established that azo dyes, such as, tartrazine are bio-transformed into metabolic intermediates capable of generating copious amount of reactive oxygen species. However, reports regarding the toxicity of tartrazine are inconsistent. Hence, the present study aimed to evaluate the toxicity of tartrazine by examining the biomarkers of oxidative stress and its apoptotic potentials in the 3rd stage instar larvae of *Drosophila melanogaster*.

Materials and Methods

Chemicals

Tartrazine CI19140 was a product of Jinzhou Tianyu Science & Technology Co., Ltd, China. Trizol®, RNAhold® and EasyScript® one-step

RT-PCR kit was obtained from TransBionovo Co., Ltd. Beijing, China. Other chemicals and reagents were of analytical standard and purchased from Sigma-Aldrich.

Raising of Flies

The flies and larvae were cultured on a standard Drosophila diet medium containing agar, fruit concentrate (apple), sugar, yeast, $MgSO_4$ and $CaCl_2$. Additional yeast suspension was provided for healthy growth of the organism. Flies were kept in darkness for the period of egg laying and harvest; otherwise, the flies were cultured in normal light/dark cycle at 25 ± 1^0C (UYSAL et al. 2015). The fruit agar is used to collect the eggs of adult female *Drosophila melanogaster*.

Experimental Design

The experiment was carried out using previously described procedures but with slight modification (RAHUL et al. 2015, UYSAL et al. 2015). The final concentration of tartrazine i.e. 250, 500, 1000 and 2000 mg/kg bw was established in diet of the respective groups, except for the control group, which contains no tartrazine. Vials were prepared and made into five different groups in triplicates. For each group, a specific concentration of sample solution was made in a total volume of 8 ml and feed was added to make up to a total of 80 ml. The prepared feed (25 ml) was placed into vials in each group and 35 µl of PBS-washed harvested *Drosophila melanogaster* eggs was pipetted into each vial. From each of the five groups, 20 developed third stage insta-larva were harvested after five days and placed in labelled 1.5 ml Eppendorf tube which was centrifuged. After that, 300 µl of homogenizing buffer (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES-NaOH, pH 7.4) was added and then homogenized. Homogenizing buffer was added to make 1000 µl. The mixture was put in refrigerated centrifuge at 10000 g for 20 minutes at 4ºC. The supernatant was extracted into Eppendorf tubes and placed on ice until required for use.

Biochemical Assays

RNA Extraction Protocol

TriPure (a brand of Trizol) reagent (RIO et al. 2010) was added into eppendorf tubes containing ~20 3rd stage insta-larvae and homogenized. Thereafter, 200 µl of chloroform was added and vortexed for 30 seconds.

After incubating at room temperature for 10 minutes, the mixture was centrifuged at 11300 rpm for 15 minutes. Two layers appeared after centrifuging, with the top colourless layer containing the RNA. The RNA was transferred to a new labelled eppendorf tubes and 250 µl of iso-propanol was added and then mixed by inversion. This was left to seat at room temperature for 10 minutes, and then centrifuged at 1200 rpm for 10 minutes and the supernatant was collected after centrifuging. After addition of 75% cold ethanol, the resulting solution was properly mixed and then centrifuged at 9100 rpm for 5 minutes. The supernatant was carefully discarded and then pellet dried in a speed vacuum for 20 minutes. The dry pellet was dissolved in RNAfree water and used for gene expression analysis.

Gene Expression Analysis

The expression level of certain apoptotic, DNA methylating and chromatin modifying genes (Table 1) were quantified using relative reverse transcriptase polymerase chain reaction (RT-PCR) techniques as described by CHAUDHRY (2006), with appropriate modifications. In brief, about 500 ng

Table 1

List of genes studied and the sequences of Gene Specific Primers

Gene code	Gene name	Primer sequence (5'->3')
HSP 27	heat shock protein 27	forward: AAAGATGGCTTCCAGGTGTG
		reverse: CCCTTGGGCAGGGTATACTT
DMILP-2	insulin like peptide-2	forward: ACTCCCGCAGAGCCTTCATA
		reverse: GCTCAACGAGGTGCTGAGTA
ANCE	angiotensin converting enzyme	forward: ATATCGCCGGACAGAACGC
		reverse: CAGAAGTCCTGTGGCAGCTT
NC	DM death regulator NEDDE-like caspase	forward: ATTGGAATGCCGAAGAGGCA
		reverse: ATACGACGAGGAGGTCACCA
DCP-1	death caspase-1	forward: CCAAAAAGGGCGCAACAACT
		reverse: TGGCAGTGAAGTAGTGCCAG
DEBCL	death executioner BCL-2	forward: CCCAATCCCTCTAACGGACG
		reverse: TGTTCAGTGCCGGGAAAACT
β-TUB	Tubulin, Beta	forward: ACCAATGCAAGAAAGCCTTG
		reverse: ATCCCCAACAACGTGAAGAC

of RNA was used for the semi-quantitative RT-PCR using the Transgen® *EasyScript*® one-step RT-PCR reagent. Briefly, the cDNA synthesis was carried out at 45ºC for 30 minutes. This was followed by 35 cycles of PCR

amplification, using gene specific primers (GSP) – Table 1, in a C1000 Touch™ Thermal Cycler (BioRad, CA, USA). The cycles consisted of 94ºC for 30 s, 5min at the annealing temperature of GSP and 1min at 72ºC. The level of transcription of the genes relative to *β*-Tubulin was quantified using Image J® software (ABRÀMOFF et al. 2004).

Lipid Peroxidation

Lipid peroxidation was measured by the method of OHKAWA et al. (1979). Briefly, larva homogenate (50 µl) was added to the reaction mixture consisting of 8% SDS (50 µl), 20% acetic acid (40 µl) and 150 µl distilled water. Reaction was initiated by adding 400 µl of 1% TBA and terminated by 10% TCA. After centrifugation at 1000 rpm for 10 minutes, the supernatant was extracted and absorbance was read at 535 nm. MDA content of the sample was calculated by using the extinction coefficient of MDA, which is $1.56 \cdot 10^5$ M^{-1} cm^{-1}.

Glutathione Content Determination

GSH content in the exposed larvae were determined using Ellman's reagent (DTNB), following a previously described procedure (SINGH et al. 2009). The assay mixture consisted of 400 µL thiol reagent, 10 µL 0.01% 5, 5'-Dithiobis-2-nitro benzoic acid (DTNB), 25 µL TCA and 125 µL of the sample. The mixture was centrifuged at 5000 rpm for 5 minutes. The supernatant was collected, and absorbance was read at 412 nm after 5 minutes.

Hydroperoxide Assay

Hydrogen peroxide assay was performed following a previously described procedure (LONG et al. 1999). 50 µL of the sample was mixed with 200 µL of distilled water, immediately followed by the addition of FOX reagent (12.5 µL), thoroughly mixed and then incubated at room temperature for 30 minutes. Absorbance was then read at 560 nm against a water blank.

Acetylcholinesterase (AChE) Assay

Acetylcholinesterase activity was measured by the method of (ELLMAN et al. 1961) as reported by JAHROMI et al. 2013a. The assay reaction mixture consisted of 5 µL acetylcholine chloride, 25 µL DTNB (10 mM), 10 µL sample, 750 µL phosphate buffer (0.1 M, pH 8.0). The reaction mixture was thoroughly mixed and incubated at room temperature for 10 minutes. The change in absorbance was monitored against a water blank at 412 nm for 3 min.

Glutathione-S-Transferase (GST) assay

Glutathione-S-transferase activity was determined following a previously described procedure (RAHUL et al. 2015). The assay reaction mixture consisted of 500 µL of 0.1 M phosphate buffer, 50 µL of 10 mM CDNB, 50 µL of 10 mM reduced glutathione and 50 µL of the sample. The reaction mixture was thoroughly mixed by inversion, followed by immediate reading of absorbance against a buffer blank at 340 nm within 5 minutes.

Thioredoxin Reductase Assay

Thioredoxin reductase activity was determined following a previously described method (HOLMGREN and BJORNSTEDT 1995) with minor modifications (SINGH et al. 2009). The reaction mixture consists of 4 µL NADPH, 316 µL thioredoxin buffer, 40 µL DTNB and 40 µL BSA. The reaction mixture was incubated in the dark, at room temperature for 10 minutes. Thereafter, 25 µL of sample was added and the resulting mixture was left to stay for approximately 3 minutes. Absorbance was then read against a buffer blank at 410 nm at every 30 seconds for 3 minutes.

Chloramine Assay

Chloramine concentrations in larval homogenates were determined to measure the formation of oxidative protein products, using a previously described method (STANLEY et al. 2010). The assay reaction mixture contains 1000 µL DTNB and 50 µL sample. The mixture was then incubated in the dark, at room temperature for 15 minutes. Absorbance was read against a water blank at 412 nm.

Statistical Analysis

Statistical analysis was performed using a software program (GraphPad Prism 8.0.1) (GraphPad Software Inc., San Diego, CA, USA). All data were presented as mean ±S.E.M. One-way ANOVA followed by post-hoc Tukey's test was used to assess the significance of the difference between control and treatment groups.

Results

Enzymatic Antioxidant Activities Following Exposure to Tartrazine

Table 2 depicts the gluthathione transferase (GST), acetylcholinesterase (AChE) and thioredoxin reductase (TR) activities in *Drosophila melanogaster* larvae after 5 days of exposure to different tartrazine concentrations. There was a non-dose dependent decrease in the activity of GST in all treated groups in comparison to the control. However, significant reduction in GST activity was observed only in the groups exposed to 500 mg/kg bw and 2000 mg/kg bw at 0.660 ±0.210 kU/mg protein and 0.372 ±0.020 kU/mg protein respectively in comparison with the control (2.869 ±1.735 kU/mg protein), corresponding to 77% and 87% decrease in GST activity respectively. Also, the tartrazine exposed groups showed a non-dose dependent decrease in AChE activity when compared with the control, though not statistically significant. Meanwhile, all tartrazine exposed groups showed a statistically significant decrease in thioredoxin reductase (TR) activity. The observed TR activity corresponds to 40% (0.003 ±0.000 kU/mg protein), 60% (0.002 ±0.000 kU/mg protein), 40% (0.003 ±0.000 kU/mg protein) and 60% (0.002 ±0.000 kU/mg protein) reduction for 250, 500, 1000 and 2000 mg/kg.bw of tartrazine respectively in comparison with the control (0.005 ±0.000 kU/mg protein).

Table 2

GST, AChE and TR activities in *Drosophila melanogaster* larvae after 5 days of exposure to tartrazine

Group	GST [kU/mg protein]	AChE [kU/mg protein]	TR [kU/mg/protein]
Control	2.869±1.735^{a}	0.411±0.195^{a}	0.005±0.000^{a}
250 mg/kg bw	1.043±0.109^{a,b}	0.288±0.040^{a}	0.003±0.000^{b}
500 mg/kg bw	0.660±0.210^{b}	0.281±0.021^{a}	0.002±0.000^{b}
1000 mg/kg bw	1.264±0.750^{a,b}	0.339±0.019^{a}	0.003±0.000^{b}
2000 mg/kg bw	0.372±0.020^{b}	0.303±0.045^{a}	0.002±0.000^{b}

Each value is mean ±S.E.M for 20 3rd stage larvae. Values within a column with different alphabet superscripts are significantly different from each other at $p < 0.05$.

Non-enzymatic Antioxidants Levels and Oxidative Stress Parameters Following Exposure to Tartrazine

Table 3 depicts glutathione (GSH), malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and chloramine levels in *Drosophila melanogaster* larvae after 5 days of exposure to different tartrazine concentration. There was

increase in GSH levels of the groups exposed to tartrazine. The increased GSH levels is non-dose dependent with statistically significant increase, compared with the control, being observed just in the groups exposed to 500 mg/kg bw and 2000 mg/kg bw at 1.110 ±0.213 nmole/mg protein and 1.247 ±0.138 nmole/mg protein respectively, which corresponds to a respective increase by 84% and 106%. Similarly, increase in MDA levels in the groups exposed to tartrazine was non-dose dependent. The groups exposed to 500 mg/kg bw, 1000 mg/kg bw and 2000 mg/kg bw of tartrazine, compared with the control, showed a statistically significant increase in malondialdehyde levels at (39.773 ±7.619 pmole/mg protein), (54.415 ±0.803 pmole/mg protein) and (52.283 ±0.328 pmole/mg protein), corresponding to 43%, 95% and 88% increase respectively. The highest level of MDA (54.415 ±0.803 pmole/mg protein) was observed at 1000 mg/kg bw of tartrazine concentration. Conversely to preceding observations, there was a dose-dependent increase in H_2O_2 levels across the tartrazine exposed groups. However, only the group exposed to 2000 mg/kg bw of tartrazine showed a statistically significant increase in H_2O_2 level (1.345 ±0.234 nmole/mg protein) when compared with the control (0.703 ±0.127 nmole/mg protein). This is equivalent to about 91% increase. In total deviation from all result patterns observed in this study, trends in chloramine levels across all groups appears irregular and there was no statistically significant effect on chloramine levels in tartrazine exposed groups compared with the control.

Table 3

GSH, MDA, H_2O_2 and Chloroamine levels present in *Drosophila melanogaster* larvae after 5 days of exposure to tartrazine

Group	GSH [nmole/mg protein]	MDA [pmole/mg protein]	H_2O_2 [nmole/mg protein]	Chloramine [pmole/mg protein]
Control	0.604 ± 0.001^{a}	27.812 ± 4.490^{a}	0.703 ± 0.127^{a}	216.545 ± 34.26^{a}
250 mg/kg bw	$0.754\pm0.049^{a,b}$	38.083 ± 3.040^{a}	0.787 ± 0.159^{a}	163.500 ± 74.45^{a}
500 mg/kg bw	$1.110\pm0.213^{b,c}$	$39.773\pm7.619^{a,b}$	$1.056\pm0.060^{a,b}$	313.687 ± 52.04^{a}
1000 mg/kg bw	$0.931\pm0.128^{a,b,c}$	54.415 ± 0.803^{c}	$1.121\pm0.107^{a,b}$	246.290 ± 19.02^{a}
2000 mg/kg bw	1.247 ± 0.138^{c}	$52.283\pm0.328^{b,c}$	1.345 ± 0.234^{b}	183.637 ± 07.63^{a}

Each value is mean ± S.E.M. for 20 *drosophila* larvae. Values within a column with different alphabet superscripts are significantly different from each other at $p < 0.05$

Expression Pattern of Apoptotic and Oxidative Stress genes

Figure 1 depicts the expression patterns of heat shock protein 27 (HSP27), inhibitors of apoptotic protein-like protein 2 (ILP2), negative cofactor 2 (NC2), angiotensin-1 converting enzyme (ANCE) and death caspase (Dcp) genes in *Drosophila melanogaster* larvae after 5 days

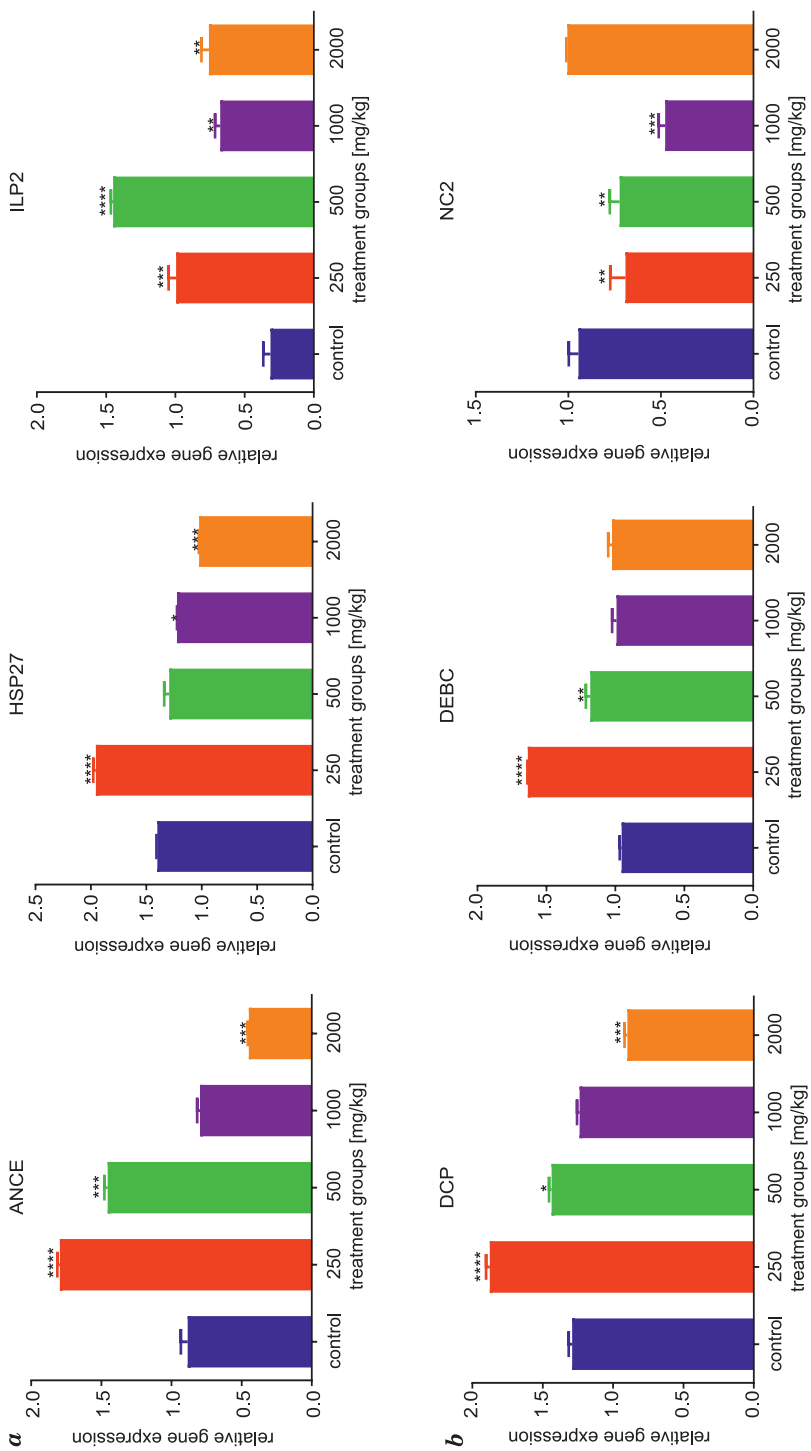

Fig. 1. Expression patterns of: *a* – oxidative stress development related genes (ANCE, HSP27, ILP2); *b* – apoptosis (DCP, DEBC, and NC2) in 3rd stage instar larvae of *Drosphilia melanogaster* after 5 days of exposure to tartrazine. Data represent mean ± S.E.M. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$; **** $p < 0.0001$ as compared to control

of exposure to different tartrazine concentration. HSP27 gene expression increased significantly at the dose 250 mg/kg bw, which dropped to a non-significant level at the dose of 500 mg/kg bw. The HSP27 gene expression decreased further to a statistically significant level with increasing concentration of tartrazine at 1000 mg/kg bw and 2000 mg/kg bw as compared with the control. On ILP2 gene expression, an irregular non-dose related increase in the gene expression was observed in all tartrazine exposed groups, though all significantly higher when compared with control. Tartrazine exposure has no statistically significant effect on NC2 gene expression except for the significant reduction observed in the group exposed to 1000 mg/kg bw tartrazine. ANCE expression increased significantly at the dose of 250 mg/kg bw, which reduced slightly at the dose of 500 mg/kg bw, however, still significantly higher when compared with the control. The ANCE gene expression further decreased accordingly with increasing concentration, initially, to almost normal level at 1000 mg/kg bw and then to a statistically significant level at 2000 mg/kg bw dose of tartrazine as compared with the control. Expression pattern of Dcp gene was strikingly similar to that observed in ANCE gene expression. The Dcp gene expression increased significantly at 250 mg/kg bw but steadily reduced and finally to a statistically significant level at 2000 mg/kg bw dose of tartrazine when compared with the control.

