Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 26(2): 489 – 509 (2022)
www.ejabf.journals.ekb.eg

Do *Thymus vulgaris* and *Rosmarinus officinalis* dietary supplementations induce cytogenetic alterations in the juvenile Nile tilapia *Oreochromis niloticus*?

Eman A. Abd El-Gawad[1*], Hasnaa A. Radwan[2], Inas S. Ghaly[2], Amany A. Abbass[1*]

1. Aquatic Animal Diseases and Management Department, Faculty of Veterinary Medicine, Benha University, Egypt
2. Cell Biology Department, National Research Centre, Giza, Egypt

[*]**Corresponding Author:** eman.mahmoud@fvtm.bu.edu.eg; amanyabbass3@fvtm.bu.edu.eg

ARTICLE INFO
Article History:
Received: March 15, 2022
Accepted: April 3, 2022
Online: April 24, 2022

Keywords:
Biochemical,
Chromosomal aberrations,
Rosemary,
Nile tilapia,
Thyme

ABSTRACT

Medicinal plants provide a cheaper and sustainable alternative to chemotherapy in aquaculture; however, its misuse could be potentially toxic to both aquatic animals and humans. This study aimed to evaluate the potential cytogenetic and biochemical effects of thyme and rosemary powder supplemented diets on the Nile tilapia juvenile. Fish (26±1.5 g) were distributed into 5 groups and fed a non-supplemented diet (control), supplemented diets with thyme at a concentration of 1% and 1.5%, and diets supplemented with rosemary at a concentration of 0.5% and 1% for 6 weeks. Cytogenetical assessment (chromosome aberrations, micronucleated erythrocytes, and DNA fragmentation) was performed after 2, 4, and 6 weeks of feeding. In addition, hepatic enzymes (aspartate aminotransferase, alkaline phosphatase, and alanine aminotransferase), renal indicators (urea and creatinine), and muscle damage biomarkers (creatine phosphokinase, [CPK] and lactate dehydrogenase, [LDH]) were analyzed after 2 and 6 weeks feeding. The clinically examined fish in the group fed with 1% rosemary showed restlessness and lethargy throughout the experiment. Postmortem examination of moribund fish revealed body darkness with excessive mucus secretion and paled liver with red patches. In addition, this group showed a mortality rate reached 18.66%. Dietary supplementation of 1.5% thyme and 1% rosemary for 6 weeks of feeding exhibited a little bit of cytogenetic effect on peripheral blood, head kidney, and liver tissue, as the recorded types of aberrations were not severe. Fish receiving 1% rosemary for 6 weeks showed significant increases in the activity of hepatic enzymes, CPK and LDH activities, as well as creatinine levels, compared to the control and other treated groups. The results of this study demonstrate that, although thyme and rosemary have beneficial effects as medicinal herbs, they may pose cytogenetic threats to cells of the Nile tilapia at an early life stage. Thyme at a dose of 1% was proven to be safe for the juvenile Nile tilapia.

INTRODUCTION

The inclusion of herbal medicine in feeds as an alternative to antibiotics has grown rapidly in the aquaculture sector and has become the focus of many scientific research (**Bilen *et al.* 2019; Yousefi *et al.* 2020**). Many plants and their bioactive components have been recorded as promising ways for controlling diseases that threaten

aquatic animals (**Zahran *et al*. 2018; Zargar *et al*. 2019; Abd El-Gawad *et al*. 2020**), promote growth (**Bilen *et al*. 2019; Naiel *et al*. 2020**), improve the immune system (**García Beltrán *et al*. 2020; Yousefi *et al*. 2020**). In addition, they act as antistress supplements (**Al-Anazi *et al*. 2015**; **Basha *et al*. 2018; Soror *et al*. 2021**).

Many aquaculturists erroneously assume that all nutraceuticals are safe and have no adverse effects. On the contrary, some researchers have shown that several plants used as food or in traditional medicine are potentially cytotoxic, genotoxic, mutagenic, and have carcinogenic effects (**Horn & Vargas, 2008; Aguilar-Santamaría *et al*. 2013**; **Oliveira *et al*. 2016**; **Askin Celik & Aslanturk, 2018**). The incidence of micronucleus in peripheral blood erythrocytes, nuclear abnormalities, chromosomal aberrations and DNA fragmentation have been used as biomarkers of cytogenotoxic effect of plants (**Urbina-Cano *et al*., 2006**; **Manju *et al*. 2013**; **Singh *et al*. 2014**). Therefore, screening the genotoxic and antigenotoxic properties of medicinal herbs should be discussed to identify the adverse effects and determine the dose at which potential hazards occur in order to inform fish cultivators that herbal plants should be used with caution.

Thyme (*Thymus vulgaris*) is one of the naturopathic medicines with a great benefit on growth, immune system, antioxidant status and resistance against fish pathogens (**Yassen *et al*. 2017; Hoseini & Yousefi, 2019; Abd El-Naby *et al*. 2020; Khalil *et al*. 2020; Tasa *et al*., 2020**). These properties are due to the presence of bioactive constituents, such as thymol (40%), carvacrol (15%), cymene, eugenol and 4-allylphenol (**Marchese *et al*. 2016; Dauqan & Abdullah, 2017**).

Rosemary (*Rosmarinus officinalis)* leaves recorded positive effects on antioxidant and immunological parameters of fish and could relieve the adverse effects of stress (**Hassan *et al*. 2018**; **Naiel *et al*. 2020**; **Yousefi *et al*. 2020**). The anti-inflammatory, antioxidant, immunostimulatory, and antimicrobial properties of rosemary are attributed to the presence of carnosic acid and rosmarinic acid, which are the main chemical constituents of the plant (**Larràn *et al*. 2001; Erkan *et al*. 2008; Charles, 2012**). Despite the reported benefits, **Maistro *et al*. (2010)** reported that *Rosmarinus officinalis* oil induced genotoxicity in mouse lymphocytes cells. Moreover, aqueous extract of *Rosmarinus officinalis* showed cytotoxic and genotoxic effect on human peripheral lymphocytes **(Askin Celik & Aslanturk, 2018**).

To the best of our knowledge, no previous studies have investigated the cytogenotoxic effects of thyme or rosemary dietary supplementation in fish. Thus, the present study aimed to assess the probable cytogenetic adverse effects and biochemical changes induced by thyme and rosemary dietary supplementations in the Nile tilapia juvenile .

MATERIALS AND METHODS

Experimental fish

The Nile tilapia juveniles (*O. niloticus)* of mean body weight (26±1.5 g) and mean length (11±0.6 cm) were sourced from fish hatchery in the Central Lab for Aquaculture Research, Abassa, Sharkia, Egypt and transported early to the wet lab at the Faculty of Veterinary Medicine, Benha University. Fish were placed in well-prepared round fiberglass tanks (500 L capacity) and subjected to two weeks acclimation, with daily monitoring to ensure proper fish health conditions and absence of any signs of diseases as defined by **Austin and Austin (1989)**. Water temperature was adjusted to 26±1°C; oxygen concentration was 6±0.4 mg/l, and pH was 7.0±0.2. Fish were hand fed to apparent satiation with commercial basal diet (30% crude protein) (Uccma feed, Egypt) twice daily at 9:00 and15:00. The left-over food and fecal matter were daily siphoned, and the third volume of tank water was exchanged twice per week. This study and all experimental protocols involving the care and use of animals were approved by the Institutional Animal Care Use Committee Research Ethics Board, Faculty of Veterinary Medicine, Benha University, Egypt (BUFVTM 04/09/2020). The experimental procedures confirmed the regulation described in the guide for the care and use of laboratory animals.

Herbal plant and feed preparation

Thyme and rosemary were purchased as dry whole plant and identified by a plant taxonomist in Botany department of the Faculty of Agriculture, Benha University, Egypt. The concentration of each medicinal plant in this study was chosen based on the results of previous study in our lab (**Yassen, 2017)**. The dry plants and commercial pellets (Uccma feed, Egypt) were grinded to fine powder using an electrical blender. The grinded commercial pellets were blended with adequate amount of distilled water containing the required dose of thyme and rosemary powder in order to form a paste. The paste was re-pelleted at room temperature (28°C) using a manual meat grinder. The pellets obtained were air dried at room temperature for one day, packaged in clean plastic container, tightly closed and kept at 4°C. Five experimental diets were prepared as follows: non-supplemented diet (control diet), thyme-supplemented diets at doses of 1% and 1.5%, and rosemary-supplemented diets at dose of 0.5% and 1%.

