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 (*Cyprinus carpio* var *koi*) 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 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 water hardness levels. Prior literature indicates that secondary biomarkers of stress 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. Evidence of this is the study of oxidative stress by Copatti et al. (2019b) and Neves et al. (2022) in different tissues of Pacu exposed to a range of hard waters. 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). 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 is an ornamental species of common carp preferred by aquarists globally for beautification. Therefore, it is generally spotted in natural conditions ponds, reservoirs, streams

86 and lakes. The species is physiologically sturdy under captive conditions as well, which makes it
87 a good model to study freshwater – water hardness interactions and associated biochemical
88 fluctuations. Therefore, the present study attempts to evaluate the effects of water hardness (75,
89 150, 225 and 300 mg $CaCO_3$/L) on biomarkers (glucose, oxidative stress and antioxidant profile)
90 in selected tissues (gill and muscle) of Koi carps (*Cyprinus carpio* var *koi*), an economically
91 important freshwater ornamental fish.

92 **Methods**

93 **Acclimation of fish**

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

99 **Experimental setup**

100 The two-week study consisted of four levels of hardness: 75 (Soft - TS), 150 (Moderate - TM),
101 225 (Hard - TH), and 300 (Very Hard - TV) mg $CaCO_3$/L, selected based on its occurrence in
102 natural systems (Stumm and Morgan 1996). Every treatment (hardness) was maintained in
103 triplicate (three tanks per treatment × two fishes per tank = six fishes per treatment). All
104 concentrations were prepared by the addition of Calcium carbonate ($CaCO_3$) to water and
105 calibrated by complexometric EDTA titration (APHA 2005). Faecal matter accumulated in the
106 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

127 reagent (15% Trichloroacetic acid, 0.38% Thiobarbituric acid and 0.25N Hydrochloric acid) in the
128 ratio of 1:2. This reaction mixture was heated in a boiling water bath for 15 minutes, cooled and
129 centrifuged at 1100 × *g* for 5 minutes. The optical density of the solution was recorded at 535 nm.

130 **Catalase (CAT)**

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

134 **Glutathione-S-Transferase (GST)**

135 GST activity was estimated by using the Habig et al. (1974) protocol. The reaction mixture
136 contained the supernatant, phosphate buffer (0.1M; pH 6.5) and 2,4-Dinitrochlorobenzene
137 (30mM). The volume was adjusted with distilled water after which the reaction was initiated by
138 adding Glutathione (0.1 M). The optical density of the solution was recorded at 340 nm.

139 **Glutathione (GSH)**

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

144 **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. Group means were analysed by 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

Glucose is the primary energy source for metabolism due to its involvement in glycolysis. Therefore, the assessment of glucose concentration would provide insight into the energy expenditure for metabolic adaptations to hardness. Flik and Verbost (1995) have mentioned that 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 strongly influences the energy reserves such as glucose to regulate fish metabolism. An environment with high hardness reduces gill permeability and loss of ions to water, thereby conserving energy (Golombieski et al. 2013). This study upholds the same rationale in the fact that glucose increased subsequently in gills of Koi carp from soft to hard waters. 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. It would not be incorrect to assume that whereas gill is conserving energy, muscles on the other hand strike balance to the metabolic needs. This assumption however would need more justification.

Oxidative stress

Secondary products of lipid peroxidation (LPO) such as Malondialdehyde (MDA) are used as biomarkers of oxidative stress (Ayala 2014). 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

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). The level of CAT activity in gills underwent reduction from soft to moderate waters of Koi carps which indicated oxidative damage due to such exposure. Since freshwater species already have less ionic presence in the surrounding environment, it might burden the gills to reduce loss of ions to water. Probably this must have decreased CAT activity, indicating that fish were inept in adjusting to concentration changes. Whereas in muscle, progressive decrease in Catalase activity from TM to TV, indicates subsided oxidative damage which might account for the protective effects of high-water hardness as suggested by (