Discussion

This present study undertaken to evaluate the toxic outcome of tartrazine administration in *Drosophila melanogaster* has shown definitive oxidative stress and apoptotic responses induced by the additive. Tartrazine elevated the oxidative stress parameters, MDA, H_2O_2, and chloramine contents in *D. melanogaster* larvae while decreasing the activities of the antioxidant enzymes, TR and GST. In addition, tartrazine modulated the expression of apoptotic and oxidative stress genes like HSP27, ILP2, ANCE and Dcp after 5 days of exposure.

Reactive oxidants are generated in biological systems both through endogenous processes and external factors including exposure to drugs, chemicals and pollutants. Due to the high reactivity of these oxidants, damage may occur to these biological systems. Increase in free radicals causes overproduction of MDA, which is a marker of oxidative stress (VAVÁKOVÁ et al. 2015). MDA is one of the final products of polyunsaturated fatty acid peroxidation (lipid peroxidation) in cells, and is usually implicated in the pathogenesis of various diseases such as atherosclerosis,

stroke and Graves' disease (AHMAD et al. 2008, PALIPOCH and KOOMHIN 2015). Tartrazine belongs to the group of azo dye food colorants and metabolized inside the body into aromatic amines by intestinal microflora. The resulting amines are able to generate ROS as part of their metabolism by the interaction of the active amino groups with nitrite or nitrate containing foods (MOUTINHO et al. 2007). In this study, tartrazine-treated groups showed a significant increase in MDA levels which suggests that high concentration of tartrazine might induce lipid peroxidation due to increase ROS, hence oxidative stress in biological system. This is consistent with reports where significant higher concentrations of MDA were observed in animals fed with synthetic dyes, such as tartrazine (EL-DESOKY et al. 2017, AMIN et al. 2010, GAO et al. 2011). In another study, tartrazine-treated rats exhibited elevated levels of MDA and nitric oxide (NO), indicating the manifestation of oxidative stress (KHAYYAT et al. 2017). In the same vein, there was a significant elevation of H_2O_2 level in the group with the highest tartrazine administration. This is an indication that high dose of tartrazine results in the generation of numerous superoxide radicals, resulting in increased oxidative stress. The elevated levels of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in this study evidently indicate that tartrazine induces oxidative stress by generating ROS in the *Drosophila melanogaster* larvae.

Antioxidant defence systems constitute mechanisms by which living organisms combat oxidative stress by mopping up generated free radicals. These antioxidants can be enzymatic or non-enzymatic. Superoxide dismutase (SOD) is an enzymatic antioxidant that acts as living organisms first line of defence against ROS by catalysing their conversion to less reactive or inert species (MITTLER et al. 2004). SOD catalyses the dismutation of superoxide to hydrogen peroxide and oxygen (KOHEN and NYSKA 2002). The end product of the dismutation reaction H_2O_2 can be removed by the activity of the enzyme catalase (ONYEKA et al. 2012). The increased concentration of H_2O_2 could be an indication of a breakdown of the enzymatic antioxidant system in the larvae. Thioredoxin reductase (TR) is an important enzymatic antioxidant that catalyses the reduction of thioredoxin in a thiol-like redox system that involves several oxidoreductases including glutathione reductase and lipoamide dehydrogenase among others (MUSTACICH and POWIS 2000). It is a conserved protein that is involved in protecting organisms against various oxidative stress (CHENGA et al. 2018). TR plays important roles in a wide range of cellular processes, such as cell growth, DNA synthesis, apoptosis regulation, and antioxidative defence in organisms (CHO et al. 2001, MUSTACICH and POWIS 2000). It protects cells through redox homeostasis against oxidative stress (CHENGA

et al. 2018, MCCARTY et al. 2015, RAHLFS et al. 2002). This study revealed a significant reduction in TR levels in all the groups administered with tartrazine. This suggested that tartrazine interferes with thioredoxin recycling, thus affecting the availability of its reduced form required for important biological processes including free radical scavenging.

Glutathione (GSH) is one of the most abundant cellular antioxidants, providing protection against reactive species. The antioxidant activity comes from the free thiol group of GSH, which is easily oxidized non-enzymatically by electrophiles and other oxidants. GSH also serves critical roles in detoxification of electrophiles (WU et al. 2004). Furthermore, GSH plays predominant role in regulation of cellular and subcellular redox state, for example through reactions with glutaredoxin and protein disulfide isomerases to organize a proper tertiary structure of proteins through thiol-disulfide exchange (FRANCO et al. 2007). Glutathione S-transferases (GST) are a large and diverse group of enzymes which catalyze the conjugation of electrophilic xenobiotic substrates with the tripeptide glutathione (GSH; g-glu-cys-gly) (MA et al. 2018). Besides, GST can also serve as nonenzymatic binding proteins (known as ligandins) interacting with various chemical compounds that include steroid, thyroid hormones, bile acids, heme, bilirubin and fatty acids (CHO et al. 2001). Also, evidence suggests that GST is involved in cellular defense against diverse groups of toxic agents that may be generated in the environment or within the cell (MA et al. 2018). GST is involved in the metabolism of drugs and xenobiotics with numerous findings suggesting it having a vital role in resistance to stress (CURTIS et al. 2010, HAYES and STRANGE 2000, ROXAS et al. 2000). In this present study, a significant elevated level of GSH and significant reduction in GST activities were observed following a high dose of tartrazine in the 3rd instar larvae of *Drosophila melanogaster*. The observed elevated level of GSH might be an adaptive response of the larvae to the exogenous molecule, tartrazine or a failure of the glutathione utilizing system in the cell. The observed reduction in GST activities is suggestive of the latter contributing to the accumulation of GSH in the cell. The reduction in GST activities could have resulted from its inhibition or depletion following increased ROS generation with subsequent oxidation of the protein. This would effectively compromise the protein activity, eventually contributing to a poor clearance of tartrazine from the system which will further subject the larvae to increased chemical stress. Our results are in consonance with similar study reflecting the damaging effect of tartrazine on antioxidant defense system. The authors reported GSH, SOD and catalase activities were reduced while MDA levels increased in tissue homogenate of rats that consumed high tartrazine. They concluded

that tartrazine adversely affects and alters biochemical markers in liver and kidney at both high and low doses (AMIN et al. 2010).

Acetylcholinesterase catalyses the hydrolysis of neurotransmitters, primarily acetylcholine, into acetate and choline, serving essential neurologic function (LIONETTO et al. 2013). It is the most important biological component of cholinergic function (JAHROMI et al. 2013b). AChE is involved in membrane integrity and changes in membrane permeability occurring for synaptic transmission and conduction (SCHMATZ et al. 2009). This present study found no significant effect of tartrazine on AChE activity in the 3rd instar larvae of *Drosophila melanogaster*. Likewise, there was no significant effect of tartrazine on chloramines levels in the larvae. The reaction of proteins with chloramines and other chlorinated oxidants results in chlorination of amino acid residues and formation of group of products generally classified as advanced oxidation protein products (AOPPs), which are surrogate markers of inflammation (ÇAKATAY et al. 2008, KORKMAZ et al. 2013, MARROCCO et al. 2017). Despite evidence in our study of tartrazine's ability to disrupt antioxidant defense system, its lack of effect on both AChE activity and on chloramines level imply that tartrazine may not be neurotoxic and inflammatory in *D. melanogaster*.

Amidst several changes in cellular activity and physiology, stressed cells remarkably produce a highly conserved set of proteins, the Heat Shock or Stress Proteins (HSPs) and certain non-coding RNAs, like the hsrω transcripts in *Drosophila* and the satellite III transcripts in humans (ARYA et al. 2007, JOLLY and LAKHOTIA 2006, LAKHOTIA 2012). Hence, this study went further to investigate the effect of tartrazine on the level of expressions of certain stress and apoptosis related genes, such as, Hsp27, ILP-2, NC2, ANCE and Dcp genes in the 3rd stage instar larvae of *Drosophila melanogaster*. These genes have been reported to be differentially expressed at different stages of development (DWIVEDI and LAKHOTIA 2016).

Hsp27 is a 27-kDa protein, which represents a key component of the cellular adaptive response that helps maintain cellular homeostasis under stress. It exerts its anti-apoptotic influence by inhibiting cytochrome c and TNF-mediated cell death (ARYA et al. 2007, PAUL et al. 2002). Increased expression of Hsp27 during stress response correlates with better survival from cytotoxic stress (BRUEY et al. 2000, CONCANNON et al. 2003, DWIVEDI and LAKHOTIA 2016, PAUL et al. 2002). Findings from this study reveal a dose-dependent effect of tartrazine on Hsp27 gene expression in 3rd stage instar larvae of *Drosophila melanogaster*. At low dose, a significant increase in Hsp27 gene expression was observed as compared to the control, indicating an adaptive response to the stress induced by tartrazine in the larvae. However, higher doses of tartrazine resulted in significant reduction in

Hsp27 gene expression in the 3rd stage instar larvae of *Drosophila melanogaster*. This reduced expression might be as a result of the overwhelming burden or toxicity of high dose of tartrazine on the adaptive response mechanism and perhaps cellular damage via induction of apoptosis. On the other hand, our results depicted significant increase in the expression level of ILP-2 genes in all 3rd stage instar larvae groups administered with tartrazine. ILP-2 belongs to a protein family known as Inhibitors of Apoptosis Protein (IAP), which inhibits apoptosis via interaction with diverse component of the caspase signaling pathways (KHALILI et al. 2016, RICHTER et al. 2001). The gradual reduction of IL-2 expression after peaking at 500 mg/kg bw tartrazine dose indicates the potential toxicity of tartrazine at higher doses.

NC2 is a protein factor that interacts with TATA-binding protein (TBP) to mediate the initiation of transcription (MASSON et al. 2008). Research data have suggested that the association of NC2 with DNA bound TBP competes with the association of transcription factor IIA (TFIIA) and transcription factor IIB (TFIIB), and thus inhibits transcription initiation (CANG_and PRELICH 2002, KAMADA et al. 2001, XIE et al. 2000). It has also been demonstrated that NC2 does not only repress, but also activates transcription, in vitro and in vivo (CANG and PRELICH 2002, GEISBERG et al. 2001). NC2 becomes limiting for TBP association with a heat inducible promoter under heat stress (MASSON et al. 2008). This present study finds no remarkable effect of tartrazine on NC2 gene expression in the 3rd stage instar larvae of *D. melanogaster*.

Dcp is an effector caspase which actively participate in programmed cell death (apoptosis) (AKAGAWA et al. 2015, HOU et al. 2008, XU et al. 2009). In the fruit fly, dcp-1 plays an essential role in nurse cell deaths during mid oogenesis under condition of starvation (LAUNDRIE et al. 2003). Dcp-1 knock-out mutants are viable without apparent adult defects, hence, dcp-1 might not be crucial for normal development (MILLS et al. 2005, MURO et al. 2006, XU et al. 2006). ANCE is a *Drosophila* homolog of the mammalian Angiotensin-converting enzyme (ACE) (HARRISON and ACHARYA 2015, KIM et al. 2017). ACE is a dipeptidyl carboxypeptidase that converts Angiotensin I to Angiotensin II (KIM et al. 2017, TAGLIAZUCCHI et al. 2016). In mammals, ACE is pivotal to the regulation of the renin-angiotensin system (RAS) on blood pressure homeostasis by generating Angiotensin II, an active vasoconstrictor that increases blood pressure (KIM et al. 2017). It also play some roles in immune response, cytokine expression, and cell proliferation (HEENEMAN et al. 2007, NAVARRO et al. 2006, PLATTEN et al. 2009).

In *Drosophila*, tissue specific expression of the ANCE gene in the germinal disc has been reported, thus indicating that ANCE might play a key

role in the fly development (HURST et al. 2003, KIM et al. 2017). However, there are reports that ANCE is dispensable for the development of *Drosophila* fly (KIM et al. 2017). In this study, the level of expression of both the Dcp and Ance genes significantly increased at the different doses of tartrazine at 250 mg/kg bw and 500 mg/kg bw. The elevated levels of Dcp gene expression is an indication of the apoptotic potentials of tartrazine while the elevation of the Ance gene expression suggests that tartrazine produces ROS, thus causing oxidative stress. Tartrazine showed no significant effect on the expression of the genes at the dose of 1000 mg/kg bw, however, at 2000 mg/kg bw the expression of both Dcp and Ance genes were reduced significantly. The observed reduction could be as a result of the high toxicity of tartrazine at the very high dose, hence disrupting the cellular adaptative response of the fly.

In investigating the apoptotic potentials of tartrazine, the present study revealed rather complex results. Tartrazine could trigger apoptotic effects as seen in the elevated expression of ANCE and Dcp at low doses. However, the antiapoptotic effect of tartrazine was also revealed via the significant reduction of ANCE and Dcp expressions at high doses. This seemingly antiapoptotic effect of tartrazine was also observed via the non-dose dependent elevation of ILP2 expression. Similar to the findings of this present study, RAPOSA et al. (2016), reported that based on the nuclear factor kappa activated B cells (NFκB) and mitogen-activated protein kinase 8 (MAPK8) expressions – tartrazine alone could contribute to apoptotic effects at low concentration (41.2 mg/kg bw) and to anti-apoptotic effects at high concentration (412 mg/kg bw). The authors concluded that artificial food colorants result in the activation of inflammatory pathways favoring the development of cancers (RAPOSA et al. 2016). Meanwhile, 500 mg/kg bw tartrazine was reported to increase apoptotic characteristics of brain tissue of rats (GAO et al. 2011).

Conclusion

In conclusion, findings from this study indicate the oxidative stress and apoptotic potential of tartrazine and by extension, other food colorants. This emphasizes the need for adequate consumers' awareness of the potential toxicity of these food colorants and proper regulation of the concentration being added into food, especially for children. Moreover, there is need for further investigation paths to accurately define the range of concentrations of these food dyes capable of eliciting these toxic effects.

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AGE AND GROWTH OF BERG LOACH (*OXYNOEMACHEILUS BERGIANUS*) IN KORDAN RIVER OF NAMAK BASIN IN IRAN

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Key words: Ageing; Alborz Province; growth pattern; Nemacheilidae.

Abstract

The age and growth of *Oxynoemacheilus bergianus* were examined in 358 specimens collected monthly from Kordan River in 2013–2014. The total length range was 32.36–74.36 mm (50.95 ±10.3 SD), and the total weight 0.31–4.06 g (1.12 ±0.68 SD). The maximum age based on otolith readings was 5^+ years for females and 4^+ years for males. The most frequent age groups were 2^+ and 3^+ in males and females, respectively. The total length–weight relation for females was $W = 0.0079TL^{2.99}$ ($r^2 = 0.92$) and for males was $W = 0.0093TL^{2.84}$ ($r = 0.92$), indicating an isometric pattern for females and a negative allometric growth for males. The Von Bertalanffy growth model was estimated as $L_t = 86.9[1\text{-}e^{-0.16(t+3.1)}]$ and $Lt = 94.3[1\text{-}e^{-0.15\ (t+2.5)}]$ for males and females, respectively. The growth performance index was estimated as 7.1 for males and as 7.2 for females, indicating a faster growth rate for females. The fastest growth rate for this species was in the first and second year of life.

Introduction

The loaches are found in most rivers throughout Iran, including Caspian Sea, Tigris, Kor and Bushehr river basins (KEIVANY et al. 2016a). Recently, six genera including *Ilamnemacheilus*, *Oxynoemacheilus*, *Paracobitis*, *Paraschistura*, *Turcinemacheilus* and *Triplophysa* with at least 40 species has been reported, many of them being endemic (ESMAEILI et al. 2017). The Nemacheilids inhabit a variety of inland waters including turbulent mountain streams to lowland salty rivers and are the second dominant fish species in the freshwater fishes of Iran. However, there is little information on their biology in Iran (TABIEE and Abdoli 2005, HEYDARNEJAD 2009, JAMALI et al. 2015, KEIVANY et al. 2016a).

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Berg loach, *Oxynoemacheilus bergianus* (Derzhavin, 1934) is an endemic widespread member of the nemacheilids reported from Caspian Sea, Urmia and Namak basins (KEIVANY et al. 2016a, ESMAEILI et al. 2014 2017). Some information on the biology of *Oxynoemacheilus bergianus* has been provided (TABATABAEI et al. 2013, KAMALOO and KEIVANY 2014, ZAMANI-FARADONBE et al. 2015, JAMALI et al. 2015), but, in general, very little is known about its distribution and biology. There are also some works on related species such as *Oxynoemacheilus kiabii* (ABBASI et al. 2013), *Paracobitis iranica* (MARMAEI et al. 2014) *Turcinoemacheilus hafezi* (PATIMAR et al. 2014), *Oxynoemacheilus angora* (HASANKHANI et al. 2014). Detailed description of its life history has not been given in the literature. Thus, providing information on the basic biological parameters of this loach species is fundamental for understanding its life history patterns and implementing effective management. Hence, the aim of this study was to examine and describe the age and growth parameters of *O. bergianus* population inhabiting Kordan River in Namak basin of Iran.

Materials and Methods

A total of 358 specimens of *Oxynoemacheilus bergianus* from Kordan River were captured monthly during September 2013 to August 2014 by dip and seine nets (1 mm mesh size). *Oxynoemacheilus bergianus* is the only species of *Oxynoemacheilus* in Kordan River. Fish samples were anesthetized in 1% clove oil and transported to the laboratory after fixation in 10% formalin for further analyses. Routine laboratory measurements, including standard (SL) and fork length (FL) to the nearest 0.01 cm and total body weight to the nearest 0.01g, were carried out. For ageing, the sagittal otoliths were removed and studied under microscope (LAGLER 1956). The sex was distinguished by examining the gonads under a stereomicroscope.

The von Bertalanffy growth parameters were calculated using $L_t = L_\infty [1\text{-}e^{-K\,(t\text{-}t_0)}]$ for FL and $W_t = W_\infty [1\text{-}e^{-K\,(t\text{-}t_0)}]^b$ for weight,

where:
L_t – the length of fish in cm at age t
L_∞ – asymptotic fish length in cm
e – the base of natural log (2.71828)
t – the fish age (year)
t_0 – the hypothetical time at which the length of the fish was zero
K – the rate at which the growth curve approaches the asymptote
W_t – the weight of the fish in g at age t
W_∞ – asymptotic weight of the fish in g and b is the constant in the length–weight relationship (RICKER 1975, SPARRE and VENEMA 1992). Pattern of growth in both sexes was determined using the Pauly's model (PAULY 1984):

$$t = \frac{\text{sd ln } L_f}{\text{sd ln } W_t} \cdot \frac{|b-3|}{\sqrt{1-r^2}} \cdot \sqrt{n-2}$$

where:

sd ln L_f and sd ln W_t – the standard deviation of the natural logarithm of the fork length and body weight, respectively

b – the slope, calculated from the length and weight relationship.

For calculating instant growth, $r = \text{Ln}(W_{(t+1)}) - \text{Ln}(W_{(t)})/\Delta t$ was used, in which r = special growth, $W_{(t+1)}$ = average weight of fish at the age of $(t+1)$, W_t = average weight of the fish at the age of t and Δt = time differences which usually equals 1.

K and t_0 were obtained according to BERTALANFFY (1938). Growth performance index (phi-prime index) φ` was computed from the equation: $\varphi` = \text{Lnk} + 2 \cdot \text{Ln}L_\infty$ (PAULY and MUNRO 1984). Condition coefficient was calculated for both sexes using the equation $K = (W/FL^3) \cdot 100$ (Ricker 1975). For comparison of two means, after Normality test, t-test, and for multiple comparison of means, one-way ANOVA, followed by Dunkan test, at 95% confidence level was used. Statistical analyses were carried out in SPSS 20 and Excel 2016 computer software.