Experimental design

The acclimatized fish were randomly distributed into fifteen experimental tanks. Each treatment group was assigned in triplicate (30 fish/ tank, N = 90 fish/ group). The first group was fed non-supplemented diet and maintained as control. The second and third groups were fed thyme-supplemented diets at a concentration of 1% and 1.5%, respectively. The last two groups received rosemary-supplemented diets at concentrations of 0.5% and 1%, respectively. The experimental groups were hand fed twice daily till satiation (9:00 and 15:00 h) for six weeks. The water quality parameters were maintained as those of the acclimatization conditions. Water exchange was done twice a week to maintain water quality parameters.

Clinical and postmortem examination

Throughout the experiment, the behavioral changes and morality in all experimental tanks were recorded daily. Postmortem examination of dead fish was carried out as described in the study of **Austin and Austin (2007)**.

Cytogenetical analysis

Three sampling were performed after 2, 4, and 6 weeks of feeding for cytogenetical investigation. At each sampling time, five fish from each replicate were taken and placed in a well-prepared glass aquarium (90 × 40 × 30 cm) (N = 15 fish/ group) and intraperitoneally injected with baker's yeast suspension at a dose of 1 ml/100 g body weight and kept for 24 hours (**Lee & Elder, 1980**). On the second day, the same fish were intramuscularly injected with freshly prepared 0.05% colchicine at a dose of 0.01 ml/g body weight. Blood, kidney and liver samples were collected for micronucleus, chromosomal aberrations and DNA fragmentation assay, respectively.

Micronucleus assay (MN)

Blood sample was collected from caudal blood vessels of anesthetized fish (3 samples/ replicate, N = 9 samples/ group), using syringe moistened with EDTA. A drop of blood was mixed with a drop of fetal calf serum, smeared directly on slide then air dried, fixed in absolute methanol for 5min, and stained with 5% Giemsa for 7min. Two thousand cells were analyzed for the frequency of MN in mature erythrocytes. The number of MN was expressed per thousand erythrocytes (**De Flora *et al*. 1993)**.

Chromosomal assessment

Chromosomal preparation of kidney tissues was carried out according to the method of **Al-Sabti (1986)** with some modification. Briefly, the anterior kidney was excised and cut into fine particles in 5 to 7ml of Roswell Park Memorial Institute (RBMI) medium, and 0.2 ml of 0.05 colchicine was added to each tube. The tubes were incubated at 37°C for 1 h and cells were centrifuged at 1000 rpm for 10 min and re-suspended in pre-warmed hypotonic solution (KCl 0.5 %) for 30min at 37°C. The sample were centrifuged and fixed in cold mixture of glacial acetic acid and methyl alcohol (1:3 v/v). After two times washing, the sediment was suspended in a small amount of the fixative. The slides were prepared by common method and stained with giemsa stain. Chromosomal analysis was performed in one hundred metaphase spreads for each fish.

DNA fragmentation test

After blood collection, fish were sacrificed, and liver tissues were collected immediately. The tissues were lysed in 0.5ml lysis buffer (10mM tris-HCL [pH 8], 1 mM EDTA, and 0.2% triton X-100) and centrifuged at 10000 rpm for 20 minutes at 4°C. The pellets were re-suspended in 0.5 ml of lysis buffer. For the pellets and supernatant, 1.5 ml of 10% trichloroacetic acid (TCA) was added and incubation was performed at 4°C for 10 minutes. The samples were centrifuged for 20 minutes at 10000 rpm at 4°C, and the pellets obtained were re-suspended in 750µl of 5% TCA, followed by incubation at 100°C for 20 minutes. Subsequently, to each sample, 2ml of DPA solution (200 mg diphenylamine in 10 ml glacial acetic acid, 150 µl of sulfuric acid and 60 µl acetaldehyde) was added and incubation was performed at room temperature for 24 hours

(**Gibb *et al*. 1997**). The proportion of fragmented DNA was calculated from absorbance reading at 600nm using the formula: DNA fragmentation% = [optical density (O.D.) fragmented DNA/O.D. fragmented DNA + O.D. of intact DNA] × 100. Genomic DNA was isolated from the liver tissue of control and treated groups, fractioned on 1.5% agarose gel, and stained with ethidium bromide according to the method of **Surzycki (2000)**.

Biochemical analysis

Blood were sampled after 2 and 6 weeks post feeding to assess some biomarkers for liver, kidneys and muscle damage. Fish at each sampling time were fasted for 24h prior to sampling and anesthetized with MS-222 (Finquel®, Argent) at a dose of 30 mg/L. Blood was drawn from the caudal blood vessels of three fish per replicate (N = 9 samples per group). The coagulated blood was centrifuged at 1000g for 10min at 4°C. The separated serum was pooled (N = 3 samples/ treatment group) and stored at -20°C until biochemical assessment.

Aspartate aminotransferase AST (U/L), and Alanine aminotransferase ALT (U/L) activities were determined by colorimetry using commercial kits (Biodiagnostic, Egypt), which depends on reduced nicotinamide adenine dinucleotide phosphate (NADPH) consumption and its conversion to nicotinamide adenine dinucleotide (NAD) at 340nm (**Reitman & Frankel, 1957)**. Alkaline phosphatase ALP (U/L) was determined at 405nm according to the method of **Kind and King (1945)**, based on the conversion of nitrophenol phosphate into nitrophenol and phosphate.

Serum urea levels (mg/dl) were quantitatively determined by measuring the absorbance at 570nm according to the method of **Henry (1964)**. Creatinine assay kits (Abcam, UK) were used for measuring serum creatinine levels (mg/dl) according to the study of **Thomas (1992**).

CPK and LDH, as muscle damage biomarker were also determined as follows: CPK was determined by measuring the concentrations of NADPH produced at 410nm according to the procedure of **Szasz *et al*. (1976)** using commercial kits (Biodiagnostic, Egypt). LDH catalyzes the conversion of L-lactate to pyruvate in the presence of NAD. The enzyme activity of LDH is proportional to the rate of production of NADH (reduced NAD). The concentration of NADH was determined by the increase in absorbance at 340nm (**Bais & Philcox, 1994)**. All blood biochemical parameters were determined by spectrophotometry (Sigma, USA).

Statistical analysis

Statistical analysis was performed with SPSS software V.16 (SPSS, Richmond, USA). Data were analyzed by one-way analysis of variance, followed by Duncan´s post hoc test for comparison between different treatments. Results were reported as mean ± standard error of mean, and differences were considered significant at the level $p < 0.05$.

RESULTS

Clinical signs and postmortem lesions

The treated fish with rosemary-supplemented diet exhibited hyperactivity (which reduced gradually), signs of distress and lethargic movements. Body darkness along with mucus secretion was observed, specially in the group fed 1% rosemary-supplemented diet. Internally, the moribund fish showed paled liver with red patches and darkness of kidneys. The rosemary-supplemented groups recorded a mortality rate of 14.66% and 18.66% in groups fed 0.5% and 1% rosemary, respectively, throughout the experiment. Meanwhile, group supplemented with thyme as well as the control group did not show any behavioral changes or mortality during the experiment.

Cytogenetic toxicity

The total chromosomal aberrations were significantly increased in all groups fed rosemary-supplemented diets throughout the sampling period when compared to the control group. In addition, oral administration of 1.5% thyme-supplemented diet for 6 weeks exhibited significant increase in the total chromosomal aberrations. Meanwhile, fish fed thyme-supplemented diets at both concentrations (1% and 1.5%) for 2 and 4 weeks showed non-significant increase in the total chromosomal aberrations when compared to the control (Table 1). The most frequent chromosomal aberrations observed were chromatid breaks, followed by deletions, fragment, gaps and aneuploidy. In the treated groups, cells with more than one chromosome aberration were not observed. Other sever types of aberrations, such as polyploidy, endomitosis, dicentric chromosome, ring and centric fusion were not observed.

