Water hardness variously influences tissue physiology of freshwater fish - *Cyprinus carpio* var *koi*: A report on glucose, oxidative stress and antioxidant biomarkers

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Abstract

Fishes endemic to freshwaters are strongly influenced by water hardness due to low ionic compositions with physiological consequences on gill and muscle. Aimed to comprehensively understand such alterations, selected tissues (gills and muscle) of freshwater fish, Koi carp were studied after sequential water hardness exposures. Secondary stress biomarkers - Glucose, Oxidative stress (Malondialdehyde – Lipid Peroxidation damage) and Antioxidative markers (Catalase, Glutathione-S-Transferase and Glutathione) were quantified after 14 days of exposure to a four-fold increase from 75 (Soft - TS), 150 (Moderate - TM), 225 (Hard - TH) to 300 (Very Hard - TV) mg $CaCO_3$/L. Although the fish showed appreciable endurance to all exposures, soft and moderate waters proved harmful to both tissues. Successive increase was observed in the glucose content especially in gills ($p < 0.05$). Hardness of 75 and 150 mg $CaCO_3$/L was concluded to be detrimental to both gill and muscle due to elevated MDA concentrations ($p < 0.05$) than corresponding higher hardness indices. Elevated CAT, GST activity and GSH levels indicated well-coordinated antioxidant enforcement. Overall, the results revealed assorted tissue-specific biomarker responses and more importantly, concentrations < 225 mg $CaCO_3$/L elicited strong oxidative impairment in both gill and muscle.

Keywords – Calcium; Carps; Gill; Muscle; Physiology; Water quality

Introduction

Hardness is perhaps the most significant physicochemical property of water because it directly or indirectly influences physiology. Aquatic environments manifest varying concentrations of water hardness due to appreciable quantities of divalent cations Calcium (Ca^{2+}), Magnesium (Mg^{2+}) and some trace cations as well (Baldisserotto 2011; Romano et al. 2020). Based on the concentration of these cations, water is classified into soft (< 75 mg $CaCO_3$/L) and hard (> 75 mg $CaCO_3$/L) (Portz et al. 2006). Both types exert their own physiological effects. Soft water poses challenges to the survival of fish, although various methods of adaptation have been reported, like compensation of calcium deficit environment through the consumed feed (Baldisserotto 2011). Similarly, hard waters can help balance ammonia, pH, and reduce mortality in animals (Baldisserotto et al. 2014). However, excessive hardness can also cause hypercalcemia, as reported in Tilapia (*Sarotherodon mossambicus*) (Wendelaar Bonga et al. 1983). Whatever the adjustment might be, these investigations are unequivocal that hardness ultimately targets tissues that lead to physiological changes including cellular rearrangements (Gonzalez et al. 1998; Gundersen and Curtis 1995).

It would not be wrong to mention that gill and muscle are favoured over other major tissues to assess hard water effects. While gills are the epicentre of Ca^{2+} homeostasis and osmoregulation (Evans et al. 2005; Wendelaar Bonga et al. 1983), muscle is susceptible to changes in constituent amino acids and ionic shifts due to water hardness (Buentello and Gatlin 2002). Both tissues have been exhaustively investigated to understand consequences of water hardness at ionic-level and associated cellular adaptations in freshwater species such as Pinfish (*Lagodon rhomboides*) and Tilapia (Carrier and Evans 1976; Flik and Verbost 1995; Wendelaar Bonga et al. 1983).

Tendency of muscle mitochondria to take up Ca^{2+} from external sources and compensate for ionic stress has been reported in *Tilapia mossambica* (Sulochana et al. 1977). These tissues localise diverse redox reactions and biomarker changes, examination of which gives worthy information about environmentally induced physiological alterations also, making it ideal to study effects of hard waters (Lushchak 2011). Though, fish would prefer water hardness to be well within range, such preference seems ecologically unrealistic since there is not constant external environment. Therefore, possibility of stress is not negatable at any given moment and relevant response is consequential to hardness levels.