Results

The length, weight, age and sex of 358 specimens (179 males, 168 females and 12 unidentified) of *O. bergianus* in Kordan River were determined during a full year (Table 1 and Table 2). The total length for males ranged between 3.62–7.07 (5.13 ± 0.92 SD), for females 3.65–7.44 (5.17 ± 0.14), 3.24–3.78 (3.52 ± 0.15) for unidentified and 3.24–7.44 (5.10 ± 1.00) for all.

Table 1

The length and weight of *O. bergianus* from Kordan River

Sex	No.	TL [cm]		Weight [g]	
		min–max	mean±sd	min–max	mean±sd
Immature	12	3.24–3.78	3.52±0.15	0.31–0.56	0.38±0.08
Male	179	3.62–7.04	5.13±0.92	0.32–2.41	1.07±0.56
Female	167	3.65–7.44	5.17±1.04	0.31–4.06	1.23±0.77
All	358	3.24–7.44	5.10±1.03	0.31–4.06	1.12±0.68

Table 2

Specific growth rate of *O. bergianus* in different ages in Kordan River

Sex	Ages					
	–	1^+	2^+	3^+	4^+	5^+
Male	CF	0.75	0.72	0.75	0.73	
	annual growth rate	0.31	0.44	0.2		
Female	CF	0.85	0.78	0.78	0.81	0.78
	annual growth rate	0.37	0.38	0.43	0.13	

The total weight for males ranged between 0.32–2.41 (1.07 ± 0.56), for females 0.31–4.06 (1.23 ± 0.77) and 0.31–0.56 (0.38 ± 0.08) for unidentified specimens. The length class 3.95–4.64 for males and 5.34–6.04 for females were the dominant classes (Fig. 1). There was a significant difference between males and females in total length and weight.

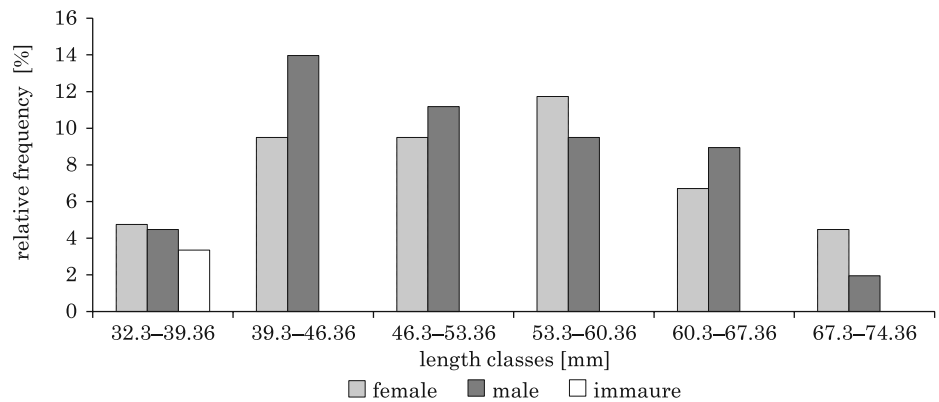

Fig. 1. The frequency for each length group of *O. bergianus* from Kordan River

Age ranged between 1^+-4^+ years in males and 1^+-5^+ years in females. Undetermined specimens belonged to 0^+ age group. The 2^+ and 3^+ year class was dominant in males and females, respectively (Table 2). Some 179 specimens (52%) were females and 168 (48%) males (Fig. 1). The sex ratio was about 1M:1.1F, which was not significantly different from 1:1 ratio ($p > 0.05$) – Table 1. Males were dominant in age groups 1–3 (Fig. 2). The length–weight relationship for females, males and all individuals was as $W = 0.0079L^{2.99}$ ($r^2 = 0.92$), $W = 0.00932L^{2.84}$ ($r^2 = 0.92$) and $W = 0.0001L^{2.89}$ ($r^2 = 0.92$), respectively, indicating an isometric growth pattern for the females and a negative allometric growth for the males and all fish, based on Pauly (1984) – Figure 3–5.

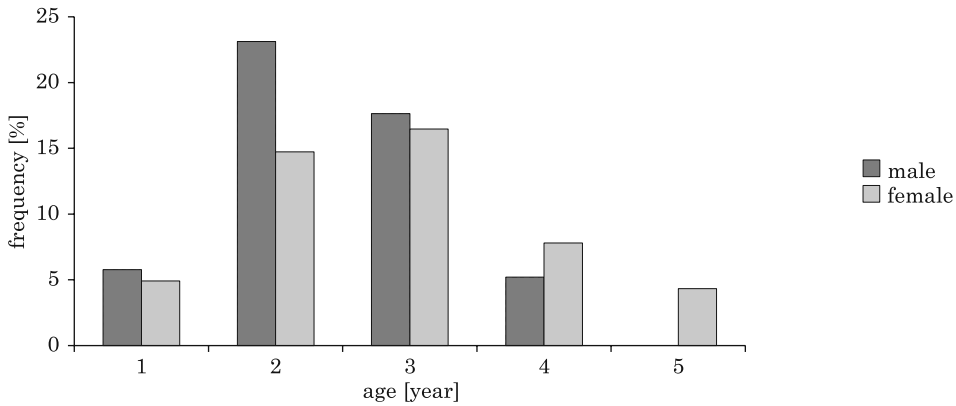

Fig. 2. The frequency for each age group of *O. bergianus* from Kordan River

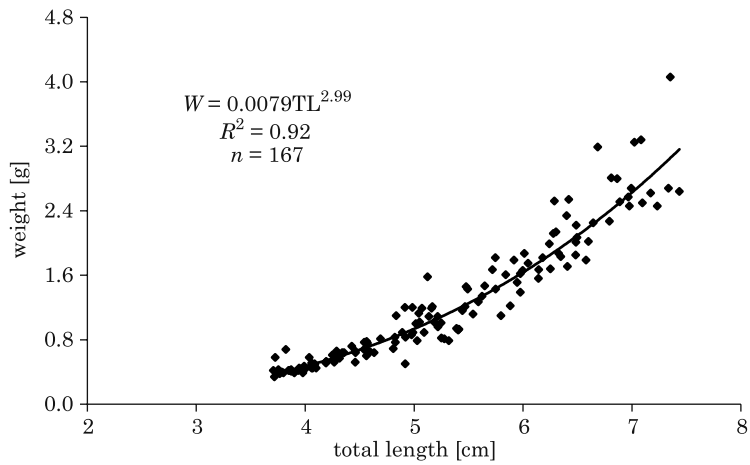

Fig. 3. Length–weight relationship of female *O. bergianus* in Kordan River

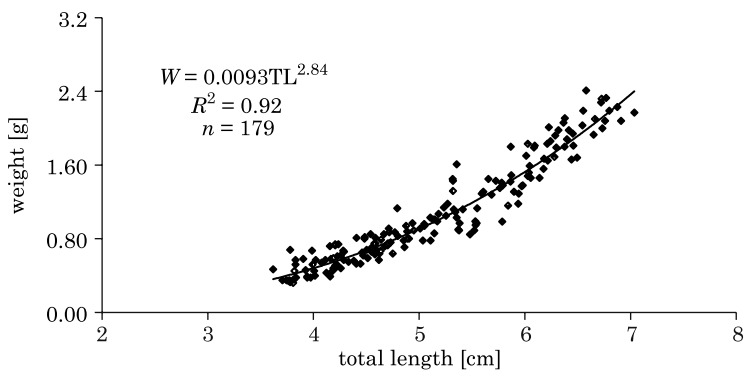

Fig. 4. Length–weight relationship of male *O. bergianus* in Kordan River

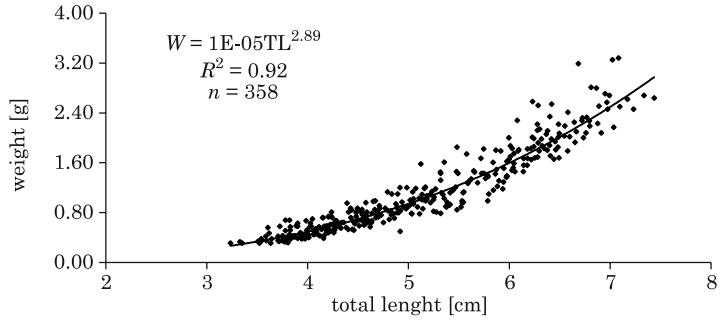

Fig. 5. Length–weight relationship of all specimens *O. bergianus* in Kordan River

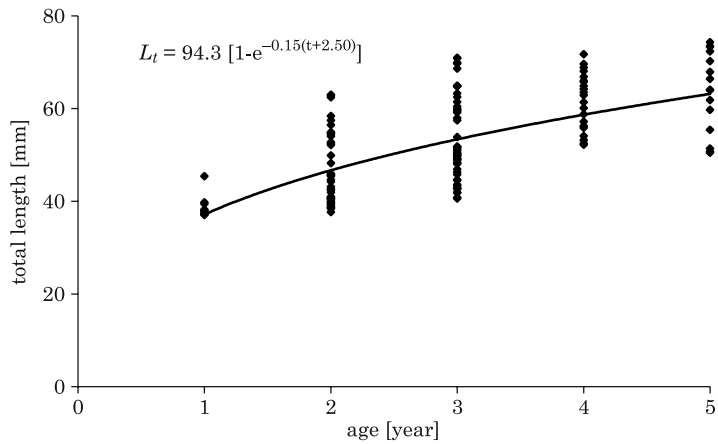

Fig. 6. Age–length relationship of female *O. bergianus* in Kordan River

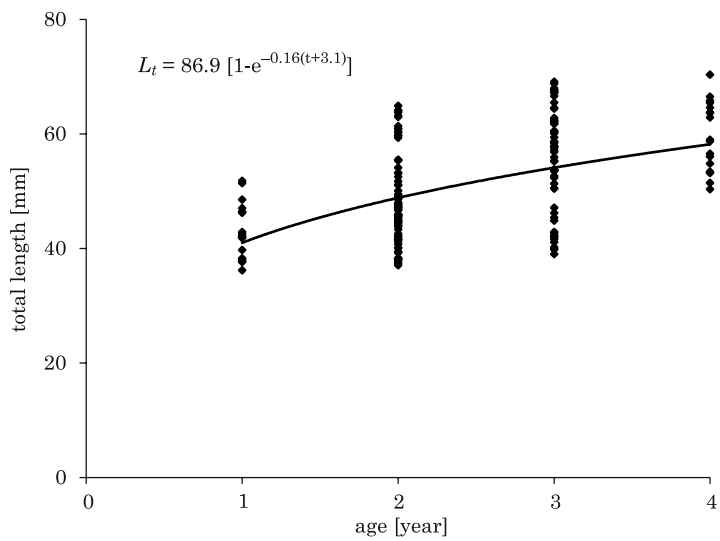

Fig. 7. Age–length relationship of male *O. bergianus* in Kordan River

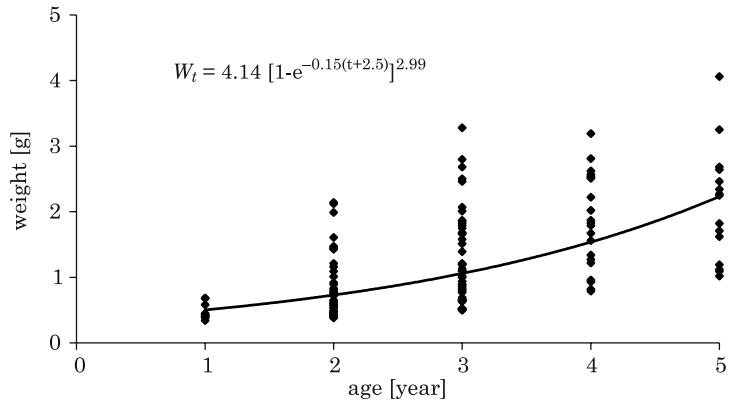

Fig. 8. Age–weight relationship of female *O. bergianus* in Kordan River

The age-length and age-weight relationships in males and females were estimated as $L_t = 8.69[1\text{-}e^{-0.16\ (t+0.3.1)}]$, $W_t = 5.47[1\text{-}e^{-0.16\ (t+3.1)}]^{2.84}$ and $L_t = 9.43[1\text{-}e^{-0.15\ (t+2.5)}]$, $W_t = 4.14[1\text{-}e^{-0.15\ (t+2.5)}]^{2.99}$, respectively (Figs. 6–8). The von Bertalanffy growth parameters for males, females, and all fish are displayed in Figure 3. Based on the growth performance index (Φ›), males showed a higher (3.84) growth rate than females (2.18). Mean total length and weight for different age groups of males and females were estimated (Fig. 9). Age-length and Age-Weight relationships of males and females are plotted in Figure 6 and Figure 7.

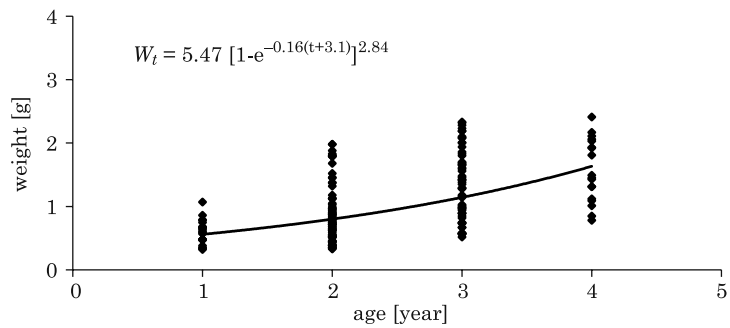

Fig. 9. Age–weight relationship of male *O. bergianus* in Kordan River

The mean condition factor was significantly different in some months. The highest value was in April for males, and in April and May for females (Fig. 10 and Fig. 11). The specific growth rate for males and females is indicated in Table 2. Growth index indicates that females grow faster than males (Table 3). The specific growth rate decreases by age in both males and females. The condition factor did not vary significantly in different ages.

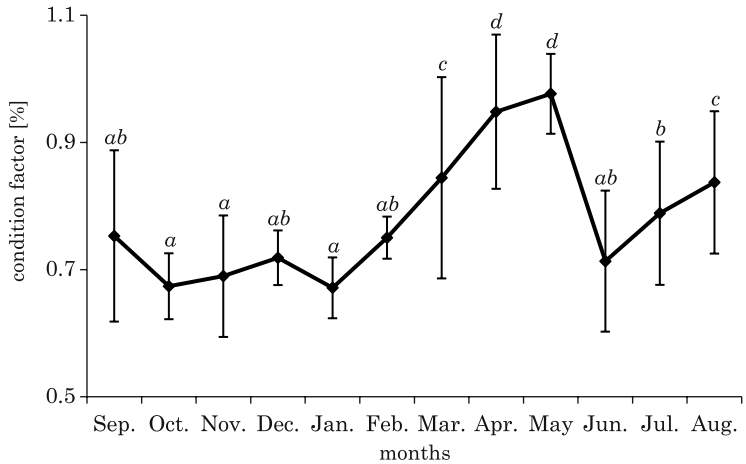

Fig. 10. Condition factor ±Sd of female *O. bergianus* in Kordan River

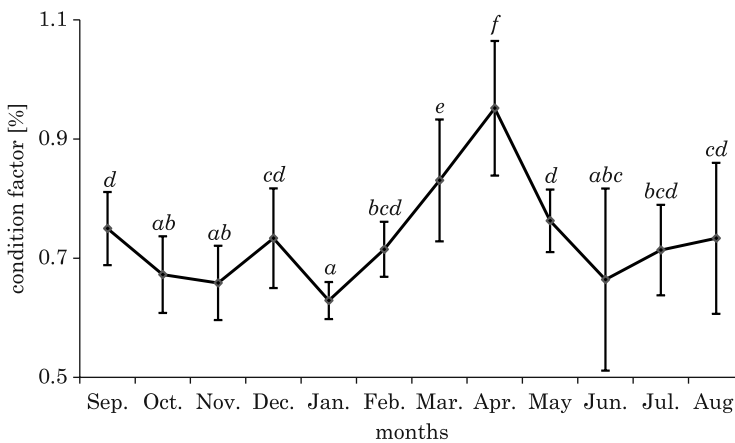

Fig. 11. Condition factor ±Sd of male *O. bergianus* in Kordan River

Table 3

Growth rate of *O. bergianus* in different sexes in Kordan River

Sex	L_∞ [cm]	t_0	k	φ
Male	8.69	-3.1	0.16	7.1
Female	9.43	-2.5	0.15	7.2

Discussion and Conclusions

This is the first study on the age and growth of *Oxynoemacheilus bergianus* in Namak basin and also one of the few studies in Iran. The sample size indicates the low frequency of the species in the river, an indication

that the species might be at risk because of ecological threats. Although there are some biological data for some nemacheilid species in Iran, there is none for some others (JAMALI et al. 2015). Like many other species, *O. bergianus* has a small size (total length and weight), not exceeding 90 mm and 6 g (ZAMANI-FARADONBE et al. 2015). Unlike Sefidrud population, females exhibit a much wider range in length and a higher maximum length than males. The total length for some other species of loaches, i.e., *Oxynoemacheilus kiabii*, *O. angora* and *Metaschistura cristata* was reported between 8.2–8.7 mm (ABBASI et al. 2013, HASANKHANI et al. 2014, PATIMAR et al. 2014). Variation in mean size (length and weight) of the population of a species could be explained on the basis of different exploitation patterns and/or ecological conditions. In this sense, while the loach is not subject to commercial exploitation, variation in the environmental conditions of the area seems to be the main factor affecting the loach population.

The maximum age of *O. bergianus* was higher than that observed in Aras River (JAMALI et al. 2015), but less than that observed in *Paracobitis malapterura* and *Paraschistura kessleri* (4^+ years for *P. malapterura* in Zarrin-Gol River (now *P. hircanica*) and *P. kessleri* in Zanglanlou River) (PATIMAR et al. 2009, 2010). The age group 2^+ was dominant in males and 3^+ in females. In some other species like *T. hafezi* and *B. barbatula* the dominant age classes were 1^+ and 2^+ (JAMALI et al. 2014, VINYOLES et al. 2010). In colder waters the age may increase to 8 years as in *B. barbatula* in Siberia (SKRYABIN 1991).

Sex ratio in *O. bergianus* was equal as in Aras population (JAMALI et al. 2015). In *O. kiabi* (ABBASI et al. 2013), this ratio was in favor of females, but in *Paracobitis malapterurus* it was in favor of males (TABIEE and ABDOLI 2005). Sex ratio varies considerably from species to species; but in the majority of species, it is close to one (NIKOLSKY 1963). However, as noted by others (PITCHER and HART 1982, SKRYABIN 1991, FERNANDEZ-DELGADO and ROSSOMANNO 1997), subsequent changes in this ratio may be explained by a number of hypotheses, including differences in habitat preference, season, sampling errors, or selective mortality. Sex ratio was also different from other species of Nemacheilidae, e.g., *Paracobitis malapterura* (PATIMAR et al. 2009), *Barbatula* (VINYOLES et al. 2010), *Paracobitis iranica* (MARMAEI et al. 2014) and *Metaschistura cristata* (Patimar et al. 2011). However, in younger age classes males were dominant and in older age classes females were dominant. In May, females were dominant as seen in *B. barbatula* (VINYOLES et al. 2010). Differing from one population to another of the same species (Table 3), males usually predominate in the younger groups because they mature earlier but live shorter (NIKOLSKY

1963, ASADOLLAH 2011, 2017). Males were longer and heavier in the early stages than females, but in later stages this was reversed.