The micronucleus assay of the potential mutagenic effect of thyme and rosemary are presented in Table (2). There was no significant increase ($p > 0.05$) in the number of micronucleated erythrocytes with thyme in all treatment groups; however, 1.5% thyme-supplemented diet for 6 weeks revealed significant increase compared to the control. Additionally, the number of micronucleated polychromatic erythrocytes was significantly increased in all groups receiving diets incorporated with rosemary compared to the control.

DNA fragmentation in the Nile tilapia fed thyme and rosemary at different concentrations for 2, 4 and 6 weeks are illustrated in Table (2). Thyme at a dose of 1% showed no significant effect ($p > 0.05$) on DNA during the experimental feeding. Concurrently, fish fed 1.5% thyme-supplemented diet for 2, 4, and 6 weeks recorded significant increases in fragmented DNA. Moreover, DNA fragmentation was significantly increased ($p < 0.05$) at all doses of rosemary.

The effects of different treatments on total genomic DNA damage are presented in Fig. (1). There was no observed DNA damage induced by 1% thyme-supplemented diet at 2, 4, and 6 weeks of feeding. However, thyme-supplemented diet at a dose of 1.5% induced a slight DNA damage. DNA damage was increased in all treatment groups fed rosemary either at 0.5% or 1% at all sampling times.

Biochemical parameters

The dietary intake of thyme at concentrations of 1% and 1.5% for 2 and 6 weeks recorded a non-significant change in activity of hepatic enzymes (AST, ALP, and ALT), compared to the control. Similarly, rosemary dietary supplementation at a dose of 0.5% revealed no significant increase in the liver enzymes status at both sampling time with respect to the control group. Meanwhile, fish receiving 1% rosemary for 6 weeks showed significant increases in the activity of liver enzymes (Table 3).

Serum urea recorded non-significant increase in all supplemented groups throughout the experiment, compared to the control. Nevertheless, creatinine level increased significantly ($p < 0.05$) in fish fed rosemary at a dose of 1% for 6 weeks, while other supplemented groups recorded no changes in creatinine level at both sampling times (Table 4).

Groups fed thyme for 6 weeks recorded significant decreases in CPK, compared to control. Similarly, rosemary at a dose of 0.5% recorded significant decrease in CPK at 2 and 6 weeks. Meanwhile, dietary administration of 1% rosemary for 6 weeks showed significant increase in CPK, compared to control. Meanwhile, there was no significant difference in LDH concentration in groups that received 1% and 1.5% thyme-supplemented diets at both sampling time. In addition, rosemary-supplemented diet at a dose of 0.5% showed no significant variation in serum LDH compared to control. However, after 6 weeks of feeding with 1% rosemary, significant variation was detected in the LDH concentration, with respect to the control and other treated groups (Table 5).

Table 1. Frequencies of chromosomal aberrations in *O. niloticus* fed control diet and supplemented diets with thyme and rosemary at different concentrations for 2, 4, and 6 weeks

Group	Feeding time	Gap	Break	Deletion	Fragment	Aneuploid	Total chromosomal aberrations
Control		0.33±0.33^{bc}	0.33±0.33^{d}	0.66±0.66^{d}	0.33±0.33^{e}	2.67±0.33^{a}	4.33±.88^{e}
Thyme 1%	2 weeks	00^{c}	0.33±0.33^{d}	0.66±0.66^{d}	0.66±0.33^{de}	3.00±0.57^{a}	4.67±0.33^{e}
	4 weeks	0.33±0.33^{bc}	0.66±0.66^{d}	1.33±0.66^{d}	0.33±0.33^{e}	2.67±0.33^{a}	5.33±0.33^{de}
	6 weeks	0.33±0.33^{bc}	0.33±0.33^{d}	1.67±0.67^{cd}	1.33±0.67^{bcde}	2.33±0.33^{a}	6.00±0.57^{de}
Thyme 1.5%	2 weeks	1.00±0.57^{abc}	1.33±0.67^{cd}	0.66±0.33 ^{d}	1..33±0.33^{bcde}	2.33±0.67^{a}	6.33±.1.67^{de}
	4 weeks	0.66±0.66^{abc}	1.67±0.67^{cd}	1.66±0.33^{cd}	1.00±0.57^{cde}	3.67±0.33^{a}	8.67±0.88^{de}
	6 weeks	1.00±0.57^{abc}	3.33 ± 0.33^{ab}	1.66±0.88^{cd}	2.33±0.33^{abc}	2.66±0.66^{a}	11.00±2.08^{bc}
Rosemary 0.5%	2 weeks	2.00±0.58^{abc}	2.33±0.33^{bc}	1.67±0.33 ^{cd}	1.33±0.33^{bcde}	4.00±1.00^{a}	11.33±1.45^{bc}
	4 weeks	2.33±1.20^{ab}	3.00±0.58^{b}	2.33±0.33^{bcd}	2.66±0.33^{ab}	4.33±0.88^{a}	12.67±2.02^{bc}
	6 weeks	2.67±0.66^{a}	2.67±0.33^{bc}	3.33±0.33^{abc}	2.33±0.33^{abc}	4.0±0.88^{a}	15.00±0.57^{ab}
Rosemary 1%	2 weeks	1.33±0.33^{abc}	3.33±0.88^{ab}	3.67±0.88 ^{ab}	1.67±0.67^{bcde}	2.66±0.33^{a}	12.67±0.88^{bc}
	4 weeks	1.33±.00^{abc}	2.66±0.33^{bc}	4.00±0.57^{ab}	2.00±0.57^{abcd}	3.33±0.88^{a}	13.33±1.20^{abc}
	6 weeks	1.66±0.33^{abc}	4.67±0.66^{a}	4.67±0.33^{a}	3.33±0.66^{a}	3.00±1.00^{a}	17.33±2.60^{a}

Data were expressed as mean ± S.E (N = 9). Mean values with different superscript letters in the same column are significantly different ($p < 0.05$).

Table 2. Rate of micronucleated erythrocytes (MN) and hepatic DNA fragmentation of *O. niloticus* fed control diet and supplemented diets with thyme and rosemary at different concentrations for 2, 4, and 6 weeks

Group	Feeding time	Micronucleus	DNA fragmentation
Control		2.00 ± 0.58^{e}	$9.46\pm0.\ 28^{g}$
Thyme 1%	2 weeks	2.33 ± 0.88^{de}	9.71 ± 0.58^{g}
	4 weeks	2.67 ± 0.88^{de}	10.0 ± 0.2^{g}
	6 weeks	2.67 ± 0.33^{de}	10.50 ± 0.63^{fg}
Thyme 1.5%	2 weeks	3.00 ± 0.58^{de}	11.50 ± 0.59^{ef}
	4 weeks	4.33 ± 0.33^{cde}	11.80 ± 0.50^{def}
	6 weeks	5.00 ± 1.00^{cd}	12.45 ± 0.18^{cde}
Rosemary 0.5%	2 weeks	5.00 ± 1.00^{cd}	12.90 ± 0.46^{cde}
	4 weeks	6.33 ± 0.88^{bc}	13.00 ± 0.32^{cde}
	6 weeks	8.67 ± 0.67^{ab}	13.20 ± 0.47^{cd}
Rosemary 1%	2 weeks	5.67 ± 0.88^{c}	13.80 ± 0.37^{bc}
	4 weeks	10.00 ± 1.15^{a}	15.0 ± 0.63^{b}
	6 weeks	10.67 ± 0.88^{a}	18.37 ± 0.58^{a}

Data were expressed as mean ± S.E (N = 9). Mean values with different superscript letters in the same column are significantly different ($p < 0.05$).