Prior literature indicates that biomarkers of secondary stress response are instrumental in understanding the effects of water hardness in fish. Investigations in Pacu (*Piaractus mesopotamicus*) (Copatti et al. 2019a; Neves et al. 2022), Silver Catfish (*Rhamdia quelen*) (Neves et al. 2017) and Kutum fingerlings (*Rutilus frisii kutum*) (Taghizadeh et al. 2013), justify the importance of glucose as marker for effects of water hardness. Even redox status also provides substantial insights into the physiological effects of water hardness in fish. Secondary products of lipid peroxidation (LPO) such as Malondialdehyde (MDA) are powerful markers of oxidative stress (Ayala 2014). Evidence to this are investigations in various tissues of Pacu exposed to a range of hard waters (Copatti et al. 2019b; Neves et al. 2022). The consequences of oxidative stress are prevented by antioxidants (Betteridge, 2000), either enzymatic (Catalase, Glutathione-S-Transferase) or non-enzymatic (Glutathione) which attempt to restore the correct prooxidant/antioxidant ratio (Lushchak, 2016). Catalase is an important antioxidant enzyme that protects cells and tissues from oxidative damage because it reduces harmful hydrogen peroxide (H_2O_2) to water (H_2O) (Betteridge 2000). Activity of GST is specific to detoxification of

xenobiotics. It conjugates GSH to various electrophiles, thereby preventing oxidative damage. After that, GSH indirectly or directly scavenges free radicals to defend the tissues from injury due to oxidative stress (Srikanth 2013). Despite broad insights offered by glucose and redox markers, it is noteworthy that investigations involving its usage have gained momentum recently (Copatti et al. 2019b; Michelotti et al. 2018). Biomarker examinations seem promising to understand the extent and efficiency of physiological adaptations due to external hardness (Limbaugh et al. 2021).

Koi carp, an ornamental variety of common carp, is popular among aquarists for aesthetical features. In general, it is naturally spotted in various water bodies such as ponds, reservoirs, streams and lakes. The species is physiologically sturdy under captive conditions as well, which qualifies it to be a worthy model to study freshwater – water hardness interactions and associated biochemical fluctuations. Therefore, the present study attempts to evaluate the effects of water hardness (75, 150, 225 and 300 mg $CaCO_3$/L) on biomarkers (glucose, oxidative stress and antioxidant profile) in gill and muscle of Koi carp.

Methods

Acclimation of fish

Juveniles (6.70 ± 0.15 g; 5.90 ± 0.12 cm) were purchased from the Ornamental Fish Research Centre, Bengaluru. Fish were randomly distributed in 50 L glass tanks filled with tap water. The tanks were maintained under natural photoperiod (≈12 Light/12 Dark) with continuous aeration (Venus Aqua AP-608A, China). Fish were fed twice a day (09:00 and 18:00) at 2% body weight with commercial feed pellets (Taiyo Grow, India).

Experimental setup

The two-week study consisted of four levels of hardness: 75 (Soft - TS), 150 (Moderate - TM), 225 (Hard - TH), and 300 (Very Hard - TV) mg $CaCO_3$/L, selected based on its occurrence in natural systems (Stumm and Morgan 1996). Every treatment (hardness) was maintained in triplicate (three tanks per treatment × two fishes per tank = six fishes per treatment). All concentrations were prepared by the addition of Calcium carbonate ($CaCO_3$) to water and calibrated by complexometric EDTA titration (APHA 2005). Faecal matter accumulated in the tank was drained out by suction pipe with subsequent renewal of the specific hardness. The tanks were covered with a mesh net to prevent the fish from escaping. Water parameters were monitored for temperature (25.1 ± 1°C), pH (7.04 ± 0.1), dissolved oxygen (6.5 ± 0.08 mg/L) and alkalinity (213 ± 0.02 mg/L) (APHA 2005).

Sampling

A total of six fish (n = 6) per treatment were sacrificed for biomarker evaluation. Individual fish were dissected for white muscle and gill. Tissues were washed with an ice-cold phosphate buffer solution (0.1M; pH 7.4) and homogenised in Potter-Elvehjem grinder. The homogenate (10% w/v) was then centrifuged at 5000 × g following which supernatant (stored at -20°C) was retained for all assays except Glutathione (homogenate precipitated with TCA before centrifugation). The absorbance values were measured on a spectrophotometer (Systronics UV-VIS 118, India).

Biochemical analyses

Glucose

Glucose was assayed according to Nelson (1944) and Somogyi (1952). The deproteinizing agent ($Ba(OH)_2$ and $ZnSO_4$) was added to the supernatant and centrifuged at 5000 × *g* for 10 minutes. Alkaline copper reagent (potassium-sodium tartrate; Na_2CO_3; $NaHCO_3$ and Na_2SO_4 in distilled water) was added to the supernatant. The mixture was heated, followed by the addition of an arseno-molybdate reagent and the optical density of the solution was recorded at 540 nm.

Malondialdehyde (MDA)

Secondary product of Lipid peroxidation (LPO) - Malondialdehyde was estimated by using the Niehaus and Samuelsson (1968) protocol. The supernatant was mixed with this TCA-TBA-HCl reagent (15% Trichloroacetic acid, 0.38% Thiobarbituric acid and 0.25N Hydrochloric acid) in the ratio of 1:2. This reaction mixture was heated in a boiling water bath for 15 minutes, cooled and centrifuged at 1100 × *g* for 5 minutes. The optical density of the solution was recorded at 535 nm.