The *b* value is often about 3 and generally between 2.5 and 3.5. The calculated values of the *b* parameter for other species of *Oxynoemacheilus* loaches ranged from 2.8 to 3.01 (GOLZARIANPOUR et al. 2011, HASANKHANI et al. 2014, JAMALI et al. 2014). Although the growth rate of about 3 indicates the isometric growth that is characteristic for adult fish, that have completed their metamorphosis, the lower *b* value of loaches also indicates a cylindrical body which prevent them from being washed out by currents (ESMAEILI et al. 2014). Even though variation in b values depends primarily on the shape and fatness of the species, the b value in fish varies according to species, sex, age, stage of maturity, season and feeding. In addition, variation in fish shape, physiological conditions, and different amounts of available food, life span or growth increment can all affect the b growth exponent (RICKER 1975, TESCH 1968, SPARRE 1992, BAGENAL and TESCH 1978, KING 1995). Length–weight relations are useful in determining weight and biomass when only length measurements are available and allow comparisons of species growth rate between different habitats and regions (HASANKHANI et al. 2013, 2014, KEIVANY et al. 2016b). The growth pattern in fishes is affected by genetics and environmental conditions.

The value of L_{∞} for females was higher than that of males which is congruent with findings of other studies (BOROŃ et al. 2008). The reason may be that females grow faster than males, and live longer (WEATHERLEY 1972, ROBOTHAM 1981, KOSTRZEWA et al. 2003, KEIVANY and SOOFIANI 2004, ZANELLA et al. 2003, BOROŃ et al. 2008, KEIVANY et al. 2012). The theoretical maximal length values (L_{∞}) were close to the size of the largest fish examined and the growth coefficient values indicated a relatively low attainment of maximal size. The differences in growth between regions can be attributed to differences in size of the largest individual sampled in each area and to the differences between populations. On the other hand, it is also possible that the variations in population parameters of the species represent epigenetic responses to different conditions (temperature and food) prevailing in different areas (BRUTON 1990). A possible reason for this difference is the lower maturity age in males compared to females. The t_0 was different from other related species (ZANELLA et al. 2003, BOROŃ et al. 2008). The condition factor is an indicator of feeding condition of a fish. Variation in this index is affected by food availability and energy requirements for reproduction (SKRYABIN 1991).

In conclusion, *Oxynoemacheilus bergianus* reaches 7.5 cm in total length, 4.1 g in total weight and a maximum of 6 years in age. The fastest growth rate for this species is in the first and second year of life. This infor-

mation could be used for sustainable propagations of the species in aquaria as a pet fish.

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QUALITATIVE CHARACTERIZATION OF FAT FRACTION FROM CRUNCHY CEREAL PRODUCTS AVAILABLE ON THE POLISH MARKET

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Key words: crunchy cereal products, fatty acids composition, fatty acids distribution, oxidative stability, hydrolytic stability.

Abstract

The paper presents the qualitative assessment of fat isolated from the selected crunchy cereal products available on the Polish market. The samples were characterized by determination of the fatty acids composition and distribution in triacylglycerol structure. Additionally, oxidative stability was identified. To estimate the quality of the fat, the acid value and the peroxide value were also determined. The results showed that the tested products were a good source of monounsaturated and polyunsaturated fatty acids, especially oleic and linoleic acid. Analyzed products showed statistically different resistance to oxidation. The longest time of oxidation induction was determined in the case of fat extracted from crunchy cereal bar covered with chocolate, while the shortest for the fat extracted from crunchy breakfast cereals with chia seed. The analysis of the peroxide and the acid values did not show any abnormalities regarding the quality of the fat tested.

Introduction

Cereal products belong to one of the most important groups of products that are considered when preparing a balanced diet. The continuous growth of consumer nutritional expectations contributes to the develop-

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ment of the market of cereal products. Among a large variety of bread and many confectionery products, such as cakes, cookies, rolls and croissants, breakfast cereals and crunchy products occupy a significant place. To crunchy products, we can include crunchy breakfast cereals and cereal bars based on oat flakes. Both, when made of the whole grain oat, are claimed to be rich in fiber, vitamins, and minerals such as phosphorus and manganese. LASKOWSKI et al. (2019) studied the food sources of energy and 28 nutrients from cereals and cereal products in the average Polish diet. The results indicated that cereals and cereal products are the source of 30.4% of total dietary energy, providing a significant percentage of manganese, carbohydrates, dietary fibre, iron, folate and copper. Polyunsaturated fatty acids (PUFA) were supplied at the level of 10–20%. For such nutrients like total fat, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) the share of cereals and cereal products contribution was below 10%. The assortment of crunchy cereal products is varied and dependent on the additives that were used in the production process, such as dried fruit, nuts, seeds, and chocolate. Oat grain, the basic raw material used for the production of crunchy products, contains the soluble fiber, protein, carbohydrates, fats, minerals and vitamins, some antioxidants, such as phytic acid, vitamin E and polyphenolic compounds (OTLES and CAGINDI 2006). In comparison to wheat and rye, oats are characterized by a high-fat content, which may range from 8 to 10% in dehusked oat grain, and about 5% in the raw one (GĄSIOROWSKI 1995). Pearl millet grain has a wide range of opportunity for utilization in production of ready-to-eat, nutritionally rich cereal bar products along with better sensory qualities. The product was found to be a source of 10.84% protein, 4.39% fat, 5.43% fiber, 3.17 mg/100g zinc, 5.13 mg/100 g iron, and 215.63 mg /100 g phosphorus (KUMARI et al. 2019). Bioactive compounds present in cereals help in preventing the risk of chronic diseases as a functional ingredient (SOFI et al. 2019). Vegetable fats occurring in cereal products have been recognized by physicians, dieticians, and nutritionists as one of the important sources of unsaturated n-3 and n-6 fatty acids (ACHREMOWICZ and SZARY-SWORST 2005). Therefore, they should be consumed by contemporary society to prevent cardiovascular diseases, which are the results of both, an inadequate diet and a lack of physical activity. It is known, that the condition of the blood circulation system depends primarily on the amount and the quality of fat consumed (CONNOR 2000, JELIŃSKA 2005). The quality of fats is influenced by many factors, such as the composition of fatty acids, its bioavailability, and resistance to oxidation. Considering the fact, that crunchy products are mostly consumed in large quantities, especially by the children, hence their fat profile can affect human health.

The aim of the study was to characterize and assess the quality of fat fraction extracted from crunchy cereal products available on the Polish market by determining such parameters as: fatty acids composition and distribution in triacylglycerol structure, oxidative and hydrolytic stability.

Materials and Methods

Determination of Fatty Acids (FA) Composition

The research material consisted of three kinds of crunchy cereal bars (covered with chocolate [1], with nuts and almonds [2], and with dried cranberries and raspberries [3]) and four crunchy breakfast kinds of cereal (banana-flavored with chocolate [4], and with chia seeds [5], with dried fruit [6], and classic [7]), all purchased in the local store.

The composition of fatty acids in crunchy cereal products was determined by gas chromatography, after prior extraction of the fat phase from the product under study. Lipid fraction was extracted from cereal products according to the procedure described by SHAHIDI et al. (1997). After mechanical grinding, 30 g of the sample was homogenized with 100 mL of hexane in a glass bottle with a screw-cap at room temperature. After 1 hour of homogenization, the content was filtered and dried with $MgSO_4$ by 30 minutes. The organic solvent was removed by the rotary evaporator at 40°C. The residue of hexane was removed by applying nitrogen flow. The fat sample was stored at -18°C until it was analyzed.

In order to determine the composition of fatty acids in the lipid fraction of crunchy cereal products, a gas chromatography (GC) method was used. Methyl esters of fatty acids were obtained by the addition of a 1M solution of KOH in methanol according to *Oleje i tłuszcze roślinne*… PN-EN ISO 5509: 2001. A Shimadzu GC 17A chromatograph equipped with a flame ionization detector and a BPX-70 capillary column of 0.22 mm (internal diameter) 30 m length and 0.25 mm film thickness was used. The oven temperature was programmed as follows: 60°C for 1 min, then it was increased by 10°C/min to 170°C; from 170°C to 230°C, it was increased by 3°C/min; then kept at 230°C for another 15 min. The temperature of the split injector was 225°C, with a split ratio of 1:100, and the detector temperature was 250°C. Nitrogen flowing with the rate of 1 ml/min was used as the carrier gas. The identification of fatty acids was carried out using the lipid standard purchased from Sigma Aldrich, Supelco Analytical, Bellefonte, PA, USA.

Determination of Fatty Acids Distribution

In order to determine the distribution of fatty acids in the lipid fraction of crunchy cereal products, the Brockerhoff method (partial hydrolysis of triacylglycerol) was carried out in the presence of an enzyme – pancreatic lipase (BROCKERHOFF 1965, HAZUKA et al. 2003). 100 mg of fat was weighed on the analytical balance into a 20 ml centrifuge tube, then 1 ml of a 1 M TRIS-HCl (pH = 8), 0.1 ml of 2.2% calcium chloride solution and 0.25 ml of 0.05% bile salt were added. The sample was mixed intensively for 30 seconds. 20 mg of pancreatic lipase was added and a mixture was mixed intensively for the next 30 seconds. The sample was then incubated in a water bath at 40°C for 3 minutes. After incubation, 1 ml of a 6 M HCl and 4 ml of diethyl ether were added to the sample and also mixed intensively for 60 seconds. The sample was then placed in the centrifuge for 5 minutes. Separated organic layer was concentrated under a nitrogen atmosphere to a volume of approx. 1 ml and the mixture was applied to a plate coated with silica gel in the dimensions 20 cm x 20 cm. The plate was placed in the chamber with the appropriate solvents system. Isolated sn-2 monoacylglycerols (MAG) were removed from the plates along with the gel and subjected to extraction. The determination of the fatty acids present in sn-2 position was done by the use of gas chromatography (HAZUKA et al. 2003).

The share of fatty acids for the sn-1 and sn-3 positions was calculated based on the knowledge of the starting composition of triacylglycerols and isolated sn-2 monoacylglycerols as followed:

$$\text{sn-1,3} = \frac{3 \cdot (\text{FA in TAG}) - (\text{FA in sn-2 MAG})}{2}$$

where:
sn-1,3 – the content of a given fatty acid in the sn-1 and sn-3 positions [%]
FA in TAG – the content of a given fatty acid in starting triacylglycerols [%]
FA in sn-2 MAG – the content of a given fatty acid in sn-2 monoacylglycerols [%].

The percentage of selected fatty acids in the sn-2 position of monoacylglycerols in relation to the total fatty acid content in all triacylglycerol positions was calculated as followed:

$$\text{sn-2} = \frac{\text{FA in sn-2 MAG}}{3 \cdot (\text{FA in TAG})}$$

Determination of Oxidative Stability

In order to determine the oxidative stability of the fat extracted from crunchy cereal products, the oxidation time was obtained by the use of pressure differential scanning calorimetry (PDSC).

For each measurement, 3–4 mg of the fat sample was weighed in an aluminum pan. The prepared sample together with the control sample (empty aluminum vessel) was placed in the measuring chamber of the Thermal Analysis DSC Q20. The measurements were carried out in isothermal conditions at 140°C and under 1400 kPa of oxygen pressure. From resulting PDSC exotherms the time to reach the peak maximum (τ_{max}) was determined and used for the assessment of the oxidative stabilities of the samples.

Determination of Peroxide and Acid Value

Both, the peroxide value (PV) and the acid value (AV) were performed according to the Polish Standards (*Oleje i tłuszcze roślinne…* PN-EN ISO: 3960: 2012, *Oleje i tłuszcze roślinne…* PN – EN ISO 660: 2010).

Statistical Analysis

All tests were performed in triplicate. The statistical analysis of the results was conducted using Statistica 10 PL (StatSoft Poland Sp. z o.o, Kraków, Poland). ANOVA analysis was also performed. The significance of differences between means were determined using Tukey's test at a significance level of $p < 0.05$.

Results and Discussion

The content of individual groups of fatty acids and composition of FA in all position of TAGs is shown in Figure 1 and Table 1. In the fat isolated from the crunchy cereal bar covered with chocolate, the highest part was saturated fatty acids (SFA), 60.07%, whereas monounsaturated fatty acids (MUFA) and polyunsaturated acids (PUFA) contributed 33.99% and 5.95%, respectively. Among saturated fatty acids, the highest proportion of stearic acid (C18:0) was found (31.67%). Unsaturated fatty acids – oleic acid (C18:1 n-9c) and linoleic acid (C18:2 n-6c) were detected in the amount of 33.53% and 5.25%, respectively – Table 1. The highest content of SFA was found in fat isolated from a crunchy cereal bar with cranberries and raspberries (67.20%) whereas MUFA and PUFA contributed 23.54%

and 9.26%, respectively (Fig. 1). Among the saturated fatty acids, the highest share of palmitic acid (C16:0) was found (25.30%). Oleic acid (C18:1 n-9c) was detected in the amount of 23.19%, while polyunsaturated fatty acid – linoleic acid (C18:2 n-6c) – at a level of 8.82% – Table 1.

Table 1

Composition of the most important fatty acids in internal and external positions of triacylglycerols (TAG) in fat extracted from three crunchy cereal bars: covered with chocolate [1], with nuts and almonds [2], with dried cranberries and raspberries [3], and the share [%] of individual fatty acids in internal position (sn-2)

Fatty acid (FA)	Composition of FA in all positions of TAGs [%]			Distribution of FA in positions, sn-2 and sn-1,3 [%]						Share of FA in sn-2 position [%]		
				sn-2			sn-1,3					
	[1]	[2]	[3]	[1]	[2]	[3]	[1]	[2]	[3]	[1]	[2]	[3]
C12:0	–	–	23.58	–	–	29.73	–	–	20.51	–	–	42.03
C14:0	–	–	–	–	–	15.25	–	–	8.08	–	–	48.55
C16:0	26.40	18.43	25.30	8.38	7.80	15.41	35.41	23.76	30.25	10.58	14.12	20.30
C18:0	31.67	15.58	4.76	5.56	3.22	3.47	44.73	21.76	5.41	5.85	6.84	24.30
C18:1 (n-9c)	33.53	53.62	23.19	70.92	70.35	25.59	14.84	45.26	21.90	70.50	43.75	36.78
C18:2 (n-6c)	5.25	7.55	8.82	10.76	14.70	8.40	2.50	3.96	9.03	68.32	64.90	31.75

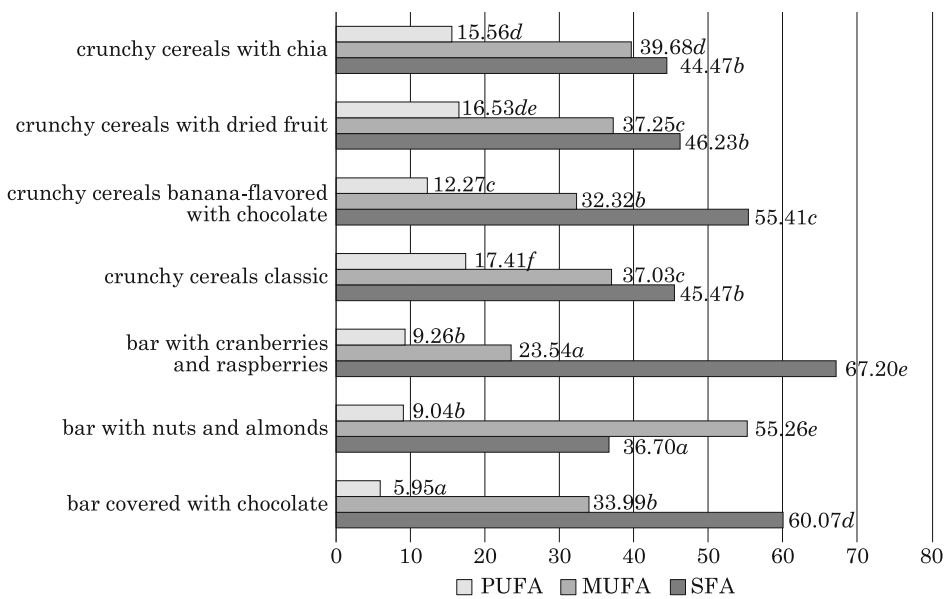

Fig. 1. The content of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in crunchy cereal products available on the Polish market. The values with the same letter for the same category of fatty acids do not differ statistically on the level $p < 0.05$

In contrary, the fat isolated from a crunchy cereal bar with nuts and almonds contained in the majority the monounsaturated fatty acid (55.26%) probably due to the addition of nuts which naturally contain a lot of MUFA (Fig. 1). Subsequent groups of fatty acids were saturated (35.70%), and polyunsaturated (9.04%). In the group of SFA, the highest share of palmitic acid (C16:0) was observed (18.43%). Oleic acid (C18:1 n-9c) was found at a level of 53.62%, while linoleic acid (C18: 2 n-6c) – 7.55% (Table 1).

Crunchy breakfast cereals were another group of products tested. Among analyzed samples, the largest share of MUFA (39.68%) was characterized in the fat from crunchy flakes with chia seeds, but PUFA (17.41%) – crunchy cereal classic. Oleic acid (C18:1 n-9c) was present in the tested sample of fat from crunchy flakes with chia seeds in amount of 40.10%, whereas linoleic acid (C18:2 n-6c) had a wading share of 14.36% – Table 2.

Table 2

Composition of the most important fatty acids in internal and external positions of triacylglycerols (TAG) in fat extracted from two crunchy breakfast cereals: banana-flavored with chocolate [4], with chia seeds [5] and share [%] of individual acids in internal position (sn-2)

Fatty acid (FA)	Composition of FA in all positions of TAGs [%]		Distribution of FA in positions, sn-2 and sn-1,3 [%]				Share of FA in sn-2 position [%]	
			sn-2		sn-1,3			
	[4]	[5]	[4]	[5]	[4]	[5]	[4]	[5]
C12:0	8.75	–	17.67	–	4.29	–	67.31	–
C14:0	4.18	–	3.21	–	4.67	–	25.60	–
C16:0	27.75	37.12	11.83	18.42	35.71	46.47	14.21	16.54
C18:0	–	4.46	–	3.62	–	4.88	–	27.06
C18:1 (n-9c)	31.72	40.10	46.17	54.61	24.50	32.85	48.52	45.39
C18:2 (n-6c)	11.55	14.36	16.86	20.12	8.90	11.48	48.66	46.70

Similar dependencies were also found in the case of crunchy cereal with dried fruit. The fat was the richest in saturated fatty acids (46.23%) with two other groups, MUFA and PUFA, resulting in 37.25% and 16.53%, respectively (Figure 1). Among the saturated fatty acids, the highest share of palmitic acid (C16:0) was found (35.19%). Oleic acid (C18:1 n-9c) was detected on a level of 36.63%, while linoleic acid (C18:2 n-6c) was present in the tested sample in the amount of 15.65% – Table 3. The fat isolated from crunchy breakfast cereal banana-flavored with chocolate was characterized by the highest share of saturated fatty acids (55.41%), among which palmitic acid dominated (C16:0) (27.75%) – Table 2.

Next groups were monounsaturated fatty acids (32.32%), and polyunsaturated acids (12.27%) – Figure 1, with the share of oleic acid (C18:1 n-9c) (31.72%) and linoleic acid (C18:2 n-6c) (11.55%), respectively (Table 2). In the fat sample extracted from crunchy cereals classic, the highest proportion of saturated fatty acids was found (45.47%). The subsequent groups observed were MUFA (37.03%) and PUFA (17.4%) with the content of oleic acid (C18:1 n-9c) (36.77%) and linoleic acid (C18:2 n-6c) (16.71%), respectively (Table 3).