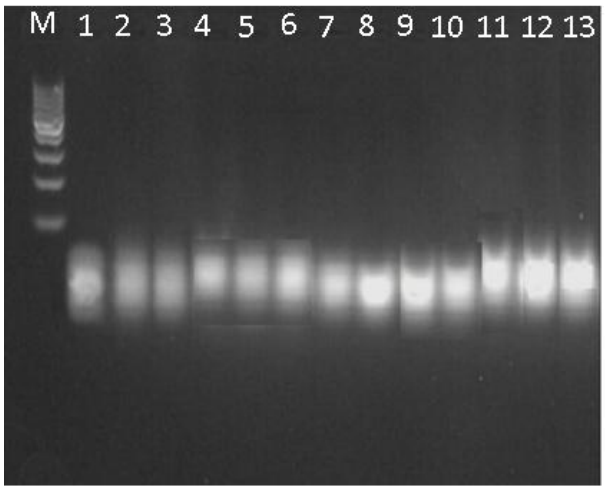

Fig. 1. Agarose gel electrophoresis of the extracted liver DNA showed no DNA damage from (L1- L4), slight massive DNA damage (L5-L7), and increased the density of the DNA damage (L8-L13). M: Marker (DNA ladder); L1 (Control), L2 (1% Thyme 2 weeks), L3 (1% Thyme 4 weeks), L4 (1% Thyme 6 weeks), L5 (1.5% Thyme 2 weeks), L6 (1.5% Thyme 4weeks), L7 (1.5% Thyme 6 weeks), L8 (0.5% Rosemary 2 weeks), L9 (0.5% Rosemary 4 weeks), L10 (0.5% Rosemary 6 weeks), L11 (1% Rosemary 2 weeks), L12 (1% Rosemary 4 weeks) and L13 (1% Rosemary 6 weeks).

Table 3. Hepatic enzymes activities in *O. niloticus* fed control diet and supplemented diets with thyme and rosemary at different concentrations for 2 and 6 weeks.

Group	Feeding time	AST (U/L)	ALP (U/L)	ALT (U/L)
Control	2 weeks	43.86±2.46^{bc}	144.74±2.24^{c}	22.38±1.8^{b}
	6 weeks	44.97±2.21^{bc}	147.74±1.51^{bc}	25.45±2.78^{b}
Thyme 1%	2 weeks	36.3±2.51^{d}	145.92±1.26^{c}	23.26±1.01^{b}
	6 weeks	42.1±3.42^{cd}	150.18±0.62^{bc}	24.52±0.87^{b}
Thyme 1.5%	2 weeks	43.02±2.17^{cd}	147.67±1.46^{bc}	25.56±1.01^{b}
	6 weeks	47.61±2.16^{bc}	151.82±1.95^{bc}	27.18±0.42^{b}
Rosemary 0.5%	2 weeks	44.57±2.5^{bc}	146.0±3.05^{c}	24.11±1.34^{b}
	6 weeks	50.96±1.52^{b}	147.15±3.75^{bc}	26.5±1.22^{b}
Rosemary 1%	2 weeks	47.46±1.48^{bc}	154.66±1.4^{b}	25.18±2.16^{b}
	6 weeks	59.28±1.89^{a}	204.63±3.37^{a}	38.86±1.41^{a}

Data were expressed as mean ± S.E (N = 3). Mean values with different superscript letters in the same column are significantly different ($p < 0.05$).

Table 4. Renal parameters (urea and creatinine) in *O. niloticus* fed control diet and supplemented diets with thyme and rosemary at different concentrations for 2 and 6 weeks

Group	Feeding time	Urea (mg/dl)	Creatinine (mg/dl)
Control	2 weeks	14.25±1.67^{b}	0.71±0.03^{bc}
	6 weeks	14.78±0.78^{ab}	0.76±0.02^{b}
Thyme 1%	2 weeks	14.08±1.07^{b}	0.55±0.02^{c}
	6 weeks	15.71±0.47^{ab}	0.63±0.08^{cd}
Thyme 1.5%	2 weeks	13.49±0.3^{b}	0.67±0.04^{bcd}
	6 weeks	14.62±0.78^{ab}	0.66±0.02^{bcd}
Rosemary 0.5%	2 weeks	14.86±0.73^{ab}	0.68±0.02^{bc}
	6 weeks	14.53±0.82^{ab}	0.66±0.05^{bcd}
Rosemary 1%	2 weeks	14.58±0.63^{ab}	0.73±0.02^{bc}
	6 weeks	17.07±1.01^{a}	0.9±0.02^{a}

Data were expressed as mean ± S.E (N = 3). Mean values with different superscript letters in the same column are significantly different ($p < 0.05$).

Table 5. Muscle damage biomarkers **(**creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) in *O. niloticus* fed control diet and supplemented diets with thyme and rosemary powder at different concentrations for 2 and 6 weeks

Groups	Feeding time	CPK (U/L)	LDH (U/L)
Control	2 weeks	163.0 ± 2.52^{c}	115.57 ± 2.46^{de}
	6 weeks	171.07 ± 0.52^{b}	121.82 ± 0.23^{bc}
Thyme 1%	2 weeks	158.33 ± 1.76^{cde}	111.89 ± 1.58^{d}
	6 weeks	161.31 ± 2.3^{cd}	118.12 ± 1.52^{cd}
Thyme 1.5%	2 weeks	153.67 ± 1.2^{ef}	112.67 ± 1.45^{de}
	6 weeks	159.0 ± 2.08^{cde}	122.75 ± 1.38^{bc}
Rosemary 0.5%	2 weeks	150.97 ± 2.05^{f}	110.18 ± 0.68^{d}
	6 weeks	155.7 ± 2.36^{def}	124.41 ± 1.39^{b}
Rosemary 1%	2 weeks	164.67 ± 1.72^{c}	115.3 ± 1.99^{de}
	6 weeks	179.17 ± 2.21^{a}	137.0 ± 3.61^{a}

Data were expressed as mean ± S.E (N= 3). Mean values with different superscript letters in the same column are significantly different ($p < 0.05$).

DISCUSSION

The use of cheap medicinal plants as a dietary additive improved the performance of fish and exhibited a protective effect against diseases **(Abd El-Gawad *et al*., 2020; Soror *et al*., 2021)**. However, there is insufficient knowledge regarding the safety dose and time duration for application of herbal plants in aquafeeds. Fishes are often used as suitable organisms for ecotoxicological studies since they play several roles in the trophic web, accumulation of toxic substances, and response to low concentration of mutagens **(Cavas & Ergene-Gozukara, 2005)**.

The present study revealed that the Nile tilapia fed 1% rosemary supplemented diet for 6 weeks exhibited behavioral and clinical signs, including lethargy, darkness with excessive mucus secretion, paled liver, and darkness of kidneys. The mortality rate was 18.66 % during the experiments. The recorded behavioral responses are consistent with those of several studies on different herbal plant in fish **(Ayotunde *et al*., 2011**; **Orji *et al*., 2014; Akinsanya *et al*., 2016)**.

The data available on genotoxic effects of medicinal plants in fish is limited. Thus, the assessment of cytotoxic and mutagenic potential of herbal plants that are toxic to fish is essential to ensure their safety use **(Singh *et al*., 2014)**. Micronuclei are cytoplasmic chromatin-containing bodies framed when acentric chromosome parts or chromosomes slack amid anaphase and neglect to end up noticeably joined into girl cell cores during cell division. Since genetic damage that culminates in chromosome breaks or spindle irregularities prompts micronucleus formation, the rate of MN fills in as a file of these sorts of abnormalities **(Ali *et al*., 2008**). The MN test detects both clastogenic and aneugenic effects **(Mersch & Beauvais, 1997**). Hence, it has been used as a biomarker of cytogenotoxicity **(Heddle *et al*., 1993)**.

The results of the present study demonstrate significant increases in the micronucleus, total chromosome aberrations, and DNA fragmentation at all sampling time in fish fed 0.5% and 1% rosemary. These results may be due to presence of phenolic compounds, which may cause cytotoxic and genotoxic damage **(Csepregi *et al*., 2020)** through inhibiting DNA polymerase that leads to an increase in sister chromatid exchange. Rosemary genetic damage has been recorded in the study of **Maistro *et al.* (2010)** who observed that 1000 and 2000 mg/kg of rosemary oil incites genotoxic and mutagenic impacts in mice. *R. officinalis* extracts increased rates of MN and chromosome aberrations on human peripheral lymphocytes cells **(Askin Celik & Aslanturk, 2018)**.

