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Physiological Responses and Histopathological Indices against Acrylamide Toxicity Treated with Metformin and Propolis in *Clarias gariepinus*

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ABSTRACT

Acrylamide (A_A), a substance extensively used in business, medical research, and waste management facilities, is thought to cause cancer in people. The *Clarias gariepinus* vertebrate fish model was used in the current investigation to assess the stress biomarkers, hematologic, and histopathological indices following exposure to (A_A). Two hundred healthy fish of *C. gariepinus* of both sexes wereclassified into four groups: the first is the negative control (CO) (without acrylamide); the second is the positive control (CA) (treated with acrylamide only) using 15mg/ l concentrations of (A_A); the third is (AM) which contains (A_A) plus metformin (Me_{Fo}) applied to treatment with (A_A) at (15mg/ l) and (Me_{Fo}) at 3g/ l, and the fourth is (AP) which contains (A_A) plus propolis (P_{Po}) applied to the treatment with (A_A) at (15mg/ l) and (P_{Po}) at 3g/ l. The hematological parameters, cortisol, glucose, and lactate dehydrogenase (LDH) were analyzed. The current data showed a significant increase in white blood cells, hemoglobin, hematocrit, cortisol, glucose, and LDH in (CA) groups when compared with (CO) group. A histopathological analysis of the kidney, spleen, and liver was performed. The groups (CA), (AM), and (AP) had noticeable structural changes in the kidney, spleen, and liver in the form of congestion, apoptosis, and vacuolations compared to the (CO) group. Administration of (A_A) resulted in serious liver, spleen, and kidney damage for *C. gariepinus* catfish. However, (P_{Po}) and (Me_{Fo}) exhibited anti-toxicity and improved kidney, spleen, and liver functions. The findings of the current study demonstrated that extensive and regular usage of (A_A) could endanger the existence and welfare of organisms, particularly fish, in aquatic environments.

INTRODUCTION

Acrylamide (A_A), formed when any carbohydrate-rich food products are cooked at high temperatures, and found in roasted coffee, has become one of the major public health issues. There have been several health risks associated with (A_A) exposure in both

animal and human models **(Ali *et al*., 2020)**. (A_A) can easily pollute aquatic systems by releasing tinctures, organic compounds, insecticides, and plastics during industrial operations **(Stadler *et al*., 2002)**. Despite considerable surface and ground contamination hazards, (A_A) cannot degrade quickly in water due to its high solubility and mobility in water systems. **Stadler *et al*. (2002)** and **Xu *et al*. (2014)** observed residues in the range of 0.5 and 600mg/ L in the flocculants used in the water treatment processes. Despite the significant usage and solubility of (A_A), which poses a risk of pollution in water systems, little is known about non-target organisms in aquatic environments **(Karthikeyan *et al*., 2022)**. Numerous studies revealed that (A_A) can cause carcinogenicity, genotoxicity, hepatotoxicity, and reproductive toxicity in animal models **(Rydberg *et al*., 2005; Matoso *et al*., 2019)**. (A_A) results in vacuolations in the cytoplasm of hepatocytes, apoptosis, and congestion of the central vein, which are signs of degenerative alterations in the liver. Additionally, it results in interstitial bleeding with the glomeruli and tubules of the kidneys deteriorating **(Ali *et al*., 2020)**. Propolis (P_{Po}) is a naturally occurring, viscous substance that honeybees gather from a variety of plants, including poplar, palm, and pine trees, as well as gums, resins, mucilage, and leaf buds. It is also considered as a balsamic, resinous product that bees make **(Bankova, 2005; Farag *et al*., 2021)**. (P_{Po}) is mostly made up of 40– 70% flavonoids and phenolic acids, 20– 35% waxes, 1- 3% essential oils, and 5% additional materials (the majority of which come from pollen or are supplied by bees) such as proteins, minerals, and polysaccharides. **(Santos *et al*., 2019)**. It functions as an antiseptic to protect the beehive from microbial illnesses and stop intruders from decomposing. Additionally, for millennia, (P_{Po}) has been utilized for therapeutic purposes in folk medicine **(Melliou *et al*., 2007; Yonar *et al*., 2011; Baltas *et al*., 2016)**. The active ingredients in (P_{Po}) have antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, anti-tumor, immunomodulatory, hepatoprotective, antidiabetic, antiherbicide and medicinal characteristic **(Abd-El-Rhman, 2009; Zhang *et al*., 2016; Anjum *et al*., 2019)**. One of the most used medications for the treatment of type 2 diabetes, metformin (Me_{Fo}), a derivative of biguanide, has been around for almost a century. (Me_{Fo}) has demonstrated efficacy in treating a variety of disorders, whether used as a single treatment or in combination with other medications **(Bailey, 2017; Blonde *et al*., 2018)**. Recent researches revealed that (Me_{Fo}) has a significant impact on several cardiovascular disease **(Lamanna *et al*., 2011)**, neurodegenerative diseases **(Patrone *et al*., 2014)**, liver diseases **(Bhat *et al*., 2015)**, malignancies' diseases **(Morales & Morris, 2015; Papanagnou *et al*., 2016)**, obesity **(Breining *et al*., 2018)**, and renal diseases **(Neven *et al*., 2018)**. Certain hematological parameters, including the number of RBCs ($\times 10^6$/ mm^3), Hb (g/ dl), hematocrit (Hct) percent, MCV (fl), MCH (pg), and WBCs ($\times 10^3$/ mm^3) revealed significant changes in the last parameters **(Alfons *et al*., 2022)**. Therefore, the objective of this research was to display harmful consequences of (A_A) in *C. gariepinus* catfish, examining its impact on the hematological parameters and the stress indicators, as well as investigating the histopathological effects on the liver, spleen, and kidney, and assessing the protective role of (P_{Po}) and (Me_{Fo}).

MATERIALS AND METHODS

1. Fish samples

Two hundred healthy fish of *C. gariepinus* of both sexes were classified into four groups with a mean total weight of 150- 200± 0.5g, and a mean total length of 28- 35± 0.5cm. The tested catfish were collected alive from a commercial fish farm which does not have any contamination history. Between 7:30 and 9:00 am, the fish were caught by seining and taken right away to the lab. The fish were held in a five round plastic tanks (width of 50cm, and height of 80cm). They were kept for 14 days in these tanks to acclimatize to laboratory conditions. The fish were fed floating pellets (commercial fish feed from Aller Aqua) containing 45% crude protein twice daily at 3% of their body weight. To prevent the buildup of harmful metabolites and rotting food, the water (dechlorinated tap) in the tanks was changed once every other day, and aired continuously with a blower. The water temperature was adjusted at 18- 22°C throughout the period of the experiment using a thermostatic heater.

2. Experimental design

2.1. Toxicity experiment

Ten fish of *C. gariepinus* were held in a non-recycled plastic tank with dimensions of 56cm x 33cm x 25cm, containing 30 liters of dechlorinated tap water for 14 days. Two experiments were conducted in three replicates: the first was the negative control (CO) (without acrylamide), and the second was positive control (CA) using 15mg/ l concentrations of acrylamide (A_A) for each aquarium. The fish were fed at 3% of body weight daily with a 50% water replacement every other day. Equal quantities were fed to the fish twice daily. The American Public Health Association's recommendations were followed to preserve the criteria governing water quality **(APHA, 1998)**.

2.2. Treated experiment

These experiments were designed in the same way as the toxicity experiment where two experiments were conducted to treat the toxicity of (A_A): the first (AM) using metformin (Me_{Fo}) with a 3g/ l concentration, and the second (AP) using propolis (P_{Po}) with a 3g/ l concentration for each aquarium.