Table 3

Composition of the most important fatty acids in internal and external positions of triacylglycerols (TAG) in fat extracted from two crunchy breakfast cereals: with dried fruit [6], classic [7] and the share [%] of individual acids in internal position (sn-2)

Fatty acid (FA)	Composition of FA in all positions of TAGs [%]		Distribution of FA in positions, sn-2 and sn-1,3 [%]				Share of FA in sn-2 position [%]	
			sn-2		sn-1,3			
	[6]	[7]	[6]	[7]	[6]	[7]	[6]	[7]
C12:0	2.99	3.35	5.81	6.36	1.58	1.85	64.77	63.28
C16:0	35.18	33.77	13.60	14.86	45.97	43.23	12.87	14.67
C18:1 n-9c	36.63	36.77	48.98	51.82	30.46	29.25	44.57	46.98
C18:2 n-6c	15.65	16.71	20.18	22.65	13.39	13.76	42.98	45.11

The share of PUFA is higher in the fat extracted from breakfast cereal than in the fat extracted from crunchy cereal bars. Among the last group the largest share of these fatty acids is found in the fat extracted from the crunchy cereal bar with cranberries and raspberries as well as nuts and almonds, the smallest in the fat extracted from the crunchy cereal bar covered with chocolate. Comparable SFA content was found in the fat extracted from crunchy breakfast cereals with dried fruit, classic and with chia seeds. Figure 1 presents that there are statistically significant differences in the content of MUFA and PUFA between the tested samples.

According to LANGE (2009) in the oat oil dominated unsaturated fatty acids with preference to linoleic (26–53%), oleic (19–48%) and α-linolenic (0.5–5%). According to WIRKOWSKA and BRYŚ (2009), who investigated the composition of fatty acids in cereal biscuits, similarly, unsaturated fatty acids contributed more with preference to oleic acid (37.8– 54.6%), linoleic acid (11.4–38.2%), and α-linolenic acid (0.2–4.3%). Our results for crunchy breakfast cereals with chia seeds are the only cereal product corresponding with mentioned research. PASZCZYK et al. (2007) investigated composition of fatty acids and trans isomers of fatty acids in fats of selected confectionery products. In the examined confectioneries the participation

of saturated fatty acids in fat in first group of products (biscuits, cakes, gingerbreads, sponge cakes and croissant) ranged from 24.4% to 81.2%, whereas that of monounsaturated fatty acids – from 14.1% to 63.2%, and polyunsaturated fatty acids – from 4.8% to 11.8%. In fat of second group of products (wafers), saturated fatty acids constituted from 35.4% to 63.4%, monounsaturated fatty acids – from 29.1% to 63.2%, and polyunsaturated fatty acids – from 3.2% to 10.0%. In our study, in all cases, except for the crunchy cereal bar with nuts and almonds, the largest amount of saturated fatty acids was detected (44.4–67.20%). Monounsaturated and polyunsaturated fatty acids were present in the range of 23.54–55.26% and 5.95–17.41%, respectively. CALIONARA et al. (2015) analyzed chemical composition and fatty acids profile of chocolates produced with different cocoa (*Theobroma cacao* L.) cultivars. They concluded, that C16:0, C18:0, C18:1n9 and C18:2n6 were, quantitatively, the most important fatty acids in all of the studied samples. The prevalence of saturated fatty acids on unsaturated fatty acids is considered to be negative from a nutritional point of view. TORRES-MORENO et al. (2015) investigated nutritional composition and fatty acids profile in cocoa beans and chocolates with different geographical origin and processing conditions. For cocoa, differences in fatty acid profile were found in C12:0, C14:0, C16:0, C16:1, C17:0, C17:1 and C18:0 whilst for chocolates only differences were found in C16:0, C18:0, C18:1 and C18:2. For all samples, C16:0, C18:0, C18:1 and C18:2 were quantitatively the most important fatty acids. Ecuadorian chocolate showed a healthier fatty acid profile having higher amounts of unsaturated FA and lower amounts of saturated fatty acids than Ghanaian chocolate. The SFA content in the human diet largely affects the serum cholesterol level. KRIS-ETHERTON (1999) showed that saturated fatty acids, such as palmitic, myristic or lauric, are responsible for hypercholesterolemic action, thereby increasing cholesterol and low-density lipoprotein (LDL) levels. SFA causes vascular clots and increases the tendency of platelets to aggregate. A diet rich in SFA increases the risk of myocardial infarction or cardiovascular disease (JIMENEZ-COLMENERO et al. 2001). In the case of fat extracted from the crunchy bar with nuts and almonds, MUFA had the largest share. In all tested products, we can observe the smallest share of polyunsaturated fatty acids. Based on the results, it can be concluded that there are statistically significant differences between SFA content in analyzed samples.

The quality of fats depends not only on the composition of fatty acids but also on the distribution of fatty acids in the triacylglycerol molecule (TAG). The distribution of fatty acids in the triacylglycerol molecule is important in the digestion tract, absorption of fats in the human body, as

well as in the process of modifying the structure of triacylglycerols. Pancreatic lipase participates in the digestion of fats in the human digestive tract. It has a specific ability due to which fatty acids are detached from the external position (sn-1 or sn-3) of triacylglycerols, which results in the formation of free fatty acids in the small intestine and sn-2 substituted monoacylglycerols (BRYŚ et al. 2011). If saturated fatty acids occupy the sn-2 position, polyunsaturated fatty acids move freely in digestion track. PUFA and their salts show a high coefficient of absorption (WIRKOWSKA et al. 2012). If, on the other hand, SFA occupy external positions (sn-1, sn-3) this is unfavorable from the nutritional point of view. Saturated fatty acids, moving freely in the human body, they tend to form hard-soluble calcium salts, which may lead to calcium deficiencies in the body (HUNTER 2001). The results of the performed analysis of fatty acid distribution in crunchy cereal products available on the Polish market are presented in Tables 1–3. The proportion of selected fatty acids in the sn-2 position of triacylglycerols in the fat extracted from three crunchy cereal bars: covered with chocolate [1], with nuts and almonds [2], and with cranberries and raspberries [3] is shown in Table 1. In two products, [1] and [2], the sn-2 position was occupied mainly by oleic acid (70.92% and 70.35%, respectively), and its share in this position was 70.50% and 43.50%, respectively. The content of linoleic acid in the sn-2 position for all crunchy cereal bars was within comparable, yet statistically different, range (8.40 –14.70%). Among the SFA group, the highest content in the sn-2 position contributed to palmitic acid in two crunchy cereal bars [1] and [2], but in case of crunchy cereal bar with cranberries and raspberries [3], the lauric acid was in the majority in that location (29.73%) and the next myristic and palmitic acid. The presence of the stearic acid was detected for all three bars, but it was rather minor importance (3.22–5.56%). Table 2 shows the share of selected fatty acids in the sn-2 position of triacylglycerols in fat extracted from two crunchy breakfast cereals: banana-flavored with chocolate [4] and with chia seeds [5]. In the largest number in the sn-2 position there was oleic acid (46.17% and 54.61%, respectively) and its share was equal 48.52% and 45.39%, respectively. The content of linoleic acid in the sn-2 position was 16.86 (banana-flavored with chocolate), with 48.66% share, and 20.12 (crunchy cereals with chia) – with 46.70% share. Among the SFAs, the highest amount in the sn-2 position was reported for lauric acid (17.67%), with 67.31% share. The highest amount in sn-2 position was found for oleic acid (54.61%), and its share in this location was as much as 45.39%. The content of linoleic acid in the sn-2 position was 20.12%, with 46.70% share. Among saturated fatty acids, palmitic acid (18.42%) was the most popular in the sn-2 position, and its share in this position was 16.54%.

The content of stearic acid in the sn-2 position was 3.62%, with 27.06% share. Table 3 shows the share of selected fatty acids in the sn-2 position of triacylglycerols in the fat extracted from next two crunchy breakfast cereals: with dried fruits [6] and classic [7]. In the largest amount in the sn-2 position there was oleic acid in both types of bar (48.98% [6] and 51.82% [7]), and its share in this position was 44.57% and 46.98%, respectively. The content of linoleic acid in the sn-2 position was also comparable, 20.18% and 22.65%, respectively, with similar share in each fat. Among the SFAs, the highest content in the sn-2 position was determined for palmitic acid in each sample (13.60% and 14.86%, respectively). The content of lauric acid in TAGs was similarly low for both crunchy cereal bars [6] and [7], 2.99% and 3.35%, respectively however, when present, it occupied mainly the sn-2 position, with 64.77% and 63.28% share respectively.

After analyzing the obtained results, fat isolated from crunchy breakfast cereals with dried fruit as well as crunchy cereals classic have a very similar fatty acid composition and distribution in sn-2 and sn-1,3 positions as well as share of a given fatty acid in sn-2 positions. In all analyzed fats, except the fat isolated from crunchy cereal bar with cranberries and raspberries, the oleic acid was noticed to have the highest percentage in the sn-2 position. Noticed distribution of saturated fatty acid in the sn-1,3 position in the triacylglycerol molecule can have a negative effect on the digestion and absorption of fats in the human body. In contrary, in the fat extracted from the crunchy cereal bar with cranberries and raspberries, there was the highest content of saturated acids in the sn-2 position. The dominant fatty acids in this position were lauric acid (29.73%), and its share in this position was as much as 42.03% and myristic acid – 15.25%, with 48.55% share. Among the unsaturated fatty acids high oleic acid content was found in the sn-2 position of triacylglycerols, and its share in this position was 36.78%.

The food quality is influenced by various processes, both biochemical and physicochemical. Oxidation contributes to undesirable changes occurring in food, which leads to a significant drop in products' quality, and even to its damage (HĘŚ and KORCZAK 2007). Pressure differential scanning calorimetry can be used to determine thermodynamic parameters and the oxidative stability of lipids. The determination of the induction time is aimed to estimate the oxidative stability of the fat being tested. In this study, the principle is assumed that the longer the induction time, the greater the oxidative stability of the test sample (THURGOOD et al. 2007). To test the resistance of fat from crunchy cereal products for oxidation, the PDSC test was performed isothermally, at 140°C. Average times of induction of crunchy cereal products are shown in Figure 2. Analyzing the ave-

rage time needed to initiate oxidation process of the fat isolated from crunchy cereal products, statistically significant differences were observed.

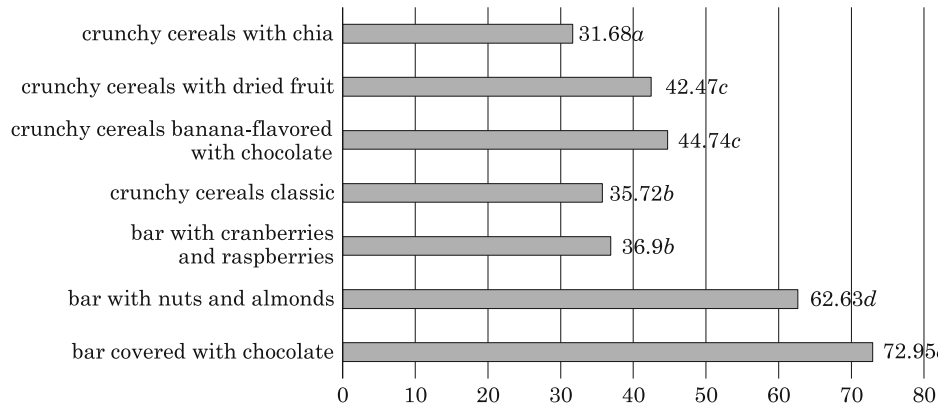

Fig. 2. Induction time [min] for fat extracted from crunchy cereal products available on Polish market. The values with the same letter do not differ statistically on the level $p < 0.05$

The longest time of induction was determined for fat extracted from crunchy cereal bar covered with chocolate (72.95 min), while the shortest time of oxidation induction was detected for the fat extracted from crunchy breakfast cereals with chia seeds (31.68 min) – Figure 2. A bit longer time was found for the fat extracted from crunchy cereal bar with cranberries and raspberries (36.90 min) and fat extracted from classic crunchy breakfast cereals (35.72 min). Time of oxidation induction of fat extracted from crunchy breakfast cereals banana-flavored with chocolate (44.74 min) and fat extracted from crunchy cereals with dried fruit (42.47 min) did not differ statistically. Unexpectedly, despite the high content of monounsaturated fatty acids in TAGs, the induction time of fat extracted from crunchy cereal bar with nuts and almonds was quite high – 62.63 min.

Peroxide value (PV) determines the degree of fat oxidation, taking into account the content of primary oxidation products. Analyzing the data on the peroxide value, it can be concluded that there were statistical differences between the obtained values for different crunchy cereal products (Fig. 3). The smallest value of the peroxide value was obtained for the fat extracted from the crunchy cereal bar with cranberries and raspberries (1.63 mEq O_2/kg fat). The highest peroxide value was determined for the fat extracted from crunchy breakfast cereals with chia seeds (6.70 mEqO_2/kg fat). The obtained values are consistent with the composition of fatty acids. A high proportion of unsaturated fatty acids in the structure of triacylglycerols induced higher values peroxide value. It is worth emphasizing that fat extracted from crunchy breakfast cereals with chia seeds was also

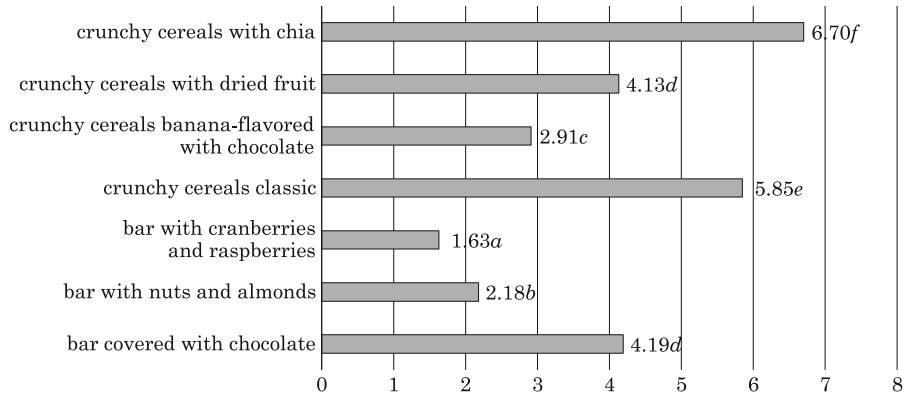

Fig. 3. Peroxide value for crunchy cereal products available on Polish market. The values with the same letter do not differ statistically on the level $p < 0.05$

distinguished by the shortest time of induction.

Codex Alimentarius informs that the peroxide value for cold-pressed oils should be lower than 10 mEq O_2/kg. All results were significantly lower than the allowed standard. WIRKOWSKA and BRYŚ (2009) in their research on the quality of the lipid fractions in cereal biscuits analyzed, inter alia, the peroxide value. The peroxide value for fat extracted from cereal biscuits was from 1 to 4 mEq O_2/kg fat. The results of the peroxide value obtained in our study are mostly comparable to the results obtained by WIRKOWSKA and BRYŚ (2009).

The acid value (AV) is a measure of the content of free fatty acids, therefore it determines the degree of fat hydrolysis. It is defined as the number of mg of potassium hydroxide needed to neutralize fatty acids present in 1 gram of fat (BERG et al. 2005). Analyzing the results on the acid value, it can be concluded that there were statistically significant differences between the individual acid values for crunchy cereal products. Results obtained

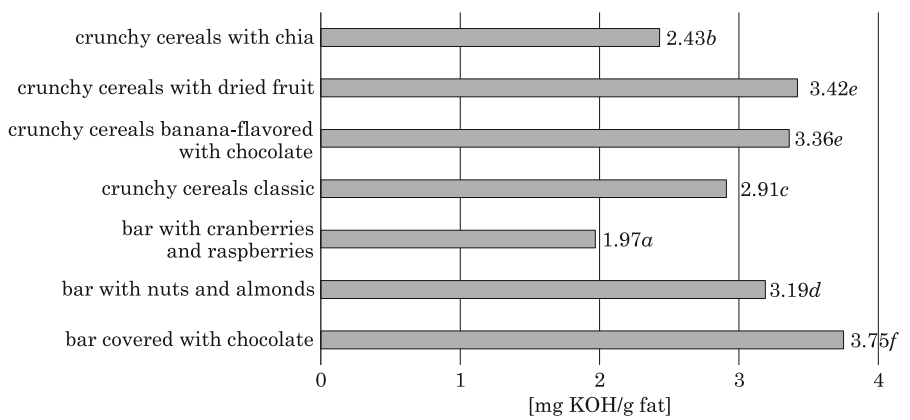

Fig. 4. Acid value for crunchy cereal products available on Polish market. The values with the same letter do not differ statistically on the level $p < 0.05$

for fats extracted from crunchy cereal products are presented in Figure 4. The lowest acid value was determined in the case of the fat extracted from the crunchy cereal bar with cranberries and raspberries (1.97 mg KOH/g fat), while the highest value for the fat extracted from the crunchy cereal bar banana covered with chocolate (3.75 mg KOH/g fat).

According to the Polish standard (*Oleje i tłuszcze roślinne…* PN-EN ISO 660:2010) the value of AV for cold-pressed oil should not exceed 4.00 mg KOH/g fat. None of crunchy cereal products exceeded the limit regarding acid value given in the Polish Standard, hence all tested crunchy products were formed from good quality grains and their processing, as well as storage conditions, were adequate.

Conclusions

The result of the present study showed that fat isolated from crunchy cereal bars and crunchy breakfast cereal are a source of favorable monounsaturated and polyunsaturated fatty acids, especially oleic and linoleic acid. The share of PUFA was higher in the fat extracted from breakfast cereal than in the fat extracted from crunchy cereal bars. Among the tested group of products the largest share of these fatty acids was found in the fat extracted from the crunchy cereal classic. Fat isolated from bar with nuts and almonds was the reachest source of monounsaturated fatty acid. In all analyzed fats, except the fat isolated from crunchy cereal bar with cranberries and raspberries, the oleic acid was noticed to have the highest percentage in the sn-2 position. Fat extracted from crunchy cereal bar covered with chocolate was characterized by greatest oxidative stability, whereas fat isolated from crunchy breakfast cereals with chia seeds was most susceptible to oxidation. Fat fractions isolated from crunchy cereal products were of acceptable hydrolytic and oxidative stability.

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EFFECT OF SHELL INJURY ON HAEMOCYTE CONCENTRATION AND SHELL REGROWTH IN GIANT AFRICAN LAND SNAILS (*Archachatina marginata*)

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Key words: shell injury, haemocyte, shell growth, Land snail, *Archachatina marginata.*

Abstract

The effect of shell injury on growth and haemocyte concentration in *Archachatina marginata* was evaluated in this study. Thirty two snails between 130–180 g were randomly divided into four treatments with eight replicate each. The four treatments included: T1 (control), T2 (1 cm shell damage), T3 (2 cm shell damage) and T4 (3 cm shell damage). Haemolymph was collected on weekly basis for four weeks. Parameters monitored were total haemocyte count and shell growth. Results showed that shell injury had significant effects ($P < 0.001$) on total haemocyte count and shell growth. It can be concluded from this study that shell injury has an influence on the immune response of the animal and that is irrespective of the level of shell damage used in this study. This humoral response is put in place to defend the animal body against any opportunistic infection that may gain entrance into the system of this animal.