In the same way, *Jatropha gossypifolia* extract in freshwater fish *Channa punctatus* **(Singh *et al*., 2014)** and *Curcuma longa* extract in Zebra fish *Danio rerio* **(Alafiatayo *et al*., 2019)** induced embryotoxicity, teratogenic and genotoxic effects. The cytogenotoxic effects of the rosemary in the present study could be responsible for further embryotoxic impacts since chromosome breaks and deletions are imperative variables of premature birth in humans and animals **(Beckman & Brent, 1984)**.

In the current study, thyme dietary supplementation at a dose of 1.5% revealed a significant increase in DNA fragmentation during the experiment, which might be due to the presence of thymol and carvacrol. **Mohamed *et al*. (2009)** postulated that the highest concentration of thyme showed a significant effect on total chromosomal aberrations and pulverization in male albino rats. Chinese hamster lung fibroblast cells, treated with 25 µM thymol caused some clastogenic DNA damage **(Ündeğer *et al*., 2009)**. **Azirak and Rencuzogullari (2008)** reported that intraperitoneally injection of both carvacrol and thymol in bone marrow cells of rats significantly induced total chromosome abnormalities. Hepatotoxicity and genotoxicity of medicinal plants were reported in the works of **Kandarkar *et al*. (1998)**, **Antunes *et al*. (1999)** and **Soliman (2001)**. This indicates that the high dose of thyme or rosemary dietary supplementation for a long term could be genotoxic and cytotoxic, making it unsafe for consumption for a long period. **Qureshi *et al*. (2015)** revealed that thyme seed extract caused serious cytotoxicity, DNA damage, and oxidative stress on cells.

Hepatic enzymes (AST, ALT, and ALP) have been assessed in several studies as an index reflecting the health status of fish **(El-Rahman *et al*., 2019; Naiel *et al*. 2020; Soror *et al*., 2021)**. Furthermore, they have been used to evaluate the potential toxicity of supplemented diets **(Bhardwaj *et al*., 2010; Hoseini *et al*., 2018)**. AST and ALT activities are important in cellular nitrogen metabolism, amino acids oxidation, and liver gluconeogenesis **(Murray *et al*., 2003**). ALP is found in red blood cells at high concentration **(Gaudet *et al*., 1975)** and acts as a good bio- indicator of hemolysis **(Mirghaed *et al*., 2017)**. High serum level of liver enzymes is indicative of pathological changes in various tissues, especially necrosis, degeneration and leakage of hepatocellular membranes **(El-Moghazy *et al*., 2014; Soleimany *et al*., 2016)**. The findings of this study revealed no significant difference in all treatment groups fed thyme at both concentrations (1% and 1.5%) and 0.5% rosemary-supplemented diets. Similarly,

studies on the nettle *Urtica dioica* in juvenile beluga **(Binaii *et al*., 2014)**, onthe rosemary in the Nile tilapia (**Naiel *et al*., 2020)** and on ginger in the Nile tilapia **(Soror *et al*., 2021)** showed that serum liver enzymes were not influenced by dietary herbal plants.

Due to the anti-radical and antioxidant properties of thyme and rosemary, their supplementation might prevent lipid peroxidation of cell membranes inhibiting the release of liver enzymes into the blood. This indicates the protective role of thyme and rosemary, both of which did not induce liver toxicity, especially at low concentrations. This explanation was supported in the study of **Naiel *et al*. (2019)** who observed that, diet supplemented with 0.5% rosemary reduced the harmful effect of aflatoxin B1 toxicity on liver function indices in the Nile tilapia. In the present study, dietary supplementation of rosemary at dose of 1% significantly increased liver enzymes activities, and this may be due to the damage of hepatic cell membranes at high dose, evidenced by DNA damage of liver tissue in this treatment. **Ozovehe (2013)** reported significant increases in ALT, AST, and ALP enzymes in African catfish *Clarias gariepinus* juvenile fed varying levels of *Moringa oleifera* leaf meal diet for 8 weeks. In the same way, **Soleimany *et al*. (2016)** showed that marshmallow *Althaea officinalis* extract dietary supplementation in common carp *Cyprinus carpio* at a dose of 10 g for 45 days significantly increased AST and ALP activities.

The present study showed that dietary administration of thyme and rosemary did not affect serum urea and creatinine in *O. niloticus*. This result coincides with that of **Naiel *et al*. (2020)** who reported no significant difference in urea and creatinine levels of *O. niloticus* fed rosemary leaf powder for 60 days. In addition, **Yılmaz *et al*. (2012)** observed that thyme-and rosemary-supplemented diets did not affect the serum uric acid and creatinine levels in sea bass *Dicentrarchus labrax.*

CPK and LDH are considered as biomarkers for screening of heart and skeletal diseases in human and fish **(Apple *et al*. 2001; Yousaf & Powell, 2012)**. LDH is a cytoplasmic biomarker enzyme present in the cytoplasm of all cells and released upon cellular degeneration such as necrosis **(Murray *et al*., 2003)**. On the other hand, CPK is mainly found in the brain, heart, and skeletal muscles and its concentration is related to the irremediable myocardial necrosis in mammals **(Ishikawa *et al*., 1997)**. In the present study, serum CPK and LDH levels in groups fed diets supplemented with thyme at both concentrations (1% and 1.5%) and rosemary at a concentration of 0.5% recorded no significant increase throughout the experiment compared to the control. Concurrently, the long administration of rosemary powder at the dose of 1% for 6 weeks showed significant increase in CPK and LDH levels when compared to control and other treated groups. **Soleimany *et al*. (2016)** recorded that oral administration of high dose of the marshmallow extract in common carp significantly increased LDH activity but did not alter CPK activity. The increased activity of CPK with high dose of rosemary dietary supplementation for a long term feeding (6 weeks) in the juvenile Nile tilapia indicates a disorder in muscle fibers.

CONCLUSION

It could be concluded that dietary inclusion of thyme or rosemary powder at doses of 1.5% and 1%, respectively, for 6 weeks could induce few cytogenotoxic changes. However, oral administration of 1% thyme for 6 weeks could be applied safely for the juvenile Nile tilapia. Thus, it is advisable that toxicological and genotoxic assays of biological active substances of medicinal plants should be performed to ascertain their safety in aquaculture application.

REFERENCES

Abd El-Gawad, E.A.; El Asely, A.M.; Soror, E.I.; Abbass, A.A. and Austin, B. (2020). Effect of dietary *Moringa oleifera* leaf on the immune response and control of *Aeromonas hydrophila* infection in Nile tilapia (*Oreochromis niloticus*) fry. Aquaculture International, 28(1): 389–402. DOI: https://doi.org/10.1007/s10499-019-00469-0

Abd El-Naby, A.S.A.; Al-Sagheer, A.A.; Negm, S.S. and Naiel, M.A. (2020). Dietary combination of chitosan nanoparticle and thymol affects feed utilization, digestive enzymes, antioxidant status, and intestinal morphology of *Oreochromis niloticus*. Aquaculture, 515: 734577.DOI: https://doi.org/10.1016/j.aquaculture.2019.734577

Aguilar-Santamaría, L.; Herrera-Arellano, A.; Zamilpa, A.; Alonso Cortés, D.; Jiménez-Ferrer, E.; Tortoriello, J. and Zúñiga-González, G. (2013). Toxicology, genotoxicity, and cytotoxicity of three extracts of Solanum chrysotrichum. J. Ethnopharmacol, 150 (1): 275-279. DOI: https://doi.org/10.1016/j.jep.2013.08.039

Akinsanya, B.; Utoh, O.U. and Ukwa, U.D. (2016). Toxicological, phytochemical and anthelminthic properties of rich plant extracts on *Clarias gariepinus*. The Journal of Basic & Applied Zoology, 74:75-86. DOI: https://doi.org/10.1016/j.jobaz.2016.09.003

Alafiatayo, A.A.; Lai, K.S.; Syahida, A.; Mahmood, M. and Shaharuddin, N.A. (2019). Phytochemical evaluation, embryotoxicity, andteratogenic effects of *Curcuma longa* extract on Zebrafish (*Danio rerio*). Evidence-Based Complementary and Alternative Medicine, 2019: 1-10. DOI: https://doi.org/10.1155/2019/3807207

Al-Anazi, M.S.; Virk, P.; Elobeid, M. and Siddiqui, M.I. (2015). Ameliorative effects of *Rosmarinus officinalis* leaf extract and vitamin C on cadmium-induced oxidative stress in Nile tilapia *Oreochromis niloticus*. Journal of Environmental Biology, 36: 1401-1408.