Catalase (CAT)

Catalase activity was measured by using the Aebi (1984) protocol. The reaction was started by adding the supernatant to an equimolar solution of H_2O_2 and phosphate buffer (50 mM; pH 7.1). A decrease in absorbance was continuously recorded at 240 nm (UV) for 3 min.

Glutathione-S-Transferase (GST)

GST activity was estimated by using the Habig et al. (1974) protocol. The reaction mixture contained the supernatant, phosphate buffer (0.1M; pH 6.5) and 2,4-Dinitrochlorobenzene

(30mM). The volume was adjusted with distilled water after which the reaction was initiated by adding Glutathione (0.1 M). The optical density of the solution was recorded at 340 nm.

Glutathione (GSH)

GSH was estimated by using the Moron et al. (1979) protocol. Homogenate was precipitated with TCA (5%) and centrifuged at 3000 × *g* for 10 minutes. The supernatant collected after centrifugation was then added to the phosphate buffer (pH 6.5) and Ellman's reagent. The optical density of the solution was recorded at 420 nm.

Protein

Total protein content of the tissues was estimated according to Lowry et al. (1951) protocol. Bovine serum albumin was used as standard. The optical density of the supernatant-reagents mixture was recorded at 660 nm.

Statistical analysis

Data was summated as Mean ± SE. Normality and homoscedasticity were evaluated using the Shapiro–Wilk and Levene tests, respectively. Inter-group comparisons were performed using One-way ANOVA followed by a post-hoc test (Tukey). Significant differences were fixed at 95 % ($p < 0.05$). GraphPad Prism (Version 5.0, USA) and JASP (Version 0.16.2, Netherlands) were used for statistical computation and visual presentations.

Results

Biomarkers in Gill

The glucose concentration increased progressively from TS to TV. Significant differences (F = 10.91; $p < 0.05$) were found between TV and the remaining treatments (Figure 1A). Soft water TS elevated the concentration of MDA, followed by a spike in TV. Only TH differed significantly (F = 21.27; $p < 0.001$) from the remaining treatments (Figure 2A). Highest antioxidant Catalase activity was observed for TH, which differed significantly (F = 50.26; $p < 0.001$) from TS, TM and TV. Also, TV differed significantly from TM and TS (F = 50.26; $p < 0.001$) (Figure 3A). GST activity for TH and TV was comparatively higher than for TS and TM. While no significant differences (F = 26.45; $p > 0.05$) were found between the low (TS and TM) and high hardness groups (TH and TV), differences were observed between treatment pairs (Figure 4A). The highest concentration of GSH was recorded for TV. Except TS that was not significant (F = 42.78; $p > 0.05$) with TM and TH, remaining treatments recorded intergroup differences (Figure 5A).

Biomarkers in White Muscle

The glucose concentration progressively increased from TS to TH but reduced at TV. Pairwise, similar concentrations and no significant differences (F = 22.98; $p > 0.05$) were observed for TS and TV and for TM and TH (Figure 1B). The highest level of MDA was observed for TM which differed significantly (F = 37.89; $p < 0.001$) from TS, TH and TV (Figure 2B). The highest CAT activity was observed in TM followed by a gradual reduction for TH and TV. Catalase was the only biomarker that varied significantly (F = 49.38; $p < 0.05$) among all treatments for white muscles (Figure 3B). No significant differences (F = 57.13; $p > 0.05$) were recorded for GST between TM and TH. The highest GST activity was recorded for TH, which differed significantly with TS and TV (F = 57.13; $p < 0.001$) (Figure 4B). Glutathione (GSH) initially increased from

TS to TM, thereafter, reducing gradually for the remaining treatments. Only significant difference ($F = 8.87$; $p < 0.05$) was observed between TM and the remaining groups (Figure 5B).