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Introduction

Land snails generally have a shell, which protects them from physical damage, predators and dehydration (ADEMOLU 2015, ADAMOWICZ and BOLACZEK 2003). Similarly, the shell houses the animal especially during unfavorable conditions. The shells are twisted into spiral levels known as whorls. The whorls are largest at the base and each one gets progressively smaller as it gets to the tip, known as the apex. The snail shell has a large opening called aperture (ADAMOWICZ and BOLACZEK 2003). Due to the current trend of intensive rearing of snails to meet up with demand, there is need for cage culture or semi-intensive rearing of this animal. During intensive rearing, snails at times try to escape from their rearing vicinity and thus fall off from some height and as such break their shell. This occurrence puts snails at a great danger depending on the site of injury. It could also lead to haemolymph loss, which may result in death of this animal if such injury is much. In most occasions, the damage to the shell calls for the process of healing which requires regrowth of the damaged part and this may be energy demanding and costly (JONATHAN 1990). Studies have also shown that the wound healing process requires the activity of macrophages which promote angiogenesis and collagen formation (LEIBOVICH and ROSS 1975, POLVERINI et al. 1977, HUNT et al. 1984, KOVACS and DIPIETRO 1994). For invertebrates like molluscs, shell formation is known to be a complex process, which involves deposition of both organic and inorganic materials (WILBUR 1983). The shell formation process comprises shell mineralization known to occur in succession of compartments (CRENSHAW 1972, SALEUDDIN and PETIT 1983). The first to be reckoned with is the mantle cavity, which secretes the molecules that form the shell, followed by the periostracum (with mostly organic layers) and the extrapallial cavity, into which the outer fold epithelium secretes a calcifying mixture of proteins, glycoproteins and calcium carbonate ($CaCO_3$) (MUTVEI 1987, FENG et al. 2000, MARIN and LUQUET 2004, DALBECK et al. 2006, MARIE et al. 2011, MARIN et al. 2012). The longitudinal section of a shell is made up of a multilayer of calcium carbonate in two or more concentric layers, which are usually covered by an external layer (SALEUDDIN and PETIT 1983). Below the periostracum is an inner nacreous layer, followed by inner primastic (MARIE et al. 2011).

During rearing of snails under intensive system, damages in shell do occur due to climbing of housing facility by this animal and such may lead to economic losses due to mortality. Therefore, is very important to understand the influence of this damage on the immune status of this animal within a specific period of time and to monitor the recovery period depen-

ding on the level of damage. The aim of this study is to evaluate the effect of shell injury on haemocyte concentration and shell regrowth in Giant African Land snails (*Archachatina marginata*).

Materials and Methods

Experimental Site

The research was carried out at the Snail Research Unit of the College of Animal Science and Livestock Production (COLANIM), Federal University of Agriculture, Abeokuta, Ogun State. Abeokuta lies between the rain forest vegetation zone of Western Nigeria on latitude 7º10'N, longitude 3º2'E and altitude 76 m above sea level. The climate is humid with a mean annual rainfall of 1.037 mm, an average temperature of 34.7ºC and an imminent average humidity of 82% throughout the year (*Google Earth* 2017).

Materials and Methods

A total of thirty – two snails (*Archachatina marginata)* between 130–180 g was purchased from a local market. The snails were kept in plastic cages (30 cm by 40 cm by 24 cm). Feeding trough, watering trough, sensitive scale, plier, Eppendorf tube, syringe and needle (5 ml), ruler, vernier caliper and concentrate feed were used during this study. Marker and masking tape were also used for proper identification.

Snails and Their Management

The plastic cages along with the plastic feeders and drinkers were cleaned before the arrival of the snails and the commencement of the experiment. Feed and water were also provided *ad libitum* throughout the period of the experiment. Four weeks was set aside for the acclimatization of the snails before the commencement of the experiment. The experiment lasted for six weeks.

Experimental Esign

Thirty-two snails used for this experiment were randomly assigned into four different treatments with 8 replicates for each treatment. The treatments were: Treatment 1: No shell damage (control); Treatment 2: 1 cm shell damage; Treatment 3: 2 cm shell damage and Treatment 4: 3 cm shell damage.

All snails in both groups were treated equally in terms of feeding and drinking water provision. The composition of feed used is given in Table 1.

Table 1

Composition of experimental diets [g/100 g]

Ingredients	Quantity [g]
Maize	50
Wheat offal	27.5
Groundnut cake	12.25
Soy bean meal	4
Bone meal	3
Oyster shell	3
Salt	0.25
Total	100

Shell Damage/Injury

The snails were cleaned with damp foam in order to remove the dirt on them. The snails were weighed on a sensitive scale before the damage of the shells. The snails in each treatment (1, 2, 3, 4) were brought out of the cages, a ruler was placed on the tip of the shell a and white board marker was used to mark out the part to be damaged as 0 cm, 1 cm, 2 cm and 3 cm. After marking out, a plier was used to cut out the part as marked to be damaged. Shell growth was measured weekly for six weeks using a Venire caliper (Fig. 1).

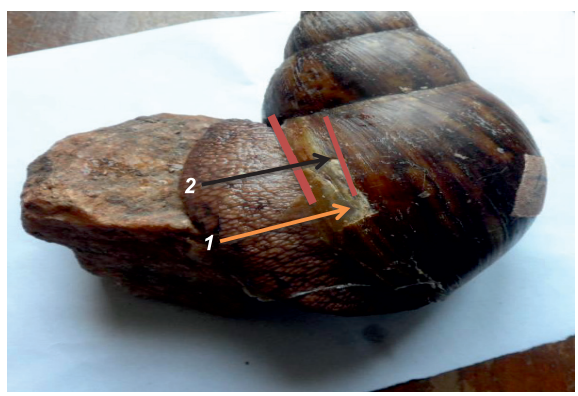

Fig. 1. Portion of shell damage and regrowth there after: *1* – fresh shell growth; *2* – length of damage

Collection of Haemolymph

Haemolymph was collected from the anterior portion of the head region after full extension of the foot muscle with the aid of syringe and needle. Haemolymph was collected from the control group and other treat-

ment levels (1 cm, 2 cm and 3 cm) immediately after shell damage and stored in eppendof tubes for haemocyte count. Haemolymph collection was also carried out on a weekly basis.

Total Haemocyte Count

Haemolymph from eight snails per treatment was selected from the four groups of snails (control, 1, 2, 3 cm). A dilution of 1 : 19 was made with the aid of 5% eosin solution, which was loaded into an improved haemocytometer. Haemocyte found in the four squares were counted. Thereafter, numbers of cells counted were multiplied by a conversion factor (50,000) to obtain the total haemocyte count.

Statistical Analysis

The data generated from this experiment were subjected to a least square analysis of variance using the SYSTAT Statistical package (SYSTAT 1992) in randomized complete block design (RCBD). Significant treatment means were separated using the Duncan multiple range test (GOMEZ and GOMEZ 1984). The model used for this experiment is stated below.

$$Yijk = \mu + Ti + Wj + (TW)ij + \sum ijk$$

where:
Yijk – dependent variable
μ – population mean
Ti – *ith* effect of level of shell damage (i = 1, 2, 3, 4)
Wj – *jth* effect of week of haemolymph collection (j = 1, 2, 3, 4)
TWij – interaction between level of shell damage and week of haemolymph collection
∑ijk – random error

Results and Discussion

The result of analysis of variance showing the effect of shell injury on haemocyte count in Giant African Land snails is summarized in Table 2.

Table 2
Analysis of variance (ANOVA) showing the effect of shell injury on the haemocyte count in Giant African Land snails (*Archachatina marginata*)

Source	Degree of freedom	Mean square
Treatment	3	330945.650***
Week	3	2425.117NS
Error	73	399960543

$P < 0.001$***

Different levels of shell damage had a significant effect on the haemocyte count ($P < 0.001$), while the effect of week on the haemocyte count during the shell damage was not significant ($P > 0.05$).

The significant effect seen in the haemocyte count is a result of anti-inflammatory responses which are very common during injury in many animal models. Allograft inflammatory factor-1 (AIF-1) which is an interferon inducible calcium-binding cytokine has been associated with inflammatory response in molluscs (LI et al. 2013). Studies have also shown that macrophages which facilitate wound healing, angiogenesis and collagen formation are found at the site of injury (LEIBOVICH and ROSS 1975, POLVERINI et al. 1977, HUNT et al. 1984, KOVACS and DIPIETRO 1994). Inflammatory response is vital to body injury, wound repair and immune response (OTTAVIAN et al. 2010). In mollusc, especially in snails, haemocytes are the analogue of various types of immune cells found in vertebrates and as such, they are known to be released whenever there are challenges in the system of this animal. Table 3 shows the least square means of the effect of shell damage on the haemocyte count in Giant African Land snails.

Table 3
Least square means showing the effect of shell injury on the haemocyte count in Giant African Land snails (*Archachatina marginata*)

Parameter	Least square means [10^6/mm^3]	S.E.M (±)
Control (undamaged shell)	345.200^a	44.719
1 cm shell damage	107.000^b	44.719
2 cm shell damage	109.400^b	44.719
3 cm shell damage	114.500^b	44.719

Means within the same column having different superscript differ significantly ($P < 0.001$)

The control group had the highest number of means compared to other levels, which were not significantly different from each other. This observation is an indication that damages of the shell at any magnitude compromises the immune status of this animal which is largely represented by total haemocyte population.

Haemocytes are known to be the chief immunoeffector cells which perform diverse immunological activities such as phagocytosis, encapsulation and cytotoxicity (RAY et al. 2013). If damages to the shell could affect the population of these cells, then it means that any other challenge at this moment of injury may be very dangerous to the survival of the animal. JONATHAN (1990) reported that experimentally shell-damaged snails had higher rate of mortality than did uninjured snails. Also, RAY et al. (2013) reported that exposure of two species of snails (*B. bengalensis* and *L. marginalis*) to cypermethrin and fenvalerate led to haemocyte density shift

and morphological damage. All these reports are testifying to the fact that both physical and chemical damage could compromise the population of haemocytes which are known to be responsible for immune activities in the system of this animal.

Table 4 shows least square means showing the effect of shell injury on the weekly haemocyte count in Giant African Land snails (*A. marginata*). Results show that haemocyte count was not significantly different ($P > 0.05$) across the three weeks of collection.

Table 4

Least square means showing the effect of shell injury on the weekly haemocyte count in Giant African Land snail (*Archachatina marginata*)

Week	Least square means [$\cdot 10^6/mm^3$]	S.E.M (±)
0	149.400	44.719
1	172.400	44.719
2	152.600	44.719
3	149.700	44.719

The implication of this observation is that a quick adjustment within the system of the animal had taken place thus nullifying the effect of damage within the three weeks of the study. Least square means showing the effect of different levels of shell damage on growth after damage is shown in Table 5.

Table 5

Least square means showing the effect of different levels of shell growth after injury

Parameter	Least square – means	S.E.M (±)
Control (no shell damage)	0.175^{c}	0.057
1 cm shell damage	0.241^{bc}	0.057
2 cm shell damage	0.347^{ab}	0.057
3 cm shell damage	0.444^{a}	0.057

Means within the same column having different superscript differ significantly ($P < 0.001$)

It is obvious that snails with 3 cm shell damage had the highest regrowth of 0.444 cm, followed by 1 cm and 2 cm shell damage which are not significantly different from each other (0.241 vs 0.347 cm) while the control had the least growth (0.175 cm). Figure 2 shows the freshly secreted shell after shell damage. The observation made in this study may be as a result of calcium and phosphorous mobilization from the body of the animal to compensate for the losses that occur during shell damage procedure. According to JONATHAN (1990), this process of shell repair is highly energy demanding. It was also reported that experimentally damaged shells grew significantly more new shell than the undamaged ones (JONATHAN 1990).

Fig. 2. Freshly secreted shell after shell damage

This assertion is in line with the observation made in this study. Mollusc shell formation has been reported to be complex and involves deposition of calcium carbonate ($CaCO_3$) which is known to be an inorganic material mixed with organic material (HARE 1963, WILBUR 1983).

Conclusion

This study has shown that shell injury has significant effect on haemocyte concentration. Irrespective of the level of shell damage used in this study, total haemocyte count was reduced compared to the control group. This observation is an evidence of immunosuppression and this call for adequate care during this period of shell injury. If adequate care is not taken during this period of injury, opportunistic infections may kill the animal as haemocytes play a crucial role in the immune defense of this animal. The implication of this study is that snail farmers should maintain a hygienic environment with adequate care during any eventuality of shell damage under intensive method of production.

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LEGAL TRADITION OR LEGAL MARASMUS? THE 'CURULE AEDILES OF LÜBECK' OR ANIMAL DEFECTS ACCORDING TO THE HISTORICAL INFLANTIC LAWS (BEFORE 1938/1940)

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Key words: veterinary medicine, civil law, warranty, legal history, Latvia, Estonia.

Abstract

The study concerns on the analysis and interpretation of the legal norms formerly applied in the territories of present-day Latvia and Estonia, in relation to civilian liability for physical defects of animals. The following trends were revealed: the hybridity of law, containing, i.a. Roman and Germanic elements; connectivity of municipal, noblemen's and peasants' law; basing the discussed provisions on the Lübeck and Roman law; persistence in organic continuity with local legal tradition; lack of willingness to change and rationalize the law in relation to changes in the surrounding world, new codifications and discoveries of the veterinary medicine.

Introduction

Trade of animals takes place regardless of the nationality of the parties to the contract of sale or the existence of their own country. This is also true for the small Baltic nations and states, like Latvia and Estonia, which first gained independence only in the 20th century. This does not mean, however, that there were no legal norms or standards regarding animal trade.

In the English literature the term *Livonia* is often used for the whole area of Inflants, the *Terra Mariana*, which is unprecise and incorrect. In the present analysis the term *Inflants* would be used in relation to territories of the current states of Latvia and Estonia, covering the following historical regions: Courland, Semigallia, Livonia (or Livland, or Swedish Livonia, now divided between Latvia and Estonia), Latgale (or Polish

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Livonia) and Estonia (northern part of the present state of Estonia). These areas are divided by a common history – and share a common history, since the Middle Ages.

Fig. 1. Current Latvian and Estonian territories as the Baltic Provinces (*Ostseegouvernements*, *Ostseeprovinzen*) under the Russian occupancy and administration in the 19th century (MEYERS KONVERSATIONS-LEXIKON 1885–1892), public domain, 1 : 2,250,000

The areas inhabited by numerous Baltic tribes were the area of territorial expansion and conquest and colonisation of other European nations: since the 12th and 13th century – the Danes (northern Estonia), and, most of all, the Germans. The feudal institutions, reflecting the Western European standards of the Holy Roman Empire, were formed by the Sword Brethren (transformed in the Livonian Order, a branch of the Teutonic Knights), the prince-archbishops of Riga and prince-bishops of Courland, Dorpat (now Tartu), Reval (now Tallinn) and Ösel-Wieck (now Saaremaa), as well as the German-speaking landlords. Also the urban populations were predominately German. It should be remembered that, for ages – lasting up to the first half of the 20th century, there was a firm social, cultural, linguistic and legal domination of Baltic Germans in the discussed areas. The languages of the legal norms were initially Latin and later on – German (MODRZYŃSKI 2017).

The development of the Protestant Reformation led to the secularization of the ecclesiastical states, establishment of the vassal Duchy of Courland and Semigallia, and incorporation of Livonia (Dutchy of Livonia – Inflants Voivodeship – so-called Polish Livonia, Wenden Voivodeship and Dorpat Voivodeship), as parts of the Polish-Lithuanian Commonwealth. In the 17th century the northern parts became dependencies of Sweden. Apart from that, it was the area of an uninterrupted Russian interest: starting from the Ruthenians in 11th century, then the Muscovites, through the full conquest by the Tsar's troops in the 18th century (forming the Baltic Provinces – Governorates of Courland, of Livonia, and of Estonia – Figure 1), and by the Soviets in 1940 and 1944 (Latvian and Estonian SSRs), to informal steps undertaken nowadays.

The aim of the present research is to examine and demonstrate how the civilian norms can be transferred and was transferred in the Baltic states, and how sticking to the legal tradition can affect animal trade in terms of physical defects.

Materials and Methods

Legal and juridical texts consisting sources of law of the historical regions of Inflants (pre-1938/1940) were tested:

– state or noblemen laws: *Statuta Curlandica,* 1617 (BIRKEL 1804), *Ritter- und Landrecht des Herzogthums Esthland* of January 17th, 1651, June 17th, 1690, and January 27th, 1699, and Livonian laws (BUDDENBROCK 1804);

– municipal law of Lübeck according to the 1586 revision, as adopted in Reval (Tallinn) and other Inflantic towns (BUNGE and MADAI 1842–1844);

the municipal law of Riga – *Statuta und Rechte der Stadt Riga*, 1673; *Polizeiordnungen* – municipal regulations of Bauske (current Bauska) of August 1st, 1635, and of Friedrichstadt (Neustädtchen, current Jaunjelgava) of January 15th, 1647;

– Rural or Peasants' Codes (*Bauerrechte*): *Gesetzbuch für die curländischen Bauern* of August 25th, 1817, *Livländische Bauerverordnung* of March 26th, 1819, and *Livländische Bauer-Verordnung* of November 13th, 1860;

– Roman law reception in the *ius commune* form (MOMMSEN and KRÜGER 1872, KRÜGER 1892, ZIMMERMANN 1996, ZIMMERMANN 2001, SIIMETS-GROSS 2012);

– and the Code of the Private Law of Livonia, Estonia and Courland, 1864.

1938 is marked as one of the temporal limits of the present study – as the year of the entry into force of the new Latvian Civil Code of January 23rd, 1937, called the *Code Ulmanis* (CL 1937. gada 28. Janvārī, LUTS-SOOTAK et al. 2019). Shortly after that, during the World War II, the Baltic Germans were evicted (*Heim ins Reich* action, 1939–1941) and the area was captured by the Soviets (according to the Molotov-Ribbentrop Pact, 1939). The previous civil law was replaced in both countries with the Soviet one in 1940 (KULL 2013). *Le monde ci-devant* ceased to exist.

Historical, historical-legal, comparative and dogmatic methods of legal analysis and interpretation were used.

Results and Discussion

Non-codified Laws

For many centuries, the Lübeck municipal law was the basis for the functioning of over 50 towns in the North-German cultural area, including in particular the Hanseatic towns on the southern coast of the Baltic Sea. Among them, Riga, Reval (now Tallinn) and Hapsal (now Haapsalu) in Inflants, Braniewo (Braunsberg), Frombork (Frauenburg) and Elblag (Elbing) in Poland, Hamburg, Wismar, Rostock and Kiel in Germany can be counted (KAHLE 1879, EBEL 1971, EBEL and SCHILLING 2001, HACH 1839, ROZENKRANZ 1962, ROZENKRANZ 1967, ROZENKRANZ 1991, TANDECKI 2001, MODRZYŃSKI 2017, STEFFENHAGEN 1875). Initially also cities like Gdańsk (Danzig) or Szczecin (Stettin) and most of the Pomeranian towns were ruled according to the Lübeck standards. They were later relocated under the Magdeburg law, incl. its Kulm variant – which, however, did not mean a complete abandonment of the application of the earlier laws (PISKORSKI 1986, ROZENKRANZ 1966, MACIEJEWSKI 2000, MODRZYŃ-

SKI 2017). The Lübeck law was the essential basis of many laws – not only municipal ones – formerly in force in the territory of present-day Latvia and Estonia.

For the greater part of their history, Inflants' laws were not codified, and included historical layers of legal norms: since the crusaders' and Danish times in the 12th century, through the German law, reception of Roman law (*ius commune*), Polish, Swedish, and, finally, Russian laws in the 19th century, including various privileges, statutes, nobility codes, municipal and rural laws, constitutions, resolutions, decrees, judgments and preliminary rulings, as well as doctrinal achievements and unwritten customary law (BUNGE 1833, BUNGE 1847, BUNGE 1849, BUNGE 1851, REY 1875, SIIMETS-GROSS 2012).