Ali, F.; El-Shehawi, A.M. and Seehy, M.A. (2008). Micronucleus test in fish genome: A sensitive monitor for aquatic pollution. African journal of biotechnology, 7(5):606-612.

Al-Sabti, K. (1986). Clastogenic effects of five carcinogenic-mutagenic chemicals on the cells of the common carp, *Cyprinus carpio* L. Comparative biochemistry and

physiology. C, Comparative pharmacology and toxicology, 85(1): 5-9. DOI: https://doi.org/10.1016/0742-8413(86)90043-5

Apple, F.S.; Murakami, M.; Panteghini, M.; Christenson, R.H.; Dati, F.; Mair, J. and Wu, A.H. (2001). International survey on the use of cardiac markers. Clinical Chemistry, 47 (3): 587–8.

Araújo, M.C.P.; Dias, F.L. and Takahashi, C.S. (1999) Potentiation by turmeric and curcumin of radiation induced chromosome aberration in Chinese hamster ovary cells. Teratogenesis, Carcinogenesis, and Mutagen, 19:9–18.

Askin Çelik, T. and Aslantürk, Ö.S. (2018). Cytotoxic and Genotoxic Effects of Aqueous Extracts of *Rosmarinus officinalis* L., *Lavandula stoechas* L. and *Tilia cordata Mill*. on *in vitro* Human Peripheral Blood Lymphocytes. Cumhuriyet Science Journal, 39(1):127-143.

Austin, B. and Austin, D.A. (1989). Method for microbiological examination of fish and shellfish. Ellis Horwood, Chichester, pp 317–327.

Austin, B. and Austin, D.A. (2007). Bacterial fish pathogens, Disease of farmed and wild fish, 4th Ed. Springer Praxis, Godalming.

Ayotunde, E.O.; Offem, E.O. and Bakeh, A.F. (2011). Toxicity of *Carica papaya* Linn: haematological and piscicidal effect on adult catfish (*Clarias gariepinus*). Journal of Fisheries and Aquatic Science, 6:291-308. DOI: 10.3923/jfas.2011.291.308

Azirak, S. and Rencuzogullari, E. (2008). The In Vivo genotoxic effects of carvacrol and thymol in rat bone marrow Cells. Environmental Toxicology, 23(6):728-35. DOI: https://doi.org/10.1002/tox.20380

Bais, R. and Philcox, M. (1994). IFCC methods for the measurement of catalytic concentration of enzymes. Part 8. IFCC method for lactate dehydrogenase (L-lactate: NAD+ oxidoreductase, EC 1.1.1.27). Journal of Automatic Chemistry, 16(5): 167–182. DOI: 10.1155/S1463924694000210

Basha, S.A.; Abd El-Gawad, E.A.; Abd EL Latif, A.A. and Abbass, A.A. (2018). Dietary effect of fenugreek (*Trigonella foenum graecum*) and liquorice (*Glycyrrhiza glabra*) on growth performance, antioxidant activities and stress resistance in *Oreochromis niloticus*. Benha Veterinary Medical Journal, 34(3): 355-370

Beckman, D.A. and Brent, R.L. (1984). Mechanisms of teratogenesis. Annual Review of Pharmacology and Toxicology, 24: 483-500. DOI: https://doi.org/10.1146/annurev.pa.24.040184.002411

Bhardwaj, S.; Srivastava, M.K.; Kapoor, U. and Srivastava, L.P. (2010). A 90 days oral toxicity of imidacloprid in female rats: morphological, biochemical and histopathological evaluations. Food and Chemical Toxicology, 48: 1185-1190. DOI: https://doi.org/10.1016/j.fct.2010.02.009

Bilen, S.; Kenanoglu, O.N.; Terzi, E.; Ozdemir, R.C. and Sonmez, A.Y. (2019). Effects of tetra (*Cotinus coggygria*) and common mallow (*Malva sylvestris*) plant extracts on growth performance and immune response in Gilthead Sea bream (*Sparus*

aurata) and European Sea bass (*Dicentrarchus labrax*). Aquaculture, 512: 734251. DOI: https://doi.org/10.1016/j.aquaculture 2019.734251

Binaii, M.; Ghiasi, M.; Farabi, S.M.V.; Pourgholam, R.; Fazli, H.; Safari, R.; Alavi, S.E.; Taghavi, M.J. and Bankehsaz, Z. (2014). Biochemical and hemato-immunological parameters in juvenile beluga (*Huso huso*) following the diet supplemented with nettle (*Urtica dioica*). Fish & Shellfish Immunology, 36: 46-51.DOI: 10.1016/j.fsi.2013.10.001

Çavaş, T. and Ergene-Gözükara, S. (2005). Induction of micronuclei and nuclear abnormalities in *Oreochromis niloticus* following exposure to petroleum refinery and chromium processing plant effluents. Aquatic Toxicology, 74(3): 264-271. DOI: https://doi.org/10.1016/j.aquatox.2005.06.001

Charles, D.J. (2012). Antioxidant Properties of Spices, Herbs and Other Sources. Springer Science & Business Media.

Csepregi, R.; Temesfői, V.; Das, S.; Alberti, Á.; Anna Tóth, C.; Herczeg, R.; Papp, N. and Kőszegi, T. (2020). Cytotoxic, Antimicrobial, Antioxidant Properties and Effects on Cell Migration of Phenolic Compounds of Selected Transylvanian Medicinal Plants. Antioxidants, 9(2), 166. DOI: 10.3390/antiox9020166

Dauqan, E.M.A. and Abdullah, A. (2017). Medicinal and functional *values of thyme (Thymus vulgaris L.)* herb. Journal of Applied Biology and Biotechnology, 5(2): 17-22. DOI: 10.7324/JABB.2017.50203

De Flora, S.; Vigano, L.; D'agostini, F.; Camoirano, A.; Bagnasco, M.; Bennicelli, C.; Melodia, F. and Arillo, A. (1993). Multiple genotoxicity biomarkers in fish exposed in situ to polluted river water. Mutation Research, 319(3): 167-177. DOI: 10.1016/0165-1218(93)90076-p.

De Marco, A.; Romanelli, M.; Stazi, M. A. and Vitagliano, E. (1986). Induction of micronucleated cells in *Vicia faba* and *Allium cepa* root tips treated with nitrilotriacetic acid (NTA). Mutation Research/Genetic Toxicology, 171(2-3): 145-148.

El-Moghazy, M.; Zedan, N.S.; El-Atrsh, A.M.; El-Gogary, M. and Tousson, E. (2014). The possible effect of diets containing fish oil (omega-3) on hematological, biochemical and histopathogical alterations of rabbit liver and kidney. Biomedicine and Preventive Nutrition, 4: 371–377. DOI: https://doi.org/10.1016/j.bionut.2014.03.005

El-Rahman, G.I.A.; Ahmed, S.A.; Khalil, A.A. and Abd-Elhakim, Y.M. (2019). Assessment of hematological, hepato-renal, antioxidant, and hormonal responses of *Clarias gariepinus* exposed to sub-lethal concentrations of oxyfluorfen. Aquatic Toxicology, 217: 105329. DOI: https://doi.org/10.1016/j.aquatox.2019.105329

Erkan, N.; Ayranci, G. and Ayranci, E. (2008). Antioxidant activities of rosemary (*Rosmarinus Officinalis L.*) extract, blackseed (*Nigella sativa L.*) essential oil, carnosic acid, rosmarinic acid and sesamol. Food chemistry, 110(1): 76-82. DOI: https://doi.org/10.1016/j.foodchem.2008.01.058