Discussion

Glucose concentration

In the present study, glucose increased steadily in gills from soft to hard waters. Since glucose primarily serves energy for metabolism through glycolysis, glucose concentration provides insights into the energy consumption for adaptations to hardness. According to Flik and Verbost (1995), freshwater fishes take up Ca^{2+} through the gills, and this transcellular movement is dependent on the surrounding Ca^{2+} concentrations, which affect the branchial permeability of the gills. Therefore, external hardness can strongly influence the energy reserves to regulate fish metabolism. Generally, an environment with high hardness reduces gill permeability (through tightening of junctions) and subsequent loss of ions to water, ultimately conserving energy (Golombieski et al. 2013), something that is evident through the study results. Contrarily, muscle showed reductions in glucose at the highest hardness level (TV), an observation also reported by Michelotti et al. (2018) in juvenile Common Snook (*Centropomus undecimalis*) exposed to 1000 mg $CaCO_3$/L. They concluded that increased energy demands for survival and growth lowered muscle glucose levels. Presumably, whereas gill conserved energy, muscle on the other hand struck balance to the metabolic needs thereby capitalising on glycolysis.

Oxidative stress

In this study, elevated MDA in the Koi carp gills exposed to soft and moderate waters indicated improper osmoregulatory functioning. Therefore, soft and moderate water hardness has detrimental effects on gills as also reported in Pacu gills (Copatti et al. 2019b). An increase in MDA of gills exposed to TV was also observed. As a deviation from the expected outcome, an explanation of this seems possible only after further investigations. MDA concentration also surged in muscles of Koi carps exposed to moderate hardness (TM), indicating oxidative damage to cell membrane fluidity and possible permeability of muscle tissues. Previous literature suggests that fishes are predisposed to peroxidation due to large amounts of Polyunsaturated Fatty Acids (PUFAs), ultimately changing lipid membrane properties (Lushchak 2011). Therefore, the present study cannot negate the probable oxidative damage in muscles exposed to moderately hard water.

Antioxidant response

Reduced activity of Catalase in TS and TM exposed gills of Koi indicated oxidative damage. This indicated failure to respond to the peroxidation damage of gills at soft and moderately hard waters. Relatively low ionic composition of freshwater environment might burden the gills to reduce loss of ions to water to maintain osmotic integrity. Therefore, the fish became inept in adjusting to concentrations < 150 mg $CaCO_3$/L. Comparatively, antioxidant activity was much stronger in muscle for the soft and moderate exposures. As for the higher levels (> 150 mg $CaCO_3$/L), Koi carps seemingly benefited from the elevated hardness levels. Protective effects of high-water hardness have been reported in Pacu by (Copatti et al. 2019a).

Elevated GST activity was noted in Koi carp gills at TH and TV, indicating that some degree of oxidative stress might have affected this tissue. Muscle also exhibited an elevated GST activity for all the exposures except soft waters (TS). This signifies that concentrations < 75 mg $CaCO_3$/L would be detrimental for freshwater fishes, with unrecoverable losses to osmoregulatory functioning. Given the intensive association between Glutathione antioxidant system, assorted expression of both GST and GSH in the investigated tissues indicated a well-coordinated response to all hardness exposures. Lower GST levels were complemented by a higher concentration of GSH for both the gills and muscle. Such type of tissue-specific antioxidant responses for the Glutathione antioxidant system has also been reported in Nile Tilapia, Sharp Tooth Catfish (*Clarias lazera*) and Common carp (*Cyprinus carpio*) (Hamed et al. 2004).

Conclusions

The results proved that sequential increase in water hardness reshuffled the physiological adjustments in both the examined tissues. Glucose proved to be an excellent marker for hard waters especially in case of gills. The study also concludes that soft and moderately hard waters can prove detrimental to freshwater fishes due to tissue oxidation induced elevated MDA concentrations. Overall, coordinated defence to oxidative stress was evident through variable expressions of CAT, GST and GSH, which might account for the adaptability of Koi carps to prolonged exposure to hardness. Since the antioxidant markers revealed multitudinous and assorted responses, an extension of this study warrants an Integrated Biomarker Response (IBR) due to sequential water hardness.

List of abbreviations -

Malondialdehyde - MDA; Catalase - CAT; Glutathione-S-Transferase - GST; Glutathione GSH; Glucose - GLU; Soft water - TS; Moderately hard water - TM; Hard water - TH; Very hard water - TV

Declaration -

Ethics approval – Animal use within this study was approved by the welfare committee of Bangalore University, India, in accordance with the national legislation.

Consent for publication – Not applicable

Availability of data and material – All relevant data is within this article. Please contact corresponding author for further queries

Competing interests – The author declares that there are no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' contributions – Author has designed and conceptualised the study.

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Figure Legends -

Figure 1 - Glucose concentration in **(A)** gill and **(B)** white muscle of Koi carps ($n = 6$) exposed to water hardness of 75 (Soft - TS), 150 (Moderate - TM), 225 (Hard - TH) and 300 (Very Hard - TV) mg $CaCO_3$/L. Superscripts among exposure groups indicate statistical significance ($p < 0.05$). (+) inside the box indicates the mean value of the exposure group.