In the examined scope a relatively complete legal system is revealed, within which two basic sources of responsibility for physical defects of animals can be distinguished: the Roman law (Justinian's Digest (MOMMSEN and KRÜGER 1872) and *ius commune*), and local statutory norms, based on the Lübeck law.

In the nobility laws the discussed problem was covered by the §§ 100, 101 and 148 of the *Statuta Curlandica* (BIRKEL 1804), Art. 9 (Book 4, Ch. 11) and Art. 5 (Book 4, Ch. 12) of the Estonian *Landrecht*, as well as the No. 293 of the Livonian laws (BUDDENBROCK 1804).

For the municipal laws, the following provisions should be indicated: Art. 6, 14, 15 and 17 (Book 3, Ch. 6) of the Lübeck law (BUNGE and MADAI 1842–1844, BUNGE 1847, BUNGE 1851, REY 1875); Art. 3 (Book 3, Ch. 11) of the laws of Riga (BUNGE 1847, REY 1875). It should be noted that the regulations of the two main Inflanitc cities – Riga and Reval were the reference point and model for the surrounding towns (BUNGE 1847). The issue of animal defects was also regulated by the 17th-century norms of the smaller towns based on the Lübeck law, but constituting elements of the Polish law *sensu largo*: §§ 4 and 6 (Ch. 24) of the *Bauske'sche Polizeiordnung*, and §§ 4 and 6 (Ch. 23) of the *Friedrichstädt'sche Polizeiordnung*.

In the 19th century, the discussed problem was regulated in the following paragraphs of the Peasants' Codes: § 98 (Courlandian), § 394 (Livonian, 1819), and § 978 (Livonian, 1860).

Auxiliary sources of law in northern and southern Inflants were: canon law, German law, Biblical (Mosaic) law, Polish and Swedish law, and – above all – the Roman law, fully adopted, just like in the Holy Roman Empire (BUNGE 1847, SIIMETS-GROSS 2012), constituting an important element of the sources of law also in the Prussian cities of the Polish-Lithuanian Commonwealth, e.g. in Elbląg (KWIATKOWSKI 2017).

Courlandian statutes established, on the basis of the principle of good faith (*bona fides*, *Treu und Glauben*), the obligation to pronounce all defects. Concealment of any imperfections of animals was prohibited. Deceitful concealment of the defect was the basis for redhibition: *rem venditam recipiet, et pretium restituet* (*die varkaufte Sache wieder zurücknehmen, und das Kaufgeld* (*den Kaufschilling*) *zurückzahlen*; return the item sold and refund the price) (BIRKEL 1804, BUNGE 1851, REY 1875). Under their rule, the limitation period for the *actio redhibitoria* was extremely short. It was the term in which not only the defect had to be detected, but also the judge had to decide: *rem vitiosam emens intra sex dies eam Judicis definitione redhibere vel retinere tenebitur* (*...nach vorgegangener richterlicher Erkenntnis...*) (BUNGE 1851, REY 1875).

The Livonian *Landrecht* did not provide for the *actio redhibitoria*, but the seller was obliged to repair damages if he knew about the defect and concealed it. Nevertheless, in the every-day practice and case-law, Aedilician warranty was applied (BUNGE 1847). This can be considered, in the author's opinion, as a manifestation of the subsidiary application of the Roman *ius commune*: redhibitory action available for 6 months, and *actio quanti minoris* – on an annual basis.

Estonian law established – in case of deceptive concealment – the liability for all defects, including evident and contractually excluded ones. It was a combination of Lübeck and Aedilician law (BUNGE 1847, MOMMSEN and KRÜGER 1872). There was a *lex specialis* related to the main defects of horses: full blindness (Fr. *amaurose*), glanders and a syndrome of behavioral disorders on the background of hydrocephalus (Pol. *wartogłowienie*, Fr. *rétivité*). Other animals were subject to the Aedilician, abstract rules of warranty. The limitation period for claims was 3 weeks.

According to the municipal norms, based on the Lübeck law, there was a rule that evident defects (*sichtbarer Mängel*) constituted a negative redhibitory premise – but not in the case of cattle (widely understood, as many animal species, not just *Bos taurus*). *Dolus* – the insidious concealment of the animal's defect by the seller gave rise to both civilian and criminal liability (BUNGE 1851, REY 1875). The main defects (*Hauptmängel*) were established only for horses, while for other animal species the liability covered all possible defects. The corollary of this was the lack of any responsibility for equine diseases other than: RAO/ COPD (recurrent airway obstruction – chronic obstructive pulmonary disease – *Pferd engbrüstig*, Fr. *pousse*), the syndrome of behavioral disorders as a result of hydrocephalus (*Pferd stetig*), and glanders (*Pferd schnöbich, rotzig*). The strengthening and confirmation of the contract of sale was a down pay-

ment (*arrha*, *Gotts-Pfennig, Gottes-Grosch*), which ensured that the contract could be contested with a claim.

Such regulations were in force in, i.a. Reval and Hapsal. They were also – almost literally – taken over to the regulations of Bauske and Friedrichstadt. They were derived directly from the Lübeck law (*Der Kayserlichen Freyen und des Heiligen Reichs Stadt Lübeck Statuta und Stadt Recht*, 1586), and were approved by the Prince of Courland and the King of Poland (BUNGE 1851).

Riga statutes, 1673, also drew on the law of Lübeck (BUNGE 1847). An open catalog of equine defects was established. Not only glanders, RAO/ COPD, blindness and behavioristic effects of hydrocephalus (*rotzige, hauptsieche, starrblinde, stätige (...) Pferde*) were treated as warranty premises. Also other, analogous defects of horses enabled the buyer for a redhibitory claim within 8 days (BUNGE 1847, REY 1875). As for cattle (in the author's opinion: *sensu largo*), which – besides – was listed in the provisions on the deposit, no major defects were set (*contra*: BUNGE 1847).

Courlandian rural law provided for a warranty period of 7 days since the animal's delivery, and Livonian Peasants' Statutes – a period of twice as long, 14 days from the delivery. The redhibitory claim was used in case of any hidden, unobtrusive (*nicht offenbar und in die Augen fallend sind*) defects in animals.

Code of the Private Law, 1864

In 1860, a draft of a Civil Code for the Baltic Governorates was developed, Art. 3698 and 3719 of which regulated the problem of animal defects (REY 1875). In 1864, a codification – or rather a compilation and systematization of the old laws – came into force (Luts-Sootak 2006, LUTS-SOOTAK et al. 2019, Siimets-Gross 2012, Giaro 2016). The *Liv-, Est- und Curländisches Privatrecht* systematized the old legal norms, but did not provide any substantial change of them.

The analyzed compilation of private law was based on almost the same – highly anachronistic – methodology as the Justinian's compilation of the VI century A.D. (MOMMSEN and KRÜGER 1872, KRÜGER 1872), with sources of individual, chaotically collected and terminologically heterogeneous provisions.

For example, Art. 3243 was based on the Justinian's Digest 18.1.45, 19.1.6.4, 19.1.13.pr.-1, 21.1.1.2 (MOMMSEN and KRÜGER 1872), Art. 9 (Book 4, Ch. 11) of the Estonian *Landrecht*, §§ 100 and 101 of the *Statuta Curlandica*, and Art. 15 (Book 3, Ch. 6) of the Lübeck law (BUNGE and MADAI 1842–1844); Art. 3244 was based, according to its *διδασκαλία*, only

on the Digest 21.1.17-8, 21.1.4.6, 21.1.6.2, 21.1.7-8, 21.1.10-14 (MOMMSEN and KRÜGER 1872); Art. 3253, 3256 and 3257 were modelled after §§ 100 and 101 of the *Statuta Curlandica* and the Digest 2.14.31, 18.1.45, 19.1.1.1, 19.1.6.4, 19.1.6.9, 19.1.13.pr., 19.1.39, 21.1.14.9-10, 21.1.19.2, 21.2.16.2, 21.1.28, 21.1.38.pr., 21.1.48.1-2, 21.2.31, 21.2.65.5 (MOMMSEN and KRÜGER 1872).

Warranty for physical defects was regulated in Art. 3243-3272 of the new Code, separately from the regulations of sales, incl. the obligations of the seller and the termination of the contract (*Auflösung des Vertrages*), as well as from warranty for legal defects (*evictio*), and for defects in real estate. Warranty regime was fully dispositive. Contractual exclusion of warranty was permissible, but ineffective in the event of an insidious concealment of the defect.

It covered all physical defects, regardless of the seller's knowledge, but depending on the significance and evidence (*in die Augen springen*) of the given defect. Defects must have been prior to the conclusion of the contract of sale (but not: pre-existing and eliminated by then), according to the Art. 3247 and 3248 of the Code, based on the Digest 21.1.1.10, 21.1.16, 21.1.54 (MOMMSEN and KRÜGER 1872) and Justinian's Code 4.58.3 (KRÜGER 1892).

According to Art. 3251, 3254 and 3255, full warranty liability was established for the seller who untruthfully ascertained and assured that there were no specific defects in the animal sold or that it has possessed some specific characteristics – but not for a mere merchant praise. This regime was based on the Roman concept of *dicta* and *promissa*, after the Justinian's Digest 4.3.37, 18.1.43.pr., 18.1.66.pr., 18.6.15, 19.1.13.3, 21.1.4.3, 21.1.1.1, 21.1.17.20, 21.1.18, 21.1.19.pr.-4 (MOMMSEN and KRÜGER 1872).

The remedies for the buyer were: *Wandelungsklage* – claim for redhibition, recission and cancellation of sale, according to Art. 3259–3262, 3271, and *Minderungsklage* – claim for estimation, i.e. price reduction, regulated in Art. 3263-3265 of the Code.

What was analyzed and discussed above was applied to all animal speciei – except horses. Equine *leges speciales* of Art. 3252 and 3258 of the Code inherited the regulations of: Art. 17 (Book 3, Ch. 6) of the Lübeck law, Art. 5 (Book 4, Ch. 12) of the Estonian *Landrecht*, Art. 3 (Book 3, Ch. 11) of the Riga statute, Art. 3 (Ch. 24) and Art. 3 (Ch. 23) of the discussed *Polizeiordnungen*, respectively, *Bauske'sche* and *Friedrichstädt'sche*.

In case of horse sale – unless the parties agreed otherwise – the warranty was limited to liability for the main defects. The number of these defects was territorially differentiated, according to the old laws, discussed above. While glanders (*Rotz*, *Schnöbe*), syndroms of hydrocephalus

(*Koller*, *Stätigkeit*) and full blindness (*Staarblindheit*) were available everywhere, the liability for RAO/COPD (*Engbrüstigkeit*) was limited only to the Estonian and Courlandian cities and towns. While in case of other animal speciei, governed by general rules, both Aedilician claims were possible to conduct, for horse defects the *Wandelungsklage* was a sole possibility for the buyer.

In addition to significant terminological heterogeneity, the biggest disadvantage of the *Privatrecht* was the huge territorial diversity of the limitation periods for warranty claims, unjustified by nothing other than local tradition and constituting a significant impediment to animal trade.

The limitation period for redhibitory claims was calculated since the date of conclusion of the contract, referred to as *Veräusserung*, or from the date on which warranty – or, in the current terminology: guarantee – ascertainments (*Gewährleistungsversprechens*) were given by the seller. The periods were: for horses – 3 weeks in Estonia (land law) and 8 days in Livonia (municipal law); for other animal species – 6 days in Courland, and 6 months in Livonia and Estonia (*sic*!). The basis for the norm of Art. 3271 were: § 148 *Statuta Curlandica,* 1617 (Birkel 1804), Art. 5 (Book 4, Ch. 12) of the Estonian *Landrecht*, Art. 3 (Book 3, Ch. 11) of the Riga law, as well as Roman legal sources: the Digest 21.1.19.6, 21.1.38.pr. (Mommsen and Krüger 1872), and the Justinian's Code 4.58.2 (Krüger 1892).

The limitation period for *quanti minoris* claims was, according to the – urely Roman – Art. 3272, one year since the conclusion of the contract (*Abschliessung des Vertrages*) or since the assurances (*Zusicherung*).

Conclusions

The above-mentioned special regulations of equine main defects were typical of Germanic customary laws, e.g. German and French customs, both written and unwritten. Also the types of defects mentioned, and limitation of the claims, like gradation depending on the significance of the defect, exclusion of the *actio quanti minoris*, are perfectly typical to the so-called German legal model of warranty (*deutschrechtliches Prinzip*). It was adopted – in the author's opinion: in an unjustified and blameworthy manner – in most of the European legal systems (Huzard 1837, Galisset and Mignon 1864, Beugnot 1836–1859, Meisner 1927, Lerche 1955, Wengersky 1988, Sommer 2000, Visser 2000–2001, Bardeleben 2013, Adamczuk 2008, Viguier 2006, Mayer 2009).

In other cases, including warranty for defects of all other animal species except horses, the Inflants' law exhibits the features of hybridity, cha-

racteristic for a mixed legal system. The laws of different genesis and history were acting in a complimentary way. Moreover, auxiliary and subsidiary ancillary Roman norms could provide a basic legal features in some factual situations, while in the others, a little different ones, the German norms should be applied. Similarly, local municipal and noblemen's laws, and *ius commune* were complementary to themselves. Premises of both Roman (Aedilician) and Lübeck laws, preserved in slightly different statutory acts, were combined and processed to build up an uniform system of civilian responsibility. This is, in the author's opinion, the justification for the – somewhat jocular – title of the current study.

Despite the flawed editorial of the *Liv-, Est- und Curländisches Privatrecht*, in the author's opinion, it could, create an effective warranty system for physical defects of animals – provided terminological and temporal harmonization, mainly due to the Roman law. The impact of the German legal tradition, the immanent part of which is the Roman law reception, is still present in both Latvian and Estonian civil laws (LUTS-SOOTAK et al. 2019, KULL 2013, KÄERDI 2003).

In the author's opinion, the historical laws of Inflants were, on the one hand, examples of the organic development and continuity of legal norms, deeply rooted in the local tradition, while on the other – outstanding examples of the legislative stagnation and lack of logical criticism, which resulted in deep anachronism of the legal norms. They could be considered as a negative embodiment of the observation made by Robert Evans-Jones on the margins of reflections on classic mixed legal systems – Scots and South African laws: *...if history seriously obstructs reason, lawyers must choose reason because the consumer has no interest in legal history for his own sake* (EVANS-JONES 2003). At the time, however, local lawyers and lawmakers did not choose *reason*. It was not only the – quite typical, even in the most developed legal systems and states – lack of willingness to change and rationalize the law in relation to new discoveries of the veterinary medicine, but even to adapt to changes in the surrounding world, including new legal trends and ideas of the age of the great European codifications.

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EFFECTS OF AQUEOUS LEAF-EXTRACTS OF CHROMOLAENA ODORATA AND TRIDAX PROCUMBENS ON DOXORUBICIN-INDUCED HEMATOLOGIC TOXICITIES IN WISTAR RATS

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Key words: *Chromolaena odorata,* doxorubicin, platelet indices, red cell indices, *Tridax procumbens*, white cell indices.

Abstract

This study investigated the influence of aqueous leaf-extracts of *Chromolaena odorata* and *Tridax procumbens* on haematological indices of doxorubicin treated rats. Doxorubicin (15 mg/kg body weight) was intra-peritoneally administered 48 h prior to sacrifice; while metformin (250 mg/kg), and the extracts (50, 75 and 100 mg/kg) were orally administered daily for 14 days. The red cells, white cells, lymphocytes and platelets counts, haematocrit, mean platelet volume, platelet distribution width, plateletcrit and platelet-larger cell ratio of Test control were significantly ($p < 0.05$) lower than those of Normal control, but not significantly lower than those of all the other groups. The mean cell haemoglobin and mean cell haemoglobin concentration of Test control were significantly ($p < 0.05$) higher than those of the other groups. The extracts had no harmful effect on the number of red cells, white cell and platelets indices, and prevented/ameliorated doxorubicin-induced haematological toxicity.

Introduction

The successful use of doxorubicin in chemotherapy has been limited, largely due to its diverse toxicities, including ocular (CARVALHO et al. 2009), cardiac (CARVALHO et al. 2009, SHOUKRY et al. 2017, AFSAR et al.

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2017, Zilinyi et al. 2018, Ahmed et al. 2019b), renal (Carvalho et al. 2009, Ahmed et al. 2019b, Bordbar et al. 2019), hepatic (Carvalho et al. 2009, Ahmed et al. 2019a, Alghorabi et al. 2019, Song et al. 2019), pulmonary (Jagetia and Lalrinpuii 2018), haematological (Sleijfer et al. 2018). Administration of doxorubicin has toxic effects on hematopoietic cells (Sleijfer et al. 2018), with the concomitant haematological toxicities such as anaemia, leukopenia, neutropenia and thrombocytopenia (Sleijfer et al. 2018). The haematological toxicity produced by doxorubicin is accompanied by reduced total erythrocytes count (Afsar et al. 2017, Fayyaz et al. 2017, Fathy et al. 2018, Khiavi et al. 2019, Islam et al. 2020), total white cells count (Afsar et al. 2017, Fayyaz et al. 2017, Fathy et al. 2018, Luthfi et al. 2018, Sleijfer et al. 2018, Khiavi et al. 2019), platelets count (Afsar et al. 2017, Fayyaz et al. 2017, Fathy et al. 2018, Luthfi et al. 2018, Sleijfer et al. 2018, Khiavi et al. 2019), lymphocytes count (Fayyaz et al. 2017, Fathy et al. 2018, Islam et al. 2020), granulocytes (neutrophils, eosinophils and basophils) count (Afsar et al. 2017, Fayyaz et al. 2017, Sleijfer et al. 2018, Khiavi et al. 2019), mid-sized cells (or monocytes) count (Fayyaz et al. 2017), mean corpuscular volume (Afsar et al. 2017), haematocrit (Afsar et al. 2017, Fathy et al. 2018, Islam et al. 2020), haemoglobin concentration (Afsar et al. 2017, Fayyaz et al. 2017, Fathy et al. 2018, Luthfi et al. 2018, Sleijfer et al. 2018, Islam et al. 2020), mean cell haemoglobin (Afsar et al. 2017) and mean cell haemoglobin concentration (Afsar et al. 2017). Fathy et al. (2018) also reported increased neutrophils and monocytes counts; while Afsar et al. (2017) reported increased lymphocytes counts.

Doxorubicin suppresses the replicating precursor cells of the bone marrow resulting in reduced production of red blood cells (Khiavi et al. 2019) and leucocytes (Sleijfer et al. 2018), and can cause blood clotting disorders, anaemia and leukopenia (Khiavi et al. 2019). Therefore, its effect on blood parameters should be closely monitored (Khiavi et al. 2019). The management of doxorubicin-induced hematotoxicity is quite essential, hence the need for the investigation of herbal medications with potential preventive and ameliorative properties.

The leaf-extracts of *Chromolaena odorata* and *Tridax procumbens* are two of such preparation from plants, with the potential for the amelioration and prevention of doxorubicin-induced haematological toxicity. The leaves and their extracts have moderately high contents of iron, magnesium, flavonoids (e.g. quercetin, catechin and ellagic acid), saponins, tannins and other polyphenolic compounds (Igboh et al. 2009, Ikewuchi and Ikewuchi 2009b, Ikewuchi 2012a,b, Ikewuchi et al. 2009, 2012, 2013, 2014a,b, 2015), all of which are known modulators of haematological indi-

ces. The anti-anaemic property of flavonoids (e.g. quercetin) was reported by SEN et al. (2005). Increases in haematocrit, haemoglobin concentration and red cell count have been reported to result from magnesium supplementation (OTHMAN et al. 2016). Iron supplementation has been shown to raise haematocrit, haemoglobin concentration, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and mean corpuscular volume, as well as red cells count and distribution width (DOGAR et al. 2013). Saponins and tannins have been reported to exhibit immunostimulatory activity and encourage lymphocytes proliferation (European Food Safety Authority 2009, DAVIDOVI et al. 2010, IKEWUCHI et al. 2011a, 2013, IKEWUCHI and IKEWUCHI 2013). Polyphenolic compounds were reported to be responsible for anti-thrombocytopenic effect of *Euphorbia hirta* (APOSTOL et al. 2012). Catechin and rutin were reported to be responsible for the anti-leukopenic and anti-thrombocytopenic activities of *Syzygium cumini* leaves (BANDIOLA and CORPUZ 2018). Ellagic acid was reported to cause increased platelet production by overexpression of cyclooxygenase pathway (ATTILIO et al. 2010).