3. Collection of blood

Following the experiment period, fish were sedated using a solution of clove powder (200mg L^{-1}), following the method of **Hedayati and Jahanbakhshi (2012)**, and two sets of three blood samples each were taken by puncturing the caudal blood vein. For immediate hematological investigation, the first set was emptied into tubes coated with EDTA. Another blood set was obtained in anticoagulant-free sterile tubes, and the serum was separated and centrifuged for 10min., then stored at -20°C, and later utilized to analyze the stress parameters.

4. Histopathological work up

Fixation and tissue processing: fish liver, kidney, and splenic tissue samples that were previousuly stored in formalin were processed using an automated tissue processor. Dehydration and a two-step initial fixation made up the procedure. Fixation involves immersing the tissue for 48 hours in 10 percent buffered formalin, followed by 30 minutes of removing the fixative with distilled water. The tissues were then put through a graded sequence of alcohol to dehydrate them (70, 90, and 100 percent). The tissue was first subjected to 70% alcohol for 120 minutes, then to 90% alcohol for 90 minutes, and finally to two cycles of 100% alcohol, each lasting one hour. The samples were then cleared in numerous changes of xylene after dehydration. It involved immersing tissue for an hour in a combination of 50% xylene and 50% alcohol, then for an additional 1.5 hours in pure xylene. The samples were then imbedded and blocked out after being impregnated with molten paraffin wax. Hematoxylin and eosin were used to stain the paraffin sections (4- 5um), following the method of **Suvarna *et al*. (2013)**. Stain sections of the analyzed tissues were inspected for any signs of pathology.

5. Hematological profile

Using an automatic cell counter (Hospitex Hema screen 18, Italy), blood parameters such as leukocyte count (WBCs), erythrocyte count (RBCs), hemoglobin level (Hb), and hematocrit value (Hct) were measured based on the suggested approach of **Dacie and Lewis (1995)**.

6. Stress-related biomarkers

To measure the amounts of glucose and cortisol, serum samples (3 samples/replicate) were used. According to a procedure previously published by **Teixeira *et al*.(2018)**, the blood glucose level was enzymatically measured by the glucose oxidase/glucose peroxidase reaction. According to **Saliu *et al*. (2017)**, cortisol levels were calculated using a commercial ELISA kit (Bio Quo Chem). Each control serum, calibrator, and tested sample's microplate wells were made in two copies. Then, 25µl of each ingredient was pumped into its corresponding well. Then, 100µl of enzyme conjugate was added to each well, and the amount was shaken between 500 and 800rpm for 30 minutes at 37°C. The plates were rinsed five times with 300µl of working washing solution, and then they were firmly tapped against absorbent paper to guarantee dryness. 3,3′, 5,5′ tetramethylbenzidine (100µl) substrate was added to each well at predetermined intervals, and they were left to work in the dark for 20– 30 minutes at room temperature. Subsequently, 150µl of stopping reagent was pipetted into each well at the same predetermined intervals and gently stirred for 5– 10 seconds. The absorbance at 450nm was determined using a microplate reader within 20 minutes of administering the stopping reagent.

7. Determination of serum lactate dehydrogenase (LDH) enzyme activity(U/l)

Using a commercial kit from Spainreact, Spain, the activity of LDH was measured using the method of **Young and Friedman (2001)**.

Principle: LDH catalyzes the reduction of pyruvate by NADH, based on the following response:

Pyruvate + NADH + H+ LDH $\longrightarrow$ L- lactate + NAD

The rate of decrease in concentration of NADPH, measured photometrically, is proportional to the catalytic concentration of LDH present in the sample.

Calculations

25 - 30° C ΔA/min × 4925 = U/L LDH

37°C ΔA/min × 9690 = U/L LDH

Units: The amount of enzyme required to change one substrate into another in a minute under standard conditions is known as an international unit (IU). Units per liter of sample (U/ L) are used to express concentration.

8. Statistical analysis

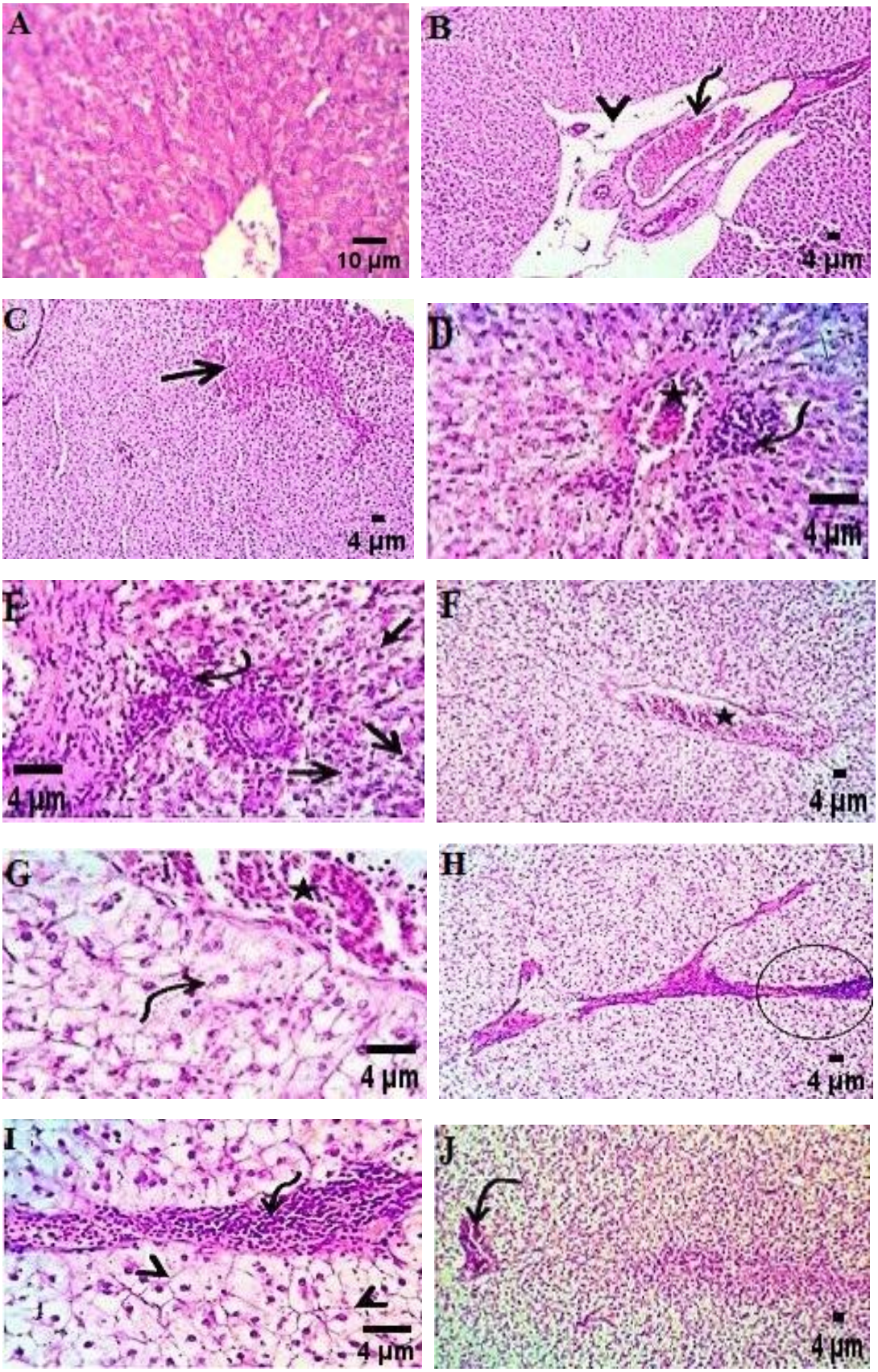
One-way analysis of variance (ANOVA) was used to evaluate the data using SPSS 18.0 (Chicago, IL, USA), and Duncan's post hoc test was used to investigate any changes in mean values at ($P< 0.05$).