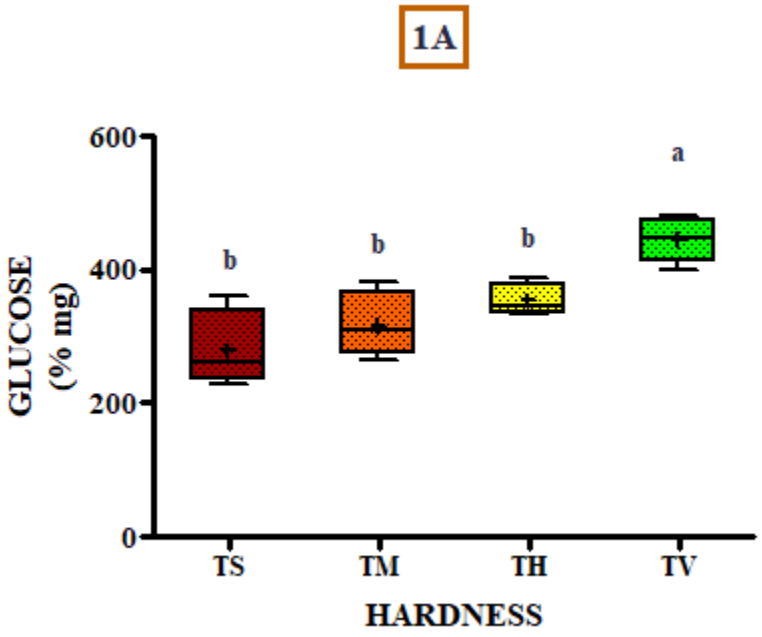
Figure 2 - MDA concentration due to Lipid Peroxidation (LPO) in **(A)** gill and **(B)** white muscle of Koi carps ($n = 6$) exposed to water hardness of 75 (Soft - TS), 150 (Moderate - TM), 225 (Hard - TH) and 300 (Very Hard - TV) mg $CaCO_3$/L. Different superscripts among exposure groups indicate significant differences ($p < 0.05$). (+) inside the box indicates the mean value of the exposure group.

Figure 3 - Catalase activity in **(A)** gill and **(B)** white muscle of Koi carps ($n = 6$) exposed to water hardness of 75 (Soft - TS), 150 (Moderate - TM), 225 (Hard - TH) and 300 (Very Hard - TV) mg $CaCO_3$/L. Different superscripts among exposure groups indicate significant differences ($p < 0.05$). (+) inside the box indicates the mean value of the exposure group.

Figure 4 - Glutathione-S-transferase (GST) activity in **(A)** gill and **(B)** white muscle of Koi carps ($n = 6$) exposed to water hardness of 75 (Soft - TS), 150 (Moderate - TM), 225 (Hard - TH) and 300 (Very Hard - TV) mg $CaCO_3$/L. Different superscripts among exposure groups indicate significant differences ($p < 0.05$). (+) inside the box indicates the mean value of the exposure group.

Figure 5 - Glutathione (GSH) in **(A)** gill and **(B)** white muscle of Koi carps ($n = 6$) exposed to water hardness of 75 (Soft - TS), 150 (Moderate - TM), 225 (Hard - TH) and 300 (Very Hard - TV) mg $CaCO_3$/L. Different superscripts among exposure groups indicate significant differences ($p < 0.05$). (+) inside the box indicates the mean value of the exposure group.

Figure 1A & B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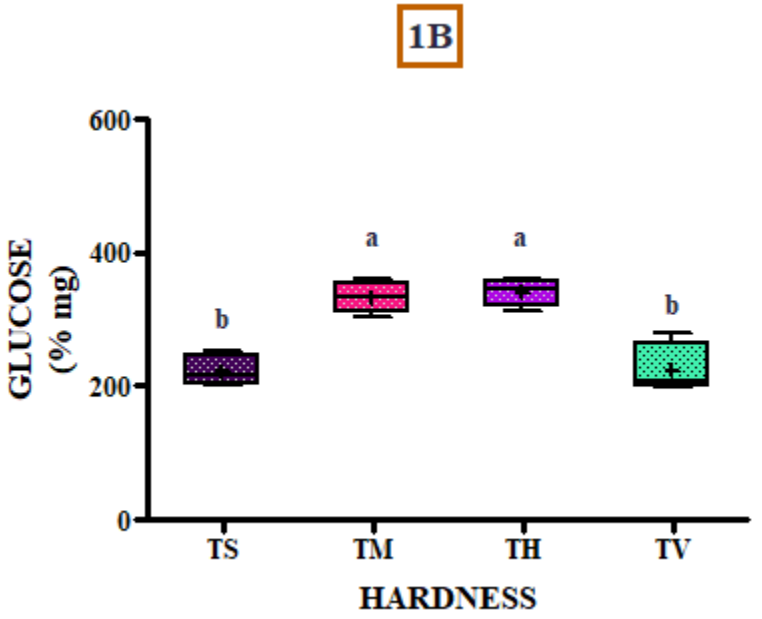
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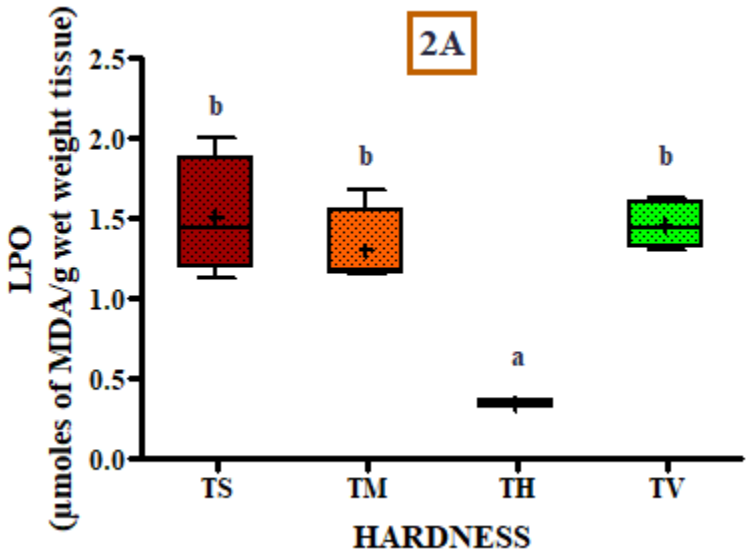
Figure 2A & B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b
TS
TM
TH
TV
HARDNESS

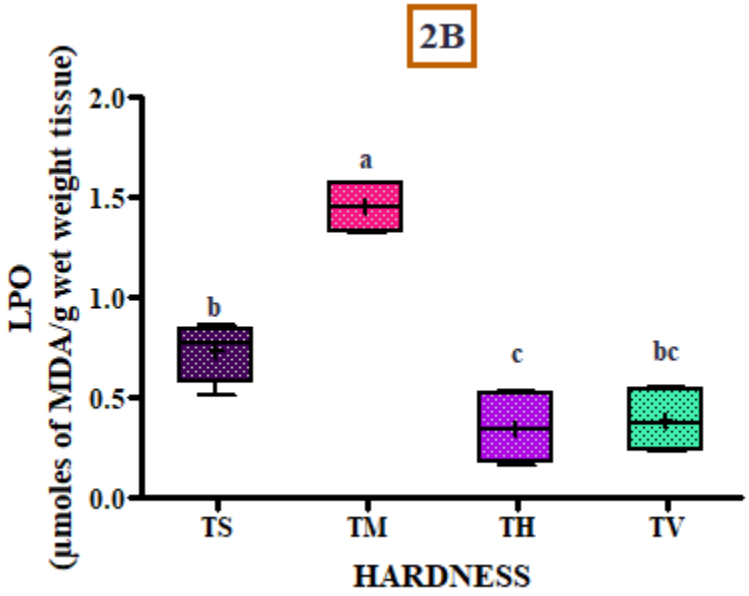
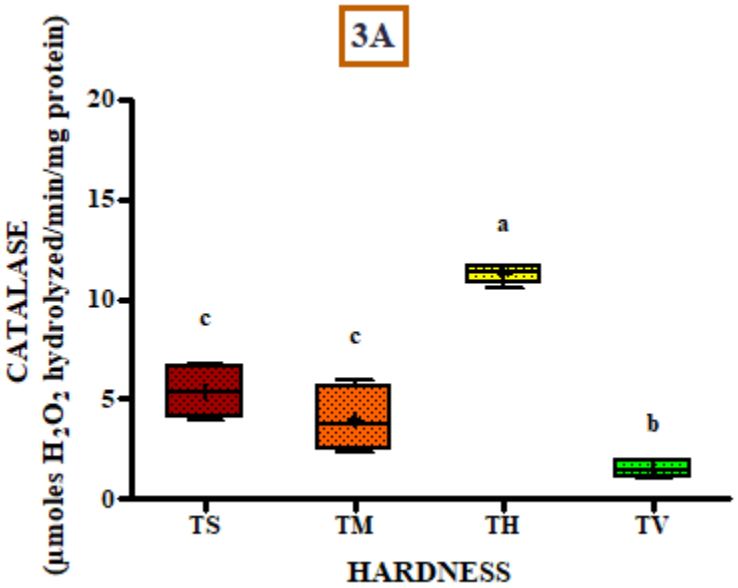

2B
LPO
(µmoles of MDA/g wet weight tissue)
2.0
1.5
1.0
0.5
0.0
b
a
c
bc
TS
TM
TH
TV
HARDNESS

Figure 3A & B


3A
CATALASE
(µmoles H_2O_2 hydrolyzed/min/mg protein)
20
15
10
5
0
c
c
a
b
TS
TM
TH
TV
HARDNESS

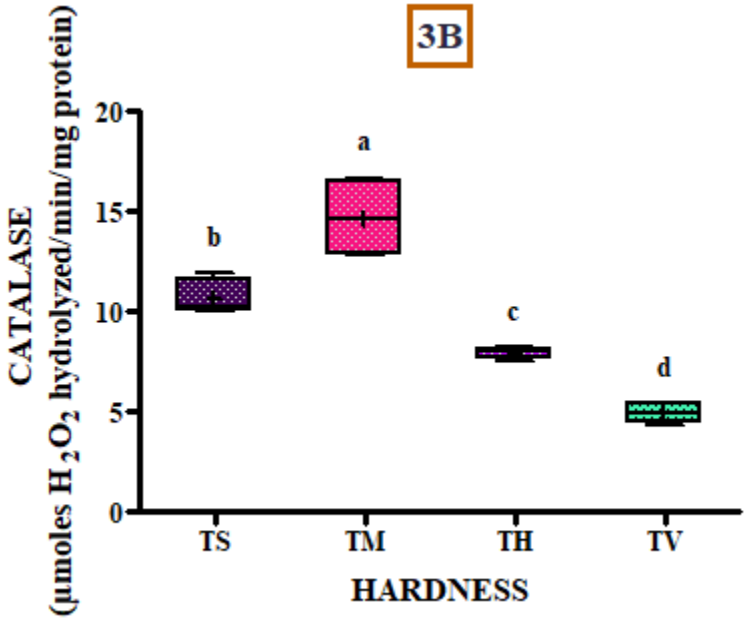
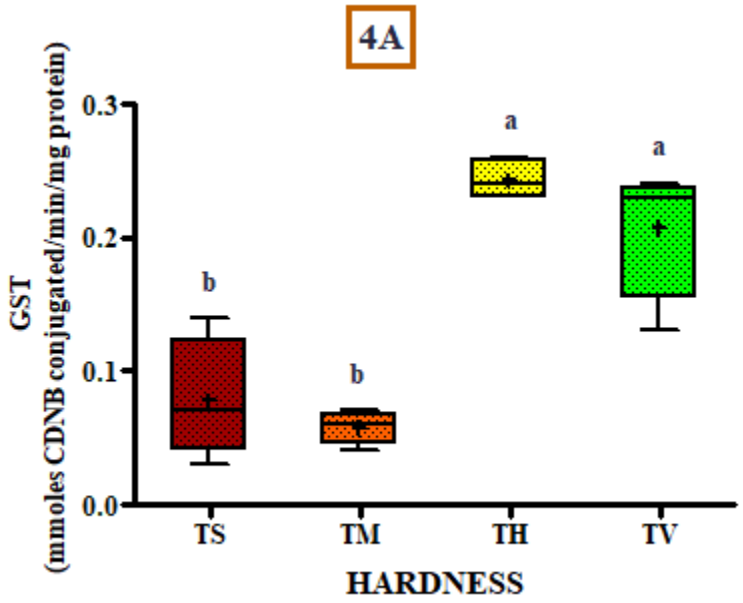

3B
CATALASE
(µmoles H_2O_2 hydrolyzed/min/mg protein)
20
15
10
5
0
b
a
c
d
TS
TM
TH
TV
HARDNESS

Figure 4A & B


4A
GST
(mmoles CDNB conjugated/min/mg protein)
0.3
0.2
0.1
0.0
b
b
a
a
TS
TM
TH
TV
HARDNESS

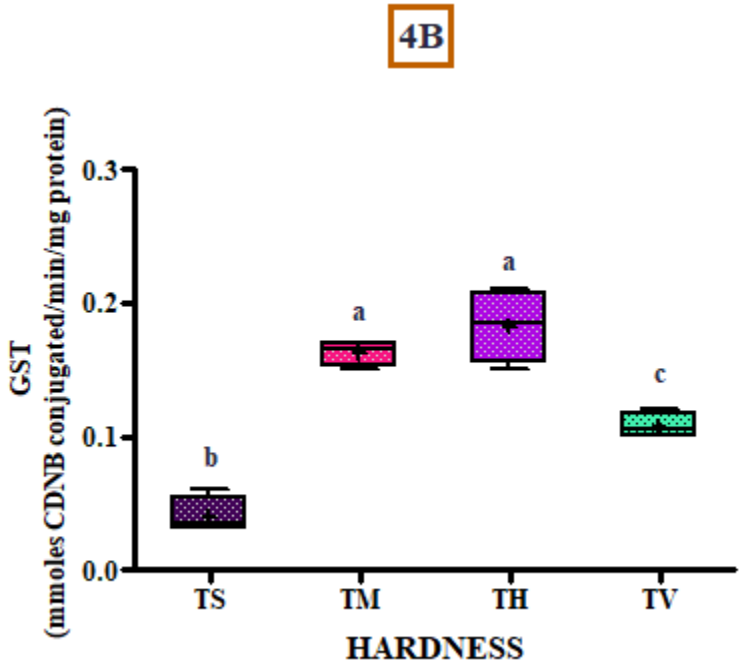
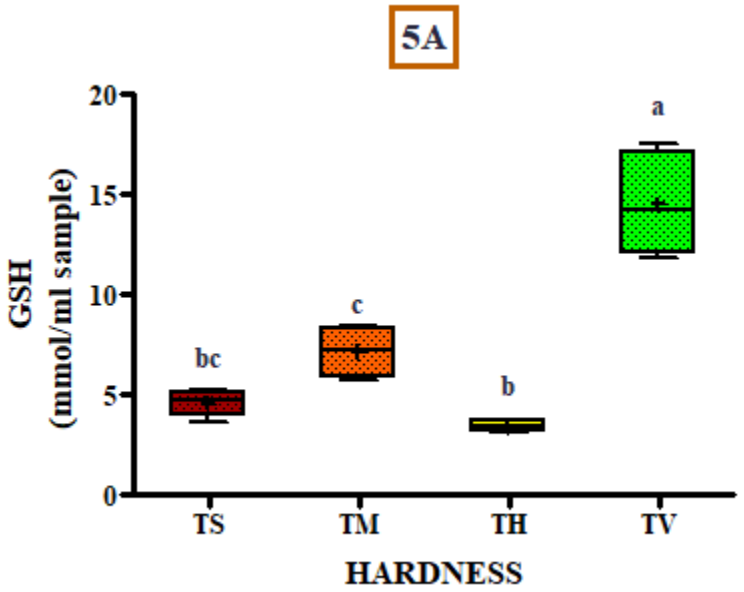
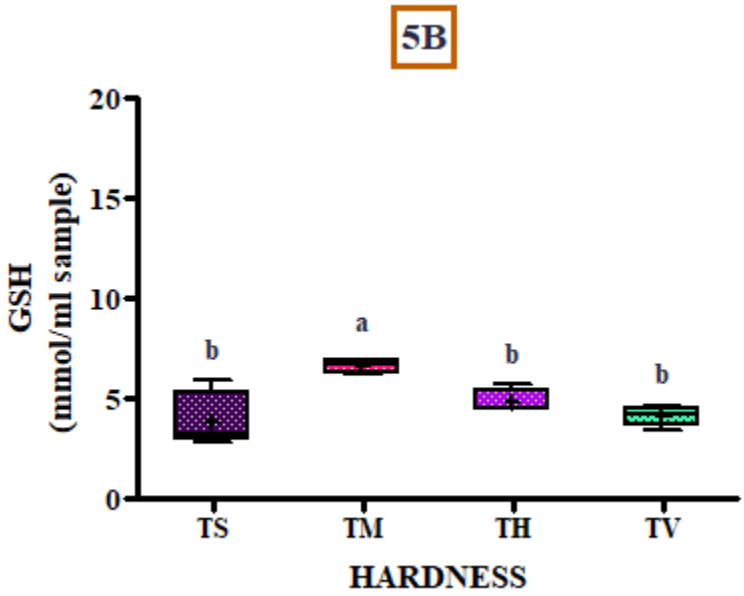

4B
GST
(mmoles CDNB conjugated/min/mg protein)
0.3
0.2
0.1
0.0
b
a
a
c
TS
TM
TH
TV
HARDNESS

Figure 5A & B


5A
GSH
(mmol/ml sample)
20
15
10
5
0
bc
c
b
a
TS
TM
TH
TV
HARDNESS


5B
GSH
(mmol/ml sample)
20
15
10
5
0
b
a
b
b
TS
TM
TH
TV
HARDNESS