Gaiani, T.F.; Carvalho, J.C.T.; Da Silva, J.M.S.F. and Maistro, E.L. (2006). Absence of Clastogenic Effects of the Extract from Medicinal Plant *Rosmarinus officinalis* L. on Wistar Rat Bone Marrow Cells. Cytologia-Tokyo, 71:101–106. DOI: https://doi.org/10.1508/cytologia.71.101

García Beltrán, J.M. González Silvera, D.; Espinosa Ruiza, C.; Campo, V.; Chupani, L.; Faggio, C. and Esteban, M.A. (2020). Effects of dietary *Origanum vulgare* on gilthead seabream (*Sparus aurata* L.) immune and antioxidant status. Fish & Shellfish Immunology, 99: 452–461. DOI: https://doi.org/10.1016/j.fsi.2020.02.040

Gaudet, M.; Racicot, J.G. and Leray, C. (1975). Enzyme activities of plasma and selected tissues in rainbow trout *Salmo gairdneri* Richardson. Journal of Fish Biology, 7: 505–512. DOI: https://doi.org/10.1111/j.1095-8649.1975.tb04625.x

Giannenas, I.; Triantafillou, E.; Stavrakakis, S.; Margaroni, M.; Mavridis, S.; Steiner, T.; Karagouni, E. (2012). Assessment of dietary supplementation with carvacrol or thymol containing feed additives on performance, intestinal microbiota and antioxidant status of rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 350-353: 26-32. DOI: https://doi.org/10.1016/j.aquaculture.2012.04.027

Gibb, R.K.; Taylor, D.D.; Wan, T.; O'Connor, D.M.; Doering, D.L. and Gerçel-Taylor, Ç. (1997). Apoptosis as a measure of chemosensitivity to cisplatin and taxol therapy in ovarian cancer cell lines. Gynecologic oncology, 65(1): 13-22. DOI: 10.1006/gyno.1997.4637.

Hassan, A.A.M.; Yacout, M.H.; Khalel, M.S.; Hafsa, S.H.A.; Ibrahim, M.A.R.; Mocuta, D.N.; Rahoveanu, A.T. and Dediu, L. (2018). Effects of some herbal plant supplements on growth performance and the immune response in Nile Tilapia (*Oreochromis Niloticus*). "Agriculture for Life, Life for Agriculture" Conference Proceeding, 1: 134–141. DOI: https://doi.org/10.2478/alife-2018-0020

Heddle, J.A.; Hite, M.; Kirkhart, B.; Mavournin, K.; MacGregor, J.T.; Newell, G.W. and Salamone, M.F. (1993). The induction of micronuclei as a measure of genotoxicity: A report of the U.S. environmental protection agency Gene-Tox program. Mutation Research, 123: 61–118. DOI: https://doi.org/10.1016/0165-1110(83)90047-7

Henry, R.J. (1964). Clinical Chemistry, Principles and Techniques. Hoeber Medical, Harper-Row Publish, New York, pp 190.

Horn, R.C. and Vargas, V.M.F. (2008) Mutagenicity and antimutagenicity of teas used in popular medicine in the Salmonella/microsome assay. Toxicology in Vitro, 22: 1043-1049. DOI: 10.1016/j.tiv.2007.12.014

Hoseini, S.M.; Mirghaed, A.T.; Iri, Y. and Ghelichpour, M. (2018). Effects of dietary cineole administration on growth performance, hematological and biochemical parameters of rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 495: 766–772. DOI: 10.1016/j.aquaculture.2018.06.073

Hoseini, S.M. and Yousefi, M. (2019). Beneficial effects of thyme (*Thymus vulgaris*) extract on oxytetracycline- induced stress response, immunosuppression,

oxidative stress and enzymatic changes in rainbow trout (*Oncorhynchus mykiss*) Aquaculture Nutrition, 25 (2): 298-309. DOI: https://doi.org/10.1111/anu.12853

Ishikawa, Y.; Saffitz, J.E.; Mealman, T.L.; Grace, A.M. and Roberts, R. (1997). Reversible myocardial ischemic injury is not associated with increased creatine kinase activity in plasma. Clinical Chemistry, 43 (3):467–475.

Kandarkar, S.V.; Sawant, S.S.; Ingle, A.D.; Deshpande, S.S. and Maru, G.B. (1998). Sub chronic oral hepatotoxicity of turmeric in mice histopathological and ultrastructural studies. Indian Journal of Experimental Biology, 36:675–679.

Khalil, S.R.; AbdElhakim, Y.; Abd El-fattah, A.H.; Farag, M.R.; Abd El-Hameed, N.E. and EL-Murr, A.E. (2020). Dual immunological and oxidative responses in *Oreochromis niloticus* fish exposed to lambda cyhalothrin and concurrently fed with Thyme powder (*Thymus vulgaris L*.): Stress and immune encoding gene expression. Fish & Shellfish Immunology, 100 : 208-218. DOI: 10.1016/j.fsi.2020.03.009

Kind, P.R.N. and King, E.J. (1954). Estimation of plasma phosphatase by determination of hydrolysed phenol with amino-antipyrine. Journal of Clinical Pathology, 7:322–326. DOI: 10.1136/jcp.7.4.322

Larrán, S.; Ringuelet, J.A.; Carranza, M.R.; Henning, C.P.; Ré, M.S.; Cerimele, E.L. and Urrutía, M.I. (2001). In vitro fungistatic effect of essential oils against *Ascosphaera apis*. Journal of essential oil research, 13(2): 122-124. DOI: 10.1080/10412905.2001.9699633

Lee, M.R. and Elder, F.F.B. (1980). Yeast stimulation of bone marrow mitosis for cytogenetic investigations. Cytogenetics and Cell Genetics, 26: 36–40. DOI: 10.1159/000131419

Maistro, E.L.; Mota, S.F.; Lima, E.B.; Bernardes, B.M. and Goulart, F.C. (2010). Genotoxicity and mutagenicity of *Rosmarinus officinalis* (Labiatae) essential oil in mammalian cells in vivo. Genetics and Molecular Research, 9(4): 2113-2122. DOI: 10.4238/vol9-4gmr857

Manju, M.; Vijayasree, A.S.; Akbarsha, M.A. and Oommen, O.V. (2013). Protective effect of dietary curcumin in *Anabas testudineus* (Bloch) with a special note on DNA fragmentation assay on hepatocytes and micronucleus assay on erythrocytes in vivo. Fish Physiology Biochemistry, 39:1323–1330

Marchese, A.; Orhan, I.E.; Daglia, M.; Barbieri, R.; Di Lorenzo, A.; Nabavi, S.F.; Gortzi, O.; Izadi, M. and Nabavi, S.M. (2016). Antibacterial and antifungal activities of thymol: A brief review of the literature. Food Chemistry, 210: 402–414. DOI: 10.1016/j.foodchem.2016.04.111.

Mersch, J. and Beauvais, M.N. (1997). The micronucleus assay in freshwater mussel, *Dreissena polymorpha* to in situ monitor genotoxicity in freshwater environments. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 393:141–149. DOI: https://doi.org/10.1016/S13835718(97)00099-5

Migliore, L.; Barale, R.; Bosco, E.; Giorgelli, F.; Minunni, M.; Scarpato, R. and Loprieno, N. (1990). Genotoxicity of methylglyoxal: cytogenetic damage in

human lymphocytes in vitro and in intestinal cells of mice. Carcinogenesis, 11(9): 1503-1507.

Mirghaed, T.A.; Ghelichpour, M.; Hoseini, S.M. and Amini, K. (2017). Hemolysis interference in measuring fish plasma biochemical indicators. Fish Physiology and Biochemistry ,34: 1143–1151. DOI 10.1007/s10695-017-0359-y

Mohammed, B.M.; Karim, K.J. And Yaseen, N.Y. (2009). Anti mutagenic effects of *Thymus syriacus* extract against the genotoxicity of gemcitabine in male albino mice. The 2nd Kurdistan Conference on Biological Sciences Journal of Duhok University, 12 (1): 216-226.