The ability of the leaf-extracts of *C. odorata* and *T. procumbens* to positively modulate haematological indices, in salt-induced hypertensive and alloxan-induced diabetic rats were reported by IKEWUCHI and coauthors (IKEWUCHI 2012a, IKEWUCHI and IKEWUCHI 2013a, IKEWUCHI et al. 2014a). The leaf-extracts of *C. odorata* and *T. procumbens* have been reported to have anti-hypertensive (IKEWUCHI et al. 2010, 2011b, 2012), anti-dyslipidaemic (IKEWUCHI and IKEWUCHI 2009a, 2011, IKEWUCHI 2012a, IKEWUCHI et al. 2011c, 2014a,b), weight reducing (IKEWUCHI and IKEWUCHI 2009a, 2011, IKEWUCHI et al. 2010, 2011c), hepato-protective (IKEWUCHI 2012b, PALANISAMY et al. 2014), anti-diabetic (IKEWUCHI 2012a, ONKARAMURTHY et al. 2013), anticancer (VISHNU and SRINIVASA 2015, ADEDAPO et al. 2016) and antioxidant (PUTRI and FATMAWATI 2019, CUI et al. 2020) activities. In this study, the influence of aqueous leaf-extracts of *Chromolaena odorata* and *Tridax procumbens* on haematological indices was investigated in doxorubicin treated rats.

Materials and Methods

Procurement of Materials

Fresh samples of *Chromolaena odorata* and *Tridax procumbens* were collected from within the University of Port Harcourt, and were duly identified as previously reported (IKEWUCHI and IKEWUCHI, 2009b, 2011a,

2013, IKEWUCHI 2012a,b, IKEWUCHI et al. 2009, 2010, 2011a,b, 2012, 2013, 2014a,b, 2015). Forty five Wistar rats (weight 120–190 g) were obtained from the Animal House of Department of Pharmacology, University of Port Harcourt, Nigeria.

Preparation of Etracts

The leaves were rid of dirt. Then 6 kg of *C. odorata* and 5.5 kg of *T. procumbens* were macerated. The resultant extracts were dried in a water bath, and their residues (127 g and 116 g, respectively) were stored for use in the assay. The resultant leaf-extracts of *C. odorata* and *T. procumbens* (hereinafter referred to as COLE and TPLE, respectively), were weighed, reconstituted in distilled water and administered to the experimental animals, according to their individual weights and dosages of their groups.

Experimental Design

All experimental procedures in this study were performed in accordance with the ethical guidelines for investigations using laboratory animals, and complied with the guide for the care and use of laboratory animals (National Research Council 2011). The animals were weighed and sorted into nine groups of five animals each, with the average differences in weight ≤ 2.5 g (FAO 1991). They were housed in cages at the Department of Pharmacology, and allowed water and feed *ad libitum*. The animals were given standard rat chow product of Top Feeds Limited Nigeria

After 1 week acclimatization, the treatment commenced and lasted for 14 days. The animals were divided into nine groups of five rats each. Group 1 was normal control, Group 2 was test control, Group 3 was administered with Metformin or Diabetmin^{TM} (metformin HCl) (dissolved in distilled water) orally daily at 250 mg/kg body weight. This group is also referred to as reference drug group. The extracts were administered to groups 4–9 in the following order respectively; 50 mg/kg (COLE-50 mg), 50 mg/kg (TPLE-50 mg), 75 mg/kg to COLE-75 mg and 75 mg/kg (TPLE-75 mg), 100 mg/kg (COLE-100 mg) and 100 mg/kg (TPLE-100 mg). The test control group was administered with doxorubicin but was not given any of the extract while the normal control group was neither given doxorubicin nor treated with the extracts. Both received distilled water in place of the extract.

On day 12, doxorubicin was dissolved in normal saline and intra-peritoneally injected (15 mg/kg), into all the groups, except the normal control which was administered normal saline in place of doxorubicin solution. The doxorubicin dosage was adopted from SONG et al. (2019). The dosages

of administration of *C. odorata* extract was adopted and modified from IKEWUCHI et al. (2012, 2014a,b); that of *T. procumbens* extract was from IKEWUCHI et al. (2011b,c); while that of metformin was from ZILINYI et al. (2018).

Collection of Blood Samples and Determination of Haematological Indices

On day 14, the animals were sacrificed under chloroform anaesthesia and blood was collected into EDTA bottles for the haematological assay. Haematological indices were determined using Medonic M^{16} Haematological Analyser (Nelson Biomedical Limited, UK).

Statistical Analysis of Data

Statistical calculations were carried out with the Excel 2010 (Data Analysis Add-in) software. All data are expressed as mean ± standard error of the mean (SEM), and were analysed using one-way analysis of variance. Significant difference of the means was determined using least significant difference test; $p < 0.05$ was considered statistically significant.

Results

The effect the leaf-extracts of *C. odorata* and *T. procumbens* on platelet indices of doxorubicin treated rats is presented in Table 1.

Table 1
Effect of the leaf extracts of *Chromolaena odorata* and *Tridax procumbens* on platelet indices

Treatments	Platelets count [$\cdot 10^9$/L]	Mean platelet volume [fL]	Platelet distribution width [fL]	Plateletcrit [%]	Platelet-larger cell ratio [%]
Normal control	853.00±280.92^a	8.40±1.48^a	13.10±2.16^a	0.78±0.31^a	22.16±9.69^a
Test control	399.40±44.70^b	6.14±0.18^b	9.48±0.23^b	0.24±0.03^b	4.72±0.62^b
Metformin	472.50±24.92^{b,c}	6.18±0.12^b	9.45±0.16^b	0.29±0.01^b	6.95±0.81^b
COLE-50 mg	524.00±55.93^{a,b,c}	6.02±0.18^b	9.28±0.25^b	0.31±0.03^b	5.54±1.02^b
COLE-75 mg	402.60±119.70^b	4.64±1.18^b	8.92±0.25^b	0.30±0.04^b	3.69±0.67^b
COLE-100 mg	482.60±79.56^{b,c}	6.16±0.17^b	9.42±0.20^b	0.29±0.05^b	6.14±0.97^b
TPLE-50 mg	475.00±47.26^{b,c}	6.46±0.35^b	10.44±0.60^b	0.31±0.03^b	8.76±2.67^b
TPLE-75 mg	739.60±96.70^c	6.40±0.14^b	9.96±0.29^b	0.47±0.05^{a,b}	7.98±1.24^b
TPLE-100 mg	629.20±69.44^{a,b,c}	6.28±0.08^b	9.54±0.11^b	0.39±0.05^b	5.64±0.41^b

Values are mean ± SEM, $n = 5$ animals, per group. Values in the same column with different superscript letters differ significantly at $p < 0.05$

Table 2

Effects of aqueous leaf extracts of *Chromolaena odorata* and *Tridax procumbens* on red cell indices of doxorubicin treated rats

Treatments	Red cells count [$\cdot 10^{12}$/L]	Mean corpuscular volume [fL]	Red cell distribution width		Haematocrit [%]	Haemoglobin concentration [g/dL]	Mean cell haemoglobin [pg]	Mean cell haemoglobin concentration [g/dL]
			absolute value [fL]	per cent [%]				
Normal control	8.19±0.39^{a}	57.52±1.35^{a}	35.80±1.09^{a}	16.94±0.48^{a,b}	47.00±1.95^{a}	16.72±0.87^{a}	19.08±0.28^{a,c}	33.24±0.61^{a}
Test control	5.63±1.60^{b}	54.12±1.53^{a,b}	34.74±4.17^{a}	19.24±3.40^{a,b}	36.95±3.57^{b,c}	13.38±3.89^{a,b}	21.68±1.47^{b}	40.98±4.11^{b}
Metformin	7.61±0.24^{a,b}	57.40±2.03^{a,b}	36.03±1.14^{a}	16.98±0.44^{a,b}	43.53±1.17^{a,b}	14.58±0.40^{a,b}	19.20±0.45^{a,c}	33.53±0.44^{a}
COLE-50 mg	7.41±0.47^{a,b}	55.12±1.35^{a,b}	35.06±1.16^{a}	17.76±0.49^{a,b}	40.68±2.20^{a,b}	14.34±0.91^{a,b}	18.84±0.34^{a,c}	34.26±0.57^{a,c}
COLE-75 mg	6.65±0.35^{a,b}	56.06±1.15^{a,b}	35.14±1.55^{a}	17.60±0.67^{a,b}	37.24±2.06^{b,c}	12.74±0.52^{a,b}	19.14±0.40^{a,c}	34.36±0.59^{a,c}
COLE-100 mg	6.35±0.57^{a,b}	56.58±0.85^{a,b}	34.58±0.70^{a}	16.18±0.60^{a}	36.00±3.37^{b,c}	12.28±1.09^{b}	19.38±0.19^{a,c}	34.28±0.36^{a,c}
TPLE-50 mg	6.06±0.58^{b}	53.38±1.70^{b}	34.36±1.46^{a}	18.70±1.76^{a,b}	32.56±3.71^{c}	11.88±0.80^{b}	19.86±0.81^{a,b}	37.46±2.45^{a,b}
TPLE-75 mg	6.59±0.67^{a,b}	54.26±0.45^{a,b}	37.62±1.34^{a}	20.54±1.48^{b}	35.70±3.67^{b,c}	13.64±0.94^{a,b}	20.94±1.46^{b,c}	39.00±2.47^{b,c}
TPLE-100 mg	6.96±0.49^{a,b}	53.70±1.76^{a,b}	33.70±1.70^{a}	17.20±0.57^{a,b}	37.66±1.81^{b,c}	12.60±0.77^{a,b}	18.18±0.25^{a}	33.96±0.81^{a,c}

Values are mean ± SEM, $n = 5$ animals, per group. Values in the same column with different superscript letters differ significantly at $p < 0.05$

Table 3

Effect of aqueous leaf extracts of *Chromolaena odorata* and *Tridax procumbens* on white cell indices of doxorubicin treated rats

Treatments	Total white cells count [$\cdot 10^9$/L]	Lymphocytes count		Granulocytes count		Mid-sized cells count	
		absolute value [$\cdot 10^9$/L]	per cent [%]	absolute value [$\cdot 10^9$/L]	per cent [%]	absolute value [$\cdot$ 10^9/L]	per cent [%]
Normal control	21.13±5.14^{a}	14.85±2.94^{a}	74.55±5.30^{a}	3.55±2.08^{a}	13.13±4.70^{a}	2.73±0.77^{a}	12.33±0.82^{a}
Test control	5.46±0.91^{b,c}	3.12±0.49^{b}	62.66±4.24^{a}	0.54±0.14^{a}	10.80±1.10^{a}	1.56±0.45^{a,b}	26.54±3.48^{a,b}
Metformin	3.78±0.72^{b}	2.43±1.01^{b}	53.03±16.10^{a}	0.23±0.11^{a}	10.50±6.58^{a}	1.13±0.45^{a,b}	36.48±17.01^{b}
COLE-50 mg	6.68±0.69^{b,c}	4.78±0.89^{b,c}	71.34±9.50^{a}	0.90±0.68^{a}	15.14±10.94^{a}	1.00±0.14^{a,b}	13.52±1.75^{a}
COLE-75 mg	4.70±1.18^{b}	3.82±1.09^{b,c}	76.64±7.17^{a}	0.28±0.10^{a}	12.48±6.41^{a}	0.60±0.27^{b}	10.64±3.52^{a}
COLE-100 mg	5.34±0.68^{b,c}	3.74±0.73^{b,c}	68.68±7.98^{a}	0.52±0.21^{a}	10.50±3.91^{a}	1.16±0.28^{a,b}	20.82±4.36^{a,b}
TPLE-50 mg	11.72±4.79^{a,b}	7.88±2.88^{a,b}	70.44±3.48^{a}	2.10±1.31^{a}	13.84±3.73^{a}	1.74±0.62^{a,b}	15.68±1.51^{a}
TPLE-75 mg	16.56±10.00^{a,c}	10.66±6.00^{a,c}	65.38±6.29^{a}	3.32±2.62^{a}	16.20±3.81^{a}	2.58±1.50^{a}	18.42±3.13^{a,b}
TPLE-100 mg	4.76±0.61^{b,c}	3.88±0.82^{b,c}	77.22±9.43^{a}	0.24±0.13^{a}	8.74±4.78^{a}	0.64±0.15^{b}	14.04±4.83^{a}

Values are mean ± SEM, $n = 5$. Values in the same column with different superscript letters differ significantly at $p < 0.05$

The platelets count of test control was significantly ($p < 0.05$) lower than those normal control and TPLE-75 mg; but not significantly lower than those of all the others. The mean platelet volume, platelet distribution width, plateletcrit and platelet-larger cell ratio of test control were significantly ($p < 0.05$) lower than those of normal control; but not significantly lower than those of all the other groups.

As shown in Table 2 the red cells count and haematocrit of test control were significantly ($p < 0.05$) lower than those of normal control, but not significantly lower than those of all the other groups. The mean cell haemoglobin and mean cell haemoglobin concentration of test control were significantly ($p < 0.05$) higher than those of all the other groups, except TPLE-50 mg and TPLE-75 mg. The haemoglobin concentration, mean corpuscular volume and red cell distribution width of test control were not significantly lower than those of all the other groups.

Table 3 shows the effect of aqueous leaf-extracts of *C. odorata* and *T. procumbens* on white cell indices of doxorubicin treated rats. The total white cells count of test control was significantly ($p < 0.05$) lower than that of normal control; but not significantly lower than those of all the other groups. The lymphocytes count of test control was significantly ($p < 0.05$) lower than those of normal control and TPLE-75 mg; but not significantly lower than those of all the other groups. The granulocytes and mid-sized cells counts of test control were not significantly lower than those of all the other groups.

Discussion

The present result is in agreement with earlier reports of doxorubicin--induced reduction in haematological parameters such as: total erythrocytes counts (Afsar et al. 2017, Fathy et al. 2018, Khiavi et al. 2019, Islam et al. 2020), total white blood cells count (Afsar et al. 2017, Fayyaz et al. 2017, Fathy et al. 2018, Luthfi et al. 2018, Sleijfer et al. 2018, Khiavi et al. 2019), platelets count (Afsar et al. 2017, Fayyaz et al. 2017, Fathy et al. 2018, Luthfi et al. 2018, Sleijfer et al. 2018, Khiavi et al. 2019), lymphocytes count (Fayyaz et al. 2017, Fathy et al. 2018, Islam et al. 2020), granulocytes (neutrophils, eosinophils, and basophils) count (Afsar et al. 2017, Fayyaz et al. 2017, Sleijfer et al. 2018, Khiavi et al. 2019), and mid-sized cells (or monocytes) count (Fayyaz et al. 2017). Others include mean corpuscular volume (Afsar et al. 2017), haematocrit (Afsar et al. 2017, Fathy et al. 2018, Islam et al. 2020), haemoglobin concentration (Fayyaz et al. 2017, Fathy et al. 2018, Luthfi et al. 2018, Sleijfer

et al. 2018, ISLAM et al. 2020), mean cell haemoglobin (AFSAR et al. 2017) and mean cell haemoglobin concentration (AFSAR et al. 2017). It however, negates the reports of doxorubicin-induced increases in lymphocytes (AFSAR et al. 2017), neutrophils and monocytes (FATHY et al. 2018).

The haemopoietic system of the test rats was beneficially affected by the extracts. The extracts mildly improved their red cell indices. Similar positive modulation of red cell indices or anti-anaemic effects of the extracts were reported by IKEWUCHI and colleagues in salt-induced hypertensive and alloxan-induced diabetic rats (IKEWUCHI 2012a, IKEWUCHI et al. 2014a). The effect of the extracts may be due to their enhancement of erythropoiesis or inhibition of doxorubicin-induced destruction of red cells, or prevention of doxorubicin-induced myelosuppresion, inhibition of hematopoietic tissues and/or defective iron metabolism (AFSAR et al. 2017, SLEIJFER et al. 2018).

The observed elevated red cells count produced by the extracts, though not dose dependent, is an affirmation of the fact that the elevated haemoglobin concentration is the product of elevated red cell mass. The capacity of the extracts to increase red cell indices in the treated animals may be attributable to the presence of magnesium, iron, and flavonoids (e.g. quercetin), hitherto reported in the leaves and their extracts (IGBOH et al. 2009, IKEWUCHI and IKEWUCHI 2009b, IKEWUCHI et al. 2009, 2012, 2013, 2015).

The extracts may have increased the white cells' count by encouraging lymphocytes proliferation (or lymphopoiesis), granulocytopoiesis and monocytopoiesis, and preventing doxorubicin-induced myelosuppresion (SLEIJFER et al. 2018). This anti-leucocytopenic (anti-lymphocytopenic, anti-granulocytopenic and anti-monocytopenic) effect of the extracts may be due to the presence of saponins and tannins, both of which were hitherto reported in the leaves and their extracts by IKEWUCHI and colleagues (IGBOH et al. 2009, IKEWUCHI, 2012a, IKEWUCHI et al. 2009, 2013, 2014a, 2015). Similar anti-leucocytopenic effect of the extracts on salt-induced hypertensive and alloxan-induced diabetic rats were previously reported by IKEWUCHI and colleagues (IKEWUCHI 2012a, IKEWUCHI and IKEWUCHI 2013, IKEWUCHI et al. 2014a). This mild/moderate increase in total white blood cells counts produced by the extracts is beneficial, because, in addition to enhancing immunological, antimicrobial and inflammatory responses (BENSON and CALIGIURI 2018, CARTY et al. 2018, DORSHKIND and RAWLINGS 2018, KHANNA-GUPTA and BERLINER 2018), they could also provide defence against the onset of acute coronary syndrome (MORENO et al. 1994, LIBBY 2001, AYALOGU et al. 2011, IKEWUCHI et al. 2011a, 2013b, IFEANACHO et al. 2020).

The extracts prevented doxorubicin-induced thrombocytopenia. They may have achieved this by enhancing platelets production and/or preventing doxorubicin toxicity on the platelets (ZUNJAR et al. 2016, AFSAR et al. 2017). Similar anti-thrombocytopenic effect by the extracts on salt-induced hypertensive and alloxan-induced diabetic rats were previously reported by IKEWUCHI and coauthors (IKEWUCHI 2012a, IKEWUCHI et al. 2014a). This anti-thrombocytopenic activity of the extracts may be due to the presence of polyphenolics (ATTILIO et al. 2010, APOSTOL et al. 2012, BANDIOLA and CORPUZ 2018). This mild/moderate increase in platelets counts evoked by the extracts indicates enhanced clotting and lowered bleeding (KAUSHANSKY 2009, IKEWUCHI et al. 2011a, 2013, CANTOR 2018, IFEANACHO et al. 2020).

In conclusion, the increase in haematocrit, red blood cells, total white blood cells, platelets and lymphocytes counts though not dose dependent, may signify the positive effects of the extracts on the haemopoietic system of experimental rats. This highlights the potential of the leaves in the management of doxorubicin-induced anaemia and immune-suppression, as well as for the improvement of the haematological abnormalities associated with doxorubicin-induced haematological toxicity.

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