RESULTS

1. Histopathological indices

1.1. Liver

Screening liver sections of *C. gariepinus* from the (CO) group revealed normal histological structure of the liver (Fig. 1A), whilst the liver of (CA) group demonstrated numerous hepatocytes with cloudy swelling and marked granular cytoplasm. Large areas showed strong necrotic changes (pyknotic nuclei). The hepato-portal veins were severely congested with portal edema. Beside intense perivascular aggregation of lymphocytes, strong hepatocellular degenerative changes mainly hydropic degeneration and individual hepatocellular apoptotic changes (Fig. 1B- E). However, the liver of (AM) group sections appeared mild dilatation and congestion of hepatic vein, small lymphocytosis, and hydropic degeneration of some hepatocytes (Fig. 1F- I). Additionally, the liver of (AP) group sections displayed mild congestion of hepato-portal veins and sinusoids, hydropic degeneration in small number of hepatocytes, minute focal necrotic changes and fine hepatic biliary hyperplasia (Fig. 1J- M).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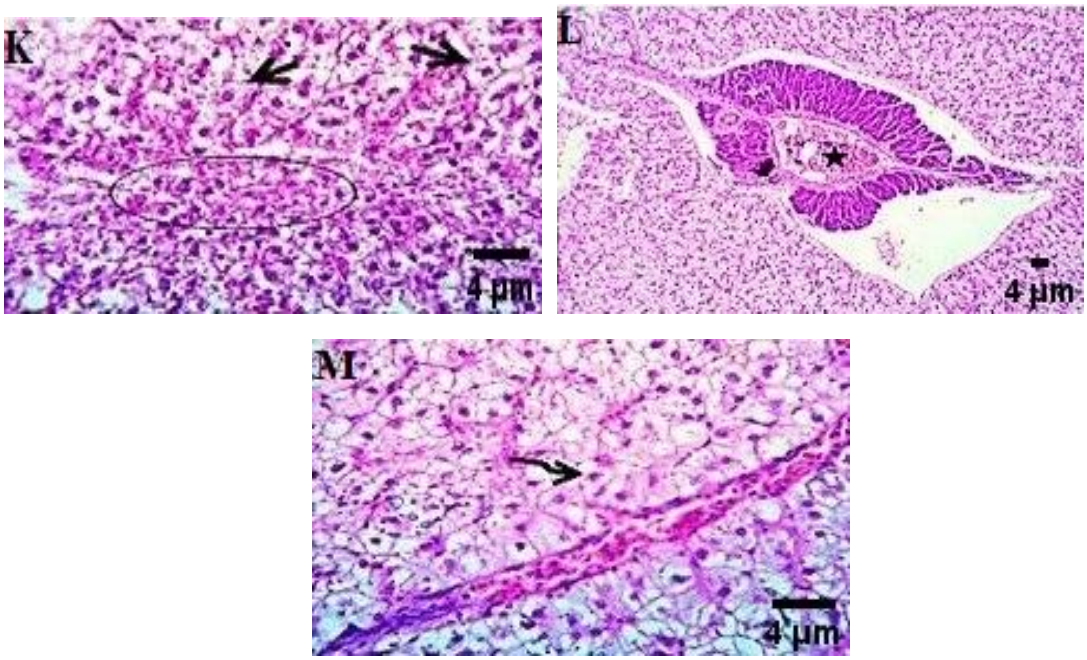
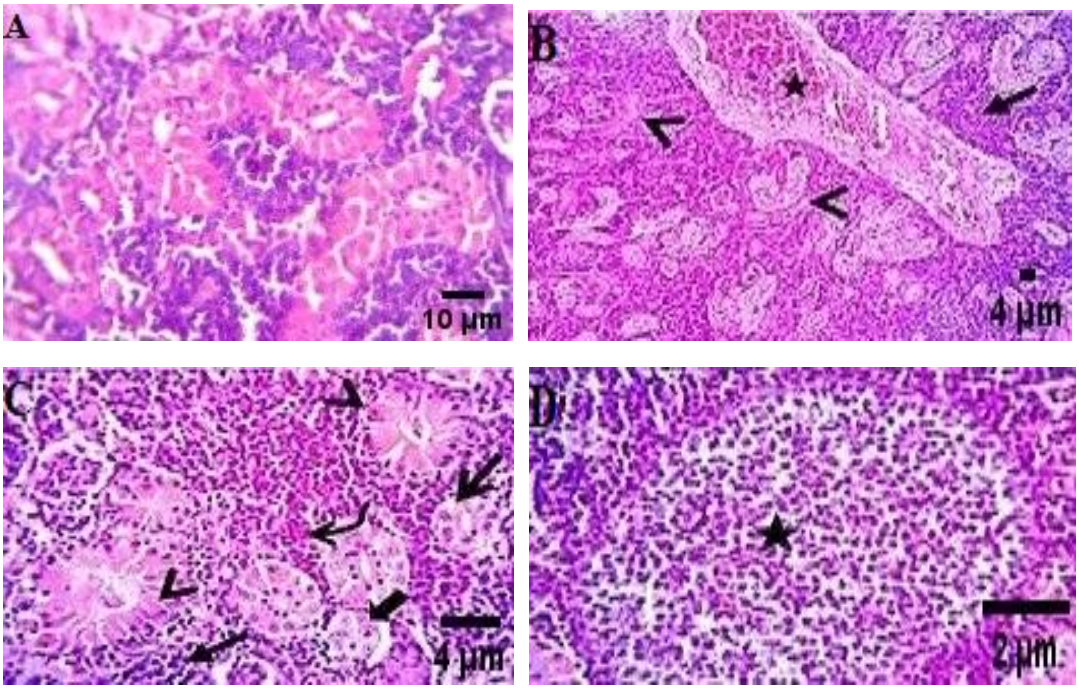


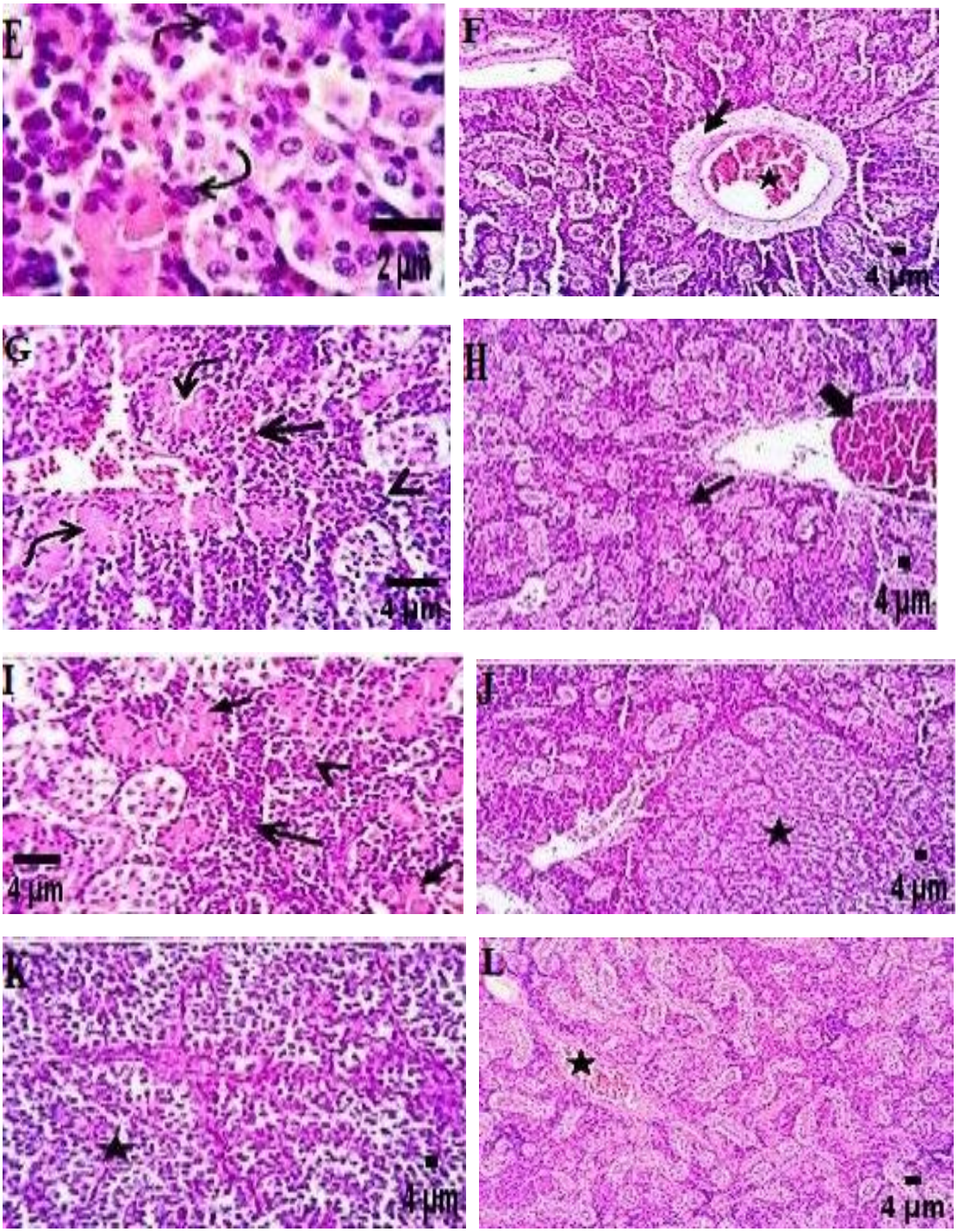
Fig. 1. Photomicrograph in **Liver** section of *Clarias gariepinus*. **(A)** Liver of negative control (CO) (without acrylamide) group showing normal histological architecture. **(B, C, D &E)** Liver of positive control (CA) (treated with acrylamide only in 15mg/ l), in **(B&C)** showing marked necrotic changes **(open arrow)**, severe congested hepato-portal veins **(curved arrow)** with strong portal edema **(arrowhead)**, and **(D&E)** showing marked congestion of hepato-portal blood vessels **(star)**, with intense perivascular aggregation of lymphocytes **(curved arrow)**, hydropic degeneration **(closed arrow)** and strong individual hepatocellular apoptotic changes **(open arrows)**. **(F, G, H & I)** Liver of (AM) group which contains (A_A) plus metformin (Me_{Fo}) applied to treatment (A_A) at (15mg/ l) and (Me_{Fo}) at 3g/ l, in **(F&G)** showing mild dilatation of hepatic vein **(stars)** and hydropic degeneration **(curved arrow)** of some hepatocytes and **(H&I)** showing mild congestion of hepato-portal veins **(circle)** with small lymphocytosis **(curved arrow)** and marked hydropic degeneration **(arrow heads)** of hepatocytes. **(J, K, L & M)** Liver of (AP) group which contains (A_A) plus propolis (P_{Po}) applied to treatment (A_A) at (15mg/ l) and (P_{Po}) at 3g/ l, in **(J&K)** showing mild congestion of hepato-portal veins **(curved arrow)**, hydropic degeneration **(open arrow)** of hepatocytes, minute focal necrotic changes **(circle)**, and **(L&M)** showing mild congestion of hepato-portal blood vessels **(star)** and hydropic degeneration **(curved arrow)** in small number of hepatocytes. **H&E X 100 (A, B, C, F, H, J & L) & 400 (D, E, G, I, K & M)**

1.2. Kidney

Examined kidney sections of *C. gariepinus* from the (CO) group demonstrated normal histological structure of the kidney (Fig. 2A), while the kidney of (CA) group sections revealed coagulative necrosis of a moderate number of renal tubules, massive interstitial lymphocytic infiltration and extravasation of erythrocytes, beside degenerative tubular changes mostly hydropic degeneration and congestion of the renal blood vessels and capillaries. Few renal tubules were regenerated, and there were multifocal renal masses formed from papillary proliferated renal tubular epithelium (papillary renal adenoma) with the presence of suspected intranuclear viral (eosinophilic) inclusions in some renal tubular epithelium and macrophages (Fig. 2B- E). Furthermore, Fig. (2F, G) displays the congestion of renal blood vessels and capillaries with perivascular edema and extravasation of erythrocytes. Coagulative necrosis in high number of renal tubules

and marked degenerative changes in the remaining tubules (hydropic degeneration) were seen. Characteristic interstitial round cells infiltration and aggregations, mostly perivascular and around the glomeruli were also encountered. However, the kidney of (AM) group sections demonstrated coagulative necrosis of a small number of renal tubules, marked interstitial lymphocytic infiltration and mild extravasation of erythrocytes, beside degenerative tubular changes mostly hydropic degeneration and congestion of the renal blood vessels and capillaries (Fig. 2H, I). A few number of renal tubules was regenerated (papillary renal adenoma) (Fig. 2J, K). Hence, Fig. (2L, M) shows mild congestion of renal blood vessels and capillaries sometimes with erythrocytic extravasation. A small number of renal tubules suffered coagulative necrosis and the remaining tubules showed hydropic degeneration. In addition to presence of marked interstitial, periglomerular and perivascular lymphocytic infiltration could be detected, while the kidney of (AP) group sections revealed mild congestion of renal blood vessels and capillaries, with few extravasations of erythrocytes. A small number of renal tubules showed coagulative necrosis. The remaining tubules showed mild cloudy swelling and hydropic degeneration (Fig. 2N, O). Nearly similar observation was displayed in Fig. (2P, Q, R, S), with mild congestion of the renal blood vessels and capillaries, and sometimes with erythrocytic extravasation. The focal renal tubular epithelium adenomatous proliferation could be detected. A small number of the renal tubules showed coagulative necrosis or degenerative changes in addition to fine hydropic degeneration.

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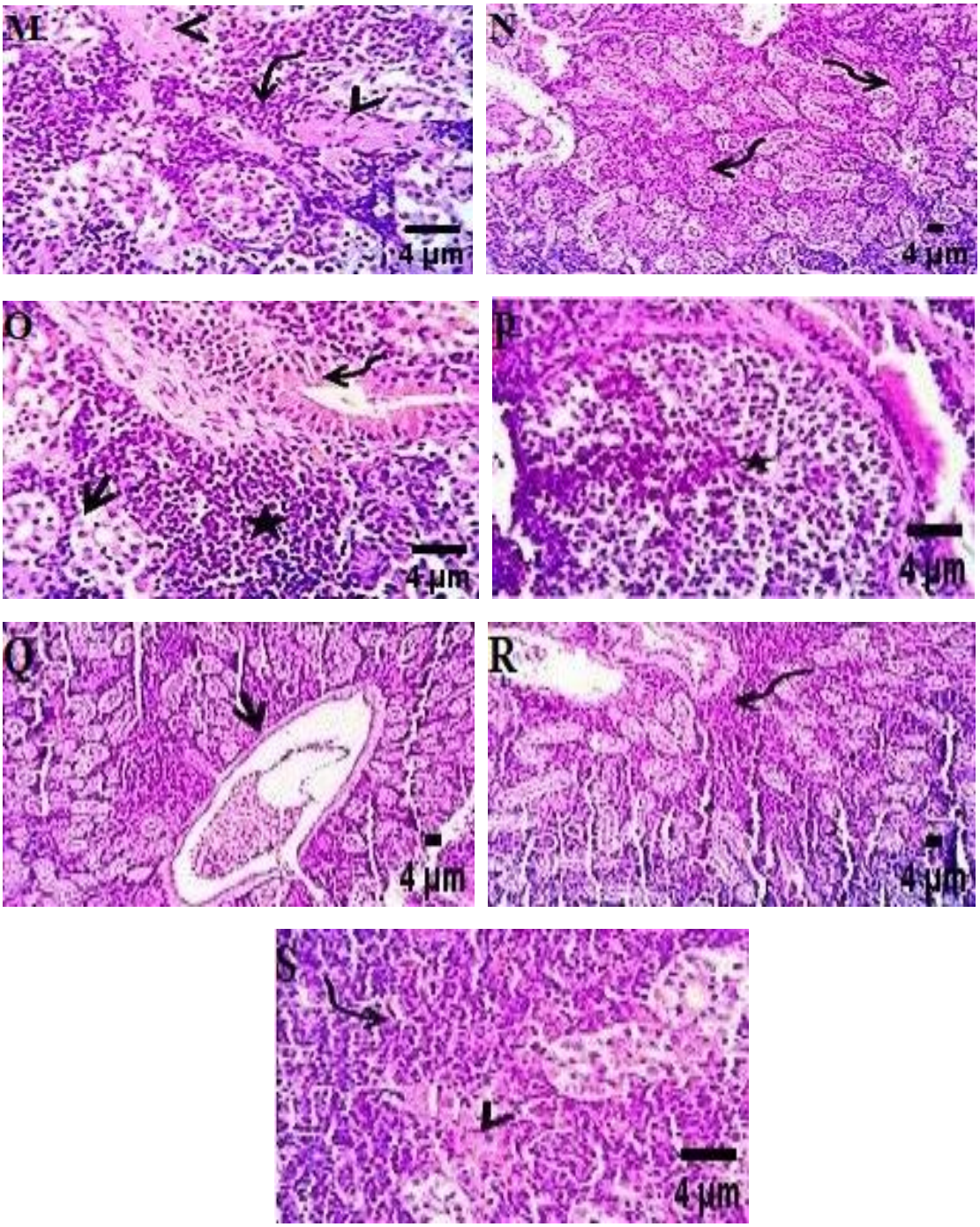
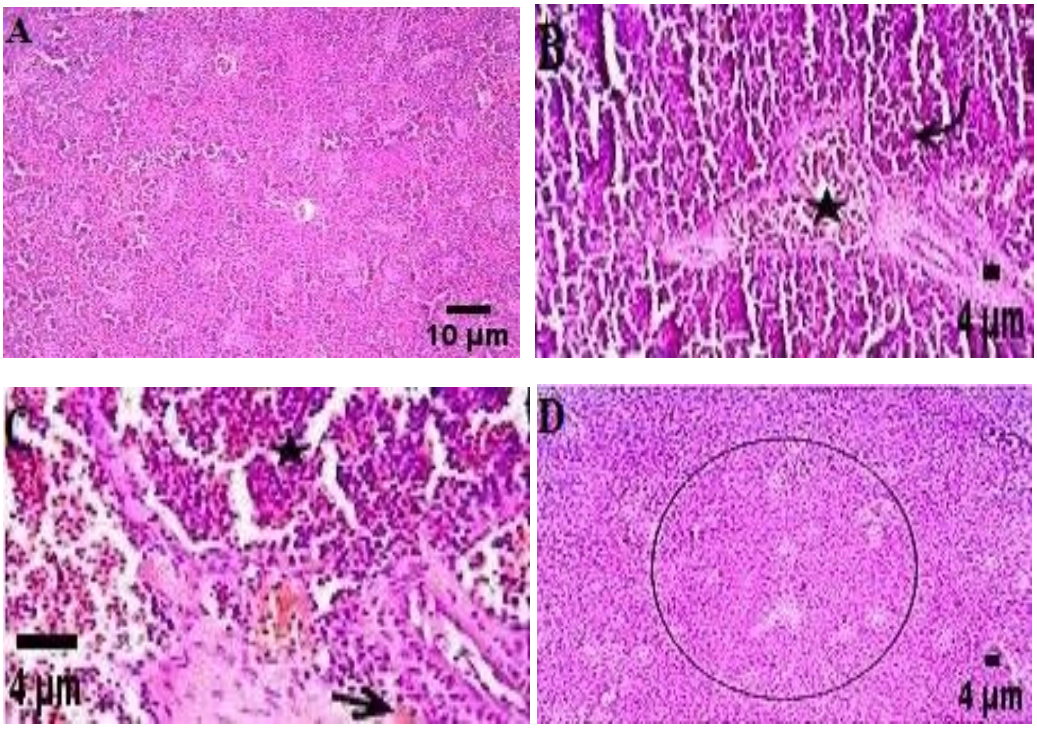


Fig. 2. Photomicrograph in **kidney** section of *Clarias gariepinus*. **(A)** Kidney of (CO) group showing normal histological architecture. **(B, C, D, E, F &G)** Kidney of (CA) group, in **(B&C)** showing coagulative necrosis **(arrow heads)** of renal tubules, massive interstitial lymphocytic infiltration **(closed arrow)** and extravasation of erythrocytes **(curved arrow)** beside degenerative tubular changes **(open arrow)** and congestion of the renal blood vessels **(star)** in addition to some regenerated renal tubules **(thick arrow)**, in **(D&E)** multifocal renal masses from papillary proliferated renal tubular epithelium (papillary renal adenoma) **(stars)** and suspected eosinophilic intranuclear viral inclusions **(curved arrows)** in some renal tubular epithelium and macrophages and in **(F&G)** showing congestion of renal blood vessels **(star)** with perivascular edema **(closed**

arrow) and extravasation of erythrocytes **(open arrow)** beside coagulative necrosis in high number of renal tubules **(curved arrows)** with characteristic interstitial round cells infiltration **(arrowhead)**. **(H, I, J, K, L & M)** Kidney sections of (AM) group, in **(H, I, J & K)** showing coagulative necrosis **(closed arrows)** of a small number of renal tubules, marked interstitial lymphocytic infiltration **(open arrow)** and mild extravasation of erythrocytes **(arrowhead)** beside congestion of the renal blood vessels **(thick arrow)** and multifocal renal masses (papillary renal adenoma) **(stars),** and **(L&M)** showing mild congestion of renal blood vessels **(star)** with coagulative necrosis of some renal tubular epithelium **(arrow heads)**, in addition to marked interstitial periglomerular and perivascular lymphocytic infiltration **(curved arrow)**. **(N, O, P, Q, R & S)** Kidney sections of (AP) group, in **(N&O)** showing coagulative necrosis **(curved arrows)** in a small number of renal tubules. The remaining tubules show mild cloudy swelling and hydropic degeneration **(arrowhead)** in addition to few interstitial and perivascular lymphocytic infiltration **(star)**, and in **(P, Q, R & S)** showing mild congestion of the renal blood vessels **(open arrow)**. Some renal tubular epithelium showing focal adenomatous proliferation **(star)**, a small number of the renal tubules showing coagulative necrosis **(arrowhead)** beside presence of some interstitial lymphocytic aggregation **(curved arrows)**. **H&E X100 (A, B, F, H, J, K, L, N, Q & R), 400 (C, G, I, M, O, P & S) & 1000 (D & E)**

1.3. Spleen

Examined spleen sections of *C. gariepinus* from the (CO) group exhibited normal histological structure of the spleen (Fig. 3A), while the spleen of (CA) group sections revealed severe congestion of splenic blood vessels and sinusoids, depletion of the lymphoid elements and the melano-macrophages from most parts of the spleen (Fig. 3B, C). On the other hand, the spleen of (AM) group section demonstrated mild depletion of hemopoietic elements (Fig. 3D). Furthermore, the spleen of (AP) group section showed fine depletion of melano-macrophage centers, mild congestion, and tiny interstitial hemorrhages (Fig. 3E).



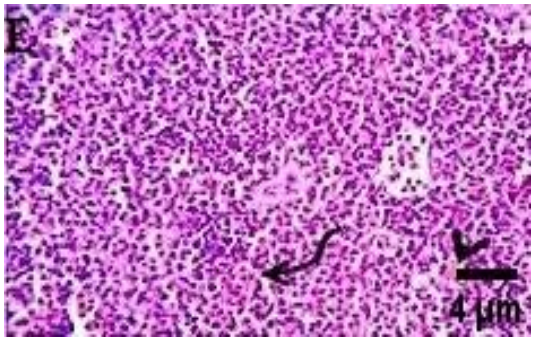


Fig. 3. Photomicrograph in **spleen** section of *Clarias gariepinus*. **(A)** Spleen of (CO) group showing normal histological architecture. **(B&C)** Spleen of (CA) group, in **(B&C)** sections revealed severe congestion of splenic blood vessels **(star)** and sinusoids **(curved arrow)** beside depletion of the lymphoid elements and melano-macrophages **(open arrow)** from most parts of the spleen. **(D)** Spleen section of (AM) group, in **(D)** showing mild depletion of hemopoietic elements **(circle)**. **(E)** Spleen section of (AP) group, in **(E)** showing fine depletion of melano-macrophage centers **(arrowhead)** beside tiny interstitial hemorrhages **(curved arrow)**. **H&E X 100 (A, B & D) & 400 (C & E)**

WBCs count exhibited in Table (1) shows that the mean level of CO is 47.67± 1.85 during (14) days, while fish intoxicated with acrylamide (A_A) in CA recorded (123.33± 5.45) on day (14) and exhibited a substantial increase at $P< 0.001$ in WBCs count in comparison to its corresponding value in CO. The obtained data of WBCs count showed a significant decrease in a group treated with AM and AP (66.00± 2.88 and 79.33± 3.18), respectively, when compared to corresponding value in CA. Hb concentration presented in Table (1) shows that the mean level of CO is 13.33± 0.43 during 14 days, while fish intoxicated with A_A in CA (15.23± 0.43) on day 14 revealed a notable increase at $P< 0.05$ in Hb concentration, compared to its corresponding value in CO. The obtained data of Hb concentration showed a significant decrease in a group treated with AP (13.20± 0.45) when compared to corresponding value in CA. Hct concentration displayed in Table (1) shows that the mean level of CO is 27.63± 2.17 during (14) days, while fish intoxicated with A_A in CA recorded a value of 43.66± 3.17 on day 14 showing a remarkable increase at $P< 0.001$ in Hct concentration when compared to its corresponding value in CO. The obtained data of Hct concentration showed a significant decrease in a group treated with (AM) and (AP) (31.86± 1.92 and 27.66± 1.88), respectively, when compared to corresponding value in CA.

2. Hematological profile

Table 1. Mean ± SE of hematological parameters *Clarias gariepinus* subjected to acrylamide and treated with Propolis & metformin doses for 14 days

Treatment	WBCs 10^3 (cell/ mm^3)	Lymph (%)	Granu. (%)	Mono. (%)	RBCs 10^6 (cell/ mm^3)	Hb (gm)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (%)	Plt.
CO	47.67 ±1.85[a]	86.00 ±3.05[a]	7.67 ±1.20[a]	6.30 ±1.85[a]	1.83 ±0.09[a]	13.33 ±0.43[a]	27.63 ±2.17[a]	150.70 ±7.74[a]	73.30 ±5.36[a]	49.12 ±5.88[a]	144 ±3.46[a]
CA	123.33 ±5.45[b]	84.00 ±2.30[a]	9.67 ±0.88[a]	6.33 ±1.45[a]	2.26 ±0.12[a]	15.23 ±0.43[b]	43.66 ±3.17[b]	195.10 ±23.59[a]	67.42 ±2.53[a]	35.35 ±3.41[a]	94.33 ±9.02[b]
AM	66.00 ±2.88[c]	90.00 ±1.52[a]	6.67 ±0.66[a]	3.33 ±1.45[a]	2.23 ±0.20[a]	14.33± 0.39[a, b]	31.86 ± 1.92[a]	144.80 ±14.27[a]	65.52 ±7.63[a]	45.30 ±3.35[a]	116.60 ±2.40[c]
AP	79.33 ± 3.18[d]	87.6 ± 2.40[a]	7.00 ±0.57[a]	5.33 ±1.85[a]	1.66 ± 0.17[a]	13.20 ±0.45[a]	27.66 ± 1.88[a]	170.13 ±23.96[a]	81.43± 10.89[a]	48.00 ±2.80[a]	134.33 ±5.81[a, c]
F ratio	**80.42**	**1.13**	**2.40**	**0.72**	**3.77**	**4.86**	**10.37**	**1.47**	**0.96**	**2.40**	**14.33**
Probability	***	NS	NS	NS	NS	*	***	NS	NS	NS	***

* Mean ($P< 0.05$), ** Mean ($P< 0.01$) and *** Mean ($P< 0.001$). (NS) Mean: Non-significant. Means followed by the same subscript in each column are not significantly different ($P> 0.05$). (CO) Mean: Negative control (without any treatment), (CA) Mean: Positive control (treated with acrylamide), (AM) Mean: *C. gariepinus* treated with acrylamide plus metformin, (AP) Mean: *C. gariepinus* treated with acrylamide plus Propolis.

3. Stress- biomarkers

Table 2. Mean ± SE of cortisol, glucose and LDH concentration in *Clarias gariepinus* subjected to acrylamide and treated with Propolis & metformin doses for 14 days

Treatment	Cortisol	Glucose	LDH
CO	20.00 ± 0.58^{a}	44.33 ± 2.33^{a}	182.33 ± 6.69^{a}
CA	45.00 ± 2.89^{b}	66.33 ± 2.33^{b}	465.33 ± 23.13^{b}
AM	30.67 ± 1.20^{c}	53.00 ± 1.53^{c}	369.66 ± 10.68^{c}
AP	30.00 ± 0.58^{c}	54.00 ± 0.58^{c}	382.66 ± 13.77^{c}
F ratio	**40.52**	**24.18**	**64.67**
Probability	***	***	***

* Mean ($P< 0.05$), ** Mean ($P< 0.01$) and *** Mean ($P< 0.001$). (NS) Mean: Non-significant. Means followed by the same subscript in each column are not significantly different ($P> 0.05$). (CO) Mean: Negative control (without any treatment), (CA) Mean: Positive control (treated with acrylamide), (AM) Mean: *C. gariepinus* treated with acrylamide plus metformin, (AP) Mean: *C. gariepinus* treated with acrylamide plus Propolis, (LDH) Mean: The enzymes lactate dehydrogenase.

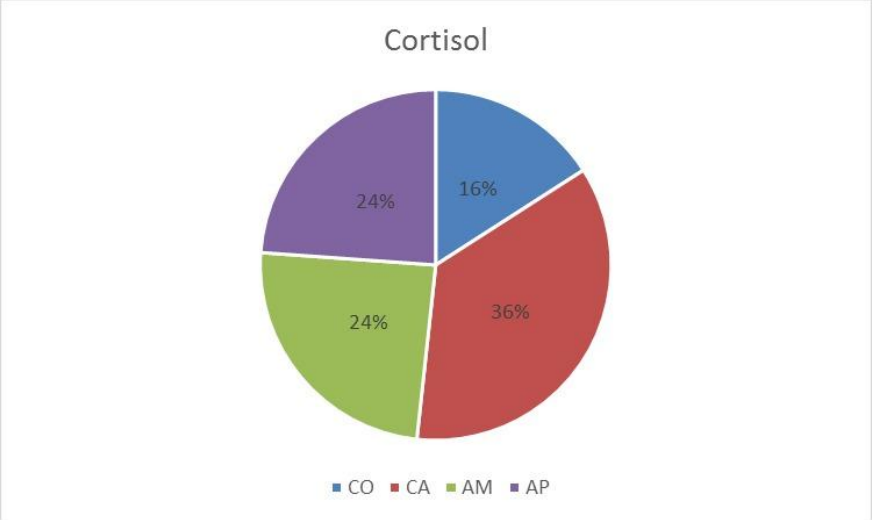


Fig. 4. Cortisol percentages in *Clarias gariepinus* subjected to acrylamide and treated with Propolis & metformin doses for 14 days against control

Serum cortisol level displayed in Table (2) shows that the mean level of (CO) is (20.00± 0.57) (ug/ dl) during (14) days, while fish intoxicated with (A_A) in (CA) recording (45.00± 2.89) at (14) days showed a notable increase at $P< 0.001$ in cortisol level in relation to its corresponding value in (CO). The obtained data of cortisol levels showed that a significant decrease in a group treated with (AM) and (AP) (30.67± 1.20 and 30.00± 0.58), respectively, when compared to corresponding value in (CA). In addition, cortisol levels revealed insignificant changes in a group treated with (AM) when

compared with (AP) (30.67± 1.20 and 30.0± 0.58), respectively. Additionally, the percentages that appear in Fig. 4 confirm the percentages of cortisol acquisition by each treatment within the *C. gariepinus* while subjected to (A_A) and treated with (P_{Po}) and (Me_{Fo}) against (CO).

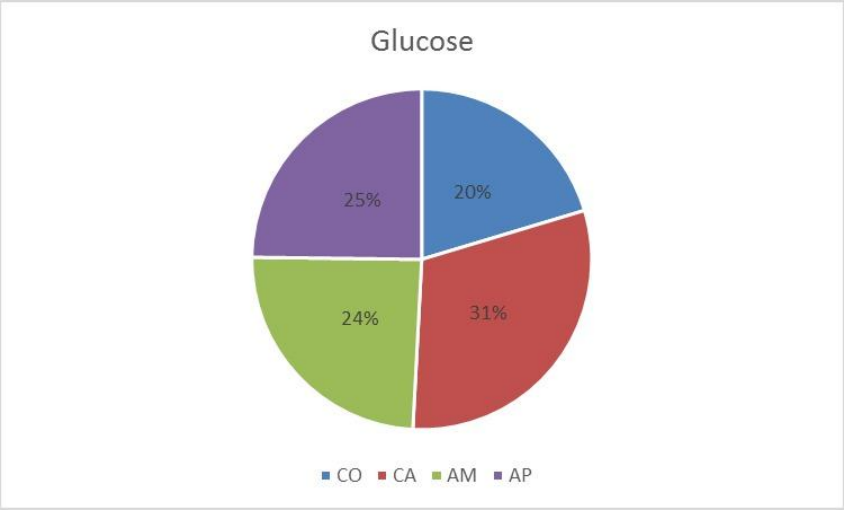


Fig. 5. Glucose percentages in *Clarias gariepinus* subjected to acrylamide and treated with Propolis & metformin doses for 14 days against control

Blood glucose levels (BG) exhibited in Table (2) shows that the mean level of (CO) is (44.33± 2.33) (mg/ dl) during (14) days, while fish intoxicated with (A_A) in (CA) recorded (66.33± 2.33) at (14) days indicated a noticeable rise at $P< 0.001$ in BG levels when compared to its corresponding value in (CO). In addition, BG levels revealed a significant decrease in a group treated with (AM) and (AP) (53.00± 1.53 and 54.00± 0.58), respectively, when compared to corresponding value in (CA). In addition, BG levels revealed insignificant changes in a group treated with (AM) when compared with (AP) (53.00± 1.53 and 54± 0.58), respectively. Therefore, the proportion that is represented in Fig. 5 decides the percentages of glucose acquisition by each treatment within the *C. gariepinus* while subjected to (A_A) and treated with (P_{Po}) and (Me_{Fo}) against (CO).

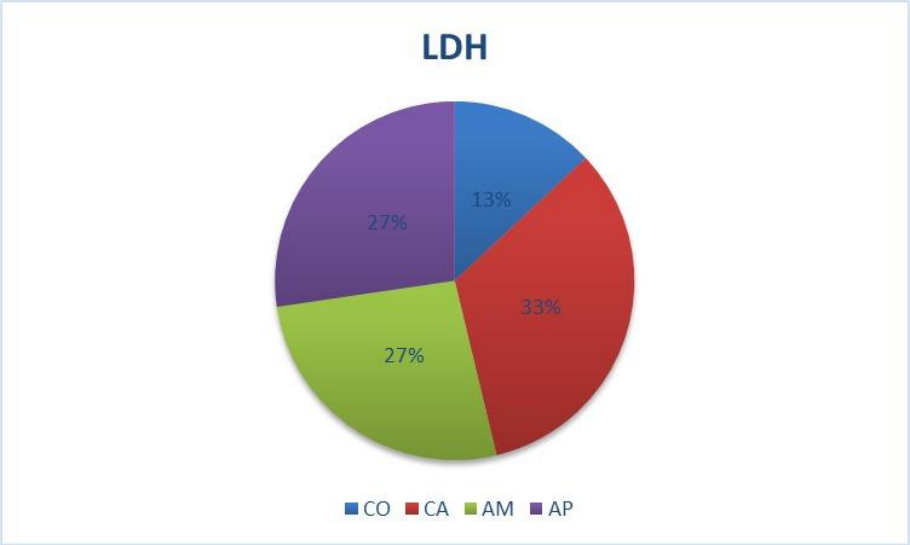


Fig. 6. LDH enzymes percentages in *Clarias gariepinus* subjected to acrylamide and treated with Propolis & metformin doses for 14 days against control

Serum LDH levels represented in Table (2) shows that the mean level of CO is 182.33± 6.69U/ L during 14 days, while fish intoxicated with (A_A) in CA recorded (465.33± 23.13) at (14) days showed a remarkable increase at $P< 0.001$ in LDH level when compared to its corresponding value in CO group. The obtained data of LDH levels showed that a significant decrease in a group treated with AM and AP (369.66± 10.68 and 382.66± 13.77), respectively, when compared to corresponding value in CA group. In addition, LDH levels revealed insignificant changes in a group treated with (AM) when compared with AP (369.66± 10.68 and 382.66± 13.77), respectively. Similarly, the proportion that is demonstrated in Fig. (6) decides the percentages of LDH enzymes acquisition by each treatment within the *C. gariepinus* while subjected to A_A and treated with P_{Po} and Me_{Fo} against CO.

DISCUSSION

Due to their toxicity and bioaccumulation in the food chain, which can cause sublethal toxic effects or fish population deaths, several environmental chemicals have been found to have contaminated aquatic environments, drawing a growing attention to this issue. In aquatic creatures like fish, these chemical substances can also interfere with the operation of the endocrine system **(Zhou *et al.*, 2000; Hontela, 2005)**. Inland water contamination issues have grown to be a local concern in recent years. Freshwater sources are polluted with a variety of pollutants due to the urban, industrial, and agricultural operations, which influence the local biota **(Abdel-Moneim *et al.*, 2012)**. The use of fish as biomarkers for the impacts of pollution and for the early detection of aquatic environmental contamination has gained more and more attention **(van der Oost *et al.*, 2003; De la Cruz-Cervantes *et al.*, 2018)**. Different outcomes in terms of oxidative stress have been found in experimental acrylamide (A_A) toxicity investigations. It is claimed that the cause of this is due to the various dose and treatment methods for A_A **(KILIÇLE *et al.*, 2020)**. There have been several health risks associated with A_A exposure in both animal and human models **(Lakshmi *et al.*, 2012; Sharma *et al.*, 2013)**.

Freshwater fish exposed to (A_A) showed changes at the tissue level in the hepatopancreas and kidney **(Larguinho *et al.*, 2014; Ibrahim & Ibrahem, 2020)**. In this regard, particular lesions that change the permeability in liver cell membranes may result from the major A_A metabolite, glycidamide, reacting with the functional groups of membrane proteins **(Sayed *et al.*, 2022)**. Due to the spleen's role as a lymphoid organ, the pathological abnormalities in splenic tissues found here may be connected to the observed depletion of immune components **(Delves & Roitt, 2000)**. It is believed that the melano-macrophage centers in the (A_A) exposed fish are involved in immunological defenses against the occurrences brought on by (A_A) consumption and (A_A) detoxification **(Komoike *et al.*, 2020)**. The biochemical results were supported by our histopathological findings in the hepatic and renal tissues, which showed that (A_A) exposed fish had severe vacuolation of the hepatocytes, compression of the sinusoids, scattered lytic necrotic areas, and hydropic degeneration and necrosis of some renal tubules. To evaluate the toxicological impacts and the status of fish health, hematological parameters are considered as helpful indicators (**García *et al.*, 2016)**. Hematological measures are frequently used to assess fish health, track stress responses, and forecast systematic correlations involving physiological adaptations of aquatic animals since they reflect fish condition more quickly than other regularly studied parameters **(Schlenk *et al.*, 2008; Bae *et al.*, 2012)**. Data from our investigation revealed a considerable increase in WBCs count of *Clarias gariepinus* intoxicated with A_A when contrasted with the equivalent values in the control (CO) group, and this could occur since fish under stress change their WBC count into leukocytosis or due to their immune system being activated. These results are in line with those of **Fink and Salibian (2005)** who hypothesized that the rise in WBCs might be a result of multipotent hematopoietic cells proliferating due to chemical poisoning. Leukocytes have a role in the control of immunological processes and the fish's protective response to stress **(Velisek *et al.*, 2012)**. When compared to the corresponding value in the CO group, the results of Hb and Hct show a significant increase in the A_A group, which could be caused by a decrease in the amount of dissolved oxygen in the water or by hemochromatosis, which is a condition caused by an excess of iron, RBCs, and hemoglobin in the body. Since hemoglobin- a blood molecule that carries oxygen from the lungs to all body tissues- contains iron, hemoglobin is considered crucial for proper blood function. Iron buildup may lead to organ problems. These results agree with the observations of **Watzek *et al.* (2012)** and **Erkmen *et al.* (2022)** who claimed that fish's hematocrit, hemoglobin, and erythrocyte counts greatly rose following exposure to A_A. These findings suggested that the fish's hemopoietic system may be considerably impacted by the hazardous chemical (A_A). Blood glucose serves as the body's primary source of energy for metabolism of catfish *C. gariepinus* **(Zubay, 1993; Hoseini *et al.*, 2016)**. Fish blood glucose measurements can reveal important details about the rate of production and consumption, metabolic health, and energy requirements of the fish **(Heath, 1995)**. Consequently, blood glucose is thought of as a stress response biomarker in *C. gariepinus* **(Abdel–Baky, 2001; Bakhshwan *et al.*, 2009)**. The recorded data demonstrated a significant rise in all (A_A) exposed fish groups when compared to the comparable values in the CO group. This might be due to a decrease in glucose utilization and/or the disruption of insulin and glucagon hormones. This result agrees with the observations of **EL-Sayed *et al.* (2013)** and **Paunescu *et al.* (2016)** who concluded that, environmental pollution-induced hyperglycemia may cause stress responses in fish by

impairing gluconeogenesis. This can lead to an increased breakdown of glycogen in the liver, and consequently raising blood glucose levels **(Holtz, 1990; Qiu *et al.*, 2017)**. Compared to the comparable values in the CO group, the acquired data revealed a substantial increase in LDH enzyme activity in all catfish *C. gariepinus* groups exposed to A_A. These results are in agreement with those of **Doubek *et al.* (2010), Acar (2018)** and **Zhao *et al.* (2022)**. The cytoplasmic enzyme LDH (lactate dehydrogenase) acts as an important metabolic key factor, which is frequently used as a marker of organ or tissue lesions in toxicology which have been utilized to show tissue damage in the catfish *C. gariepinus* **(Holmes & Goldberg, 2009; Hoseini *et al.*, 2015)**. When compared to the comparable values in the CO group, the collected data revealed a remarkable increase in cortisol activity in all catfish *C. gariepinus* groups subjected to A_A. These results coincide with multiple earlier investigations, wherein exposure to several aquatic chemicals has been shown to raise fish blood cortisol levels (**Li *et al.*, 2008; Garcia-Santos *et al.*, 2013)**.

CONCLUSION

According to the findings of the present study, the survival and well-being of species, particularly fish, in aquatic environments may be threatened by the frequent and widespread use of acrylamide. Due to the widespread use of A_A, the findings of the current study also showed that the parameters under study provide crucial biomonitoring tools for the assessment of pollutant effects in aquatic ecosystems. As a result, precautions should be made to protect both human and environmental health. Therefore, we advise against using (A_A) containing products and against cooking dishes made of potatoes and grains at high temperatures for an extended period. Since it poses numerous health risks, we should adopt (A_A) mitigation techniques to reduce exposure.

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