Murray, R.K.; Granner, D.K.; Mayes, P.A. and Rodwell, V.W. (2003). Harper's illustrated biochemistry. 26th Edition. McGraw-Hill, Medical Publishing Division

Naiel, M.A.E.; Ismael, N.E.M.; Negm, S.S.; Ayyat, M.S. and Al-Sagheer, A.A. (2020). Rosemary leaf powder–supplemented diet enhances performance, antioxidant properties, immune status, and resistance against bacterial diseases in Nile Tilapia (*Oreochromis niloticus*). Aquaculture, 526: 735370. DOI: https://doi.org/10.1016/j.aquaculture.2020.735370

Naiel, M.A.E.; Ismael, N.E.M. and Shehata, S.A. (2019). Ameliorative effect of diets supplemented with rosemary (*Rosmarinus officinalis*) on aflatoxin B1 toxicity in terms of the performance, liver histopathology, immunity and antioxidant activity of Nile Tilapia (*Oreochromis niloticus*). Aquaculture, 511: 734264.DOI: https://doi.org/10.1016/j.aquaculture.2019.734264

Oliveira, A.M.; Nascimento, M.F.; Ferreira, M.R.A.; Moura, D.F.; Souza, T.G.S.; Silva, G.C.; Ramos, E.H.S.; Paiva, P.M.G.; Medeiros, P.L.; Silva, T.G.; Soares, L.A.L., Chagas, C.A. Souza, I.A. and Napoleão, T.H. (2016). Evaluation of acute toxicity, genotoxicity and inhibitory effect on acute inflammation of an ethanol extract of *Morus alba* L. (Moraceae) in mice. Journal of Ethnopharmacology, 194: 162-168. DOI: 10.1016/j.jep.2016.09.004

Orji, O.U.; Ibiam, U.A. and Aja, P.M. (2014). Acute toxicity studies of the lyophilized aqueous extract of *Psychotria microphylla* leaf on *Clarias gariepinus* juveniles. International Journal of Biology and Biological Sciences 3 (4): 38-44.

Ozovehe, B.N. (2013). Growth Performance, Haematological indices and jome biochemical enzymes of juveniles *Clarias gariepinus* (Burchell 1822) fed varying levels of *Moringa oleifera* leaf meal diet. Journal of Aquaculture Research and Development, 4:166. DOI: 10.4172/2155-9546.1000166

Qureshi, S.T.; Chandio, P.; Noman, A.; Parveen, A. and Soomro, Y. (2015). Cytotoxic and Genotoxic and Oxidative Effects of Aqueous Extracts of Some Frequently Used Medicinal Plants in Pakistan. Botany Research International, 8 (1): 29-35. DOI: 10.5829/idosi.bri.2015.8.1.516

Reitman, S. and Frankel, S. (1957). Colorimetric determination of serum transaminases. American Journal of clinical Pathology, 28:56–63. DOI: 10.1093/ajcp/28.1.56

Singh, P.; Dabas, A.; Srivastava, R.; Nagpure, N.S. and Singh, A. (2014). Evaluation of Genotoxicity Induced by Medicinal Plant *Jatropha gossypifolia* in Freshwater Fish *Channa punctatus* (Bloch). Turkish Journal of Fisheries and Aquatic Sciences, 14: 421-428. DOI: 10.4194/1303-2712-v14_2_12

Soleimany, V.; Banaee, M.; Mohiseni, M.; Nematdoost Hagi, B. and Mousavi Dehmourdi, L. (2016). Evaluation of pre-clinical safety and toxicology of Althaea officinalis extracts as naturopathic medicine for common carp (*Cyprinus carpio*). Iranian journal of Fisheries Sciences, 15(2): 613-629.

Soliman, M.I. (2001) Genotoxicity Testing of Neem Plant (*Azadirachta indica* A. Juss.) using the *Allium cepa* chromosomes aberration assay. Journal of Biological Sciences 1(11): 1021-1027. DOI: 10.3923/jbs.2001.1021.1027

Soror, E.I.; El Asely, A.M.; Abdel-Gawad, E.A.; Radwan, H.A. and Abbass, A.A. (2021). Recuperative effects of honeybee pollen, ginger (*Zingiber officinale*), and *Moringa oleifera* in Nile tilapia (*Oreochromis niloticus* L.) after sub-lethal exposure to dimethoate. Aquaculture, 530: 735886. https://doi.org/10.1016/j.aquaculture.2020.735886

Surzycki, S. (2000). In Basic techinques in molecular biology preparation of genomic DNA from animal cells. Springer Verlag Publications, Berlin- Heidelberg., pp. 40-44

Szasz,G.; Gruber, W. and Bernt, E. (1976). Creatine kinase in serum: 1. determination of optimum reaction conditions. Clinical Chemistry, 22: 650–656.

Tasa, H.; Imani, A.; Moghanlou, K.S.; Nazdar, N. and Moradi-Ozarlou, M. (2020). Aflatoxicosis in fingerling common carp (*Cyprinus carpio*) and protective effect of rosemary and thyme powder: Growth performance and digestive status. Aquaculture, 527: 735437. DOI:https://doi.org/10.1016/j.aquaculture.2020.735437

Thomas, L. (1992). Labor und Diagnose. 4th edn. Medizinische Verlagsgesellschaft, Marburg

Ündeğer, Ü.; Başaran, A.; Degen, G.H. and Başaran, N. (2009). Antioxidant activities of major thyme ingredients and lack of (oxidative) DNA damage in V79 Chinese hamster lung fibroblast cells at low levels of carvacrol and thymol. Food and Chemical Toxicology, 47 (8): 2037-2043. DOI: https://doi.org/10.1016/j.fct.2009.05.020

Urbina-Cano, P.; Morales, B.L.; Herrera, M.A.; Rivera, J.R.; Magna, M.L.; Sanroman, R. and Rivera, A. (2006). DNA damage in mouse lymphocytes exposed to curcumin and copper. J Appl Genet 7:377–382

Yassen, D.A. (2017). Control of motile aeromonas septicemia using some herbal plants in cultured *Oreochromis niloticus*. M.Vsc thesis (Fish Diseases and Management). Fac.Vet.Med. Benha University.

Yassen, D.A.; Abd El Gawad, E.A. and Abbass, A.A. (2017). Effect of herbal plants *Thymus vulgaries* and *Rosmarinus officinale* on growth performance and protection of *Oreochromis niloticus* against *Aeromonas hydrophila* infection. Benha Journal of Applied Sciences, (2): 9-17.

Yılmaz, S.; Ergün, S. and Celik, E.S. (2012). Effects of herbal supplements on growth performance of sea bass (*Dicentrarchus labrax*): change in body composition and some blood parameters. Journal of BioScience and Biotechology, 1(3): 217-222.

Yousaf, M.N. and Powell, M.D. (2012). The Effects of heart and skeletal muscle inflammation and cardiomyopathy syndrome on creatine kinase and lactate dehydrogenase levels in Atlantic salmon (*Salmo salar* L.) The Scientific World Journal 2012:741302. DOI: 10.1100/2012/741302

Yousefi, M.; Shabunin, S.V.; Vatnikov, Y.A.; Kulikov, E.V.; Adineh, H.; Hamidi, M.K. and Hoseini, S.M. (2020). Effects of lavender (*Lavandula angustifolia*) extract inclusion in diet on growth performance, innate immunity, immune-related gene expression, and stress response of common carp, *Cyprinus carpio*. Aquaculture, 515: 734588. DOI: https://doi.org/10.1016/j.aquaculture.2019.734588

Zahran, E., Abd El-Gawad, E.A. and Risha, E. (2018). Dietary *Withania sominefera* root confers protective and immunotherapeutic effects against *Aeromonas hydrophila* infection in Nile tilapia (*Oreochromis niloticus*). Fish & Shellfish Immunology, 80:641–650. DOI: 10.1016/j.fsi.2018.06.009

Zargar, A.; Rahimi-Afzal, Z.; Soltani, E.; Mirghaed, A.T.; Ebrahimzadeh-Mousavi, H.A.; Soltani, M. and Yuosefi, P. (2019). Growth performance, immune response and disease resistance of rainbow trout (*Oncorhynchus mykiss*) fed *Thymus vulgaris* essential oils. Aquaculture Research, 50: 3097–3106. DOI: https://doi. org/ 10.1111/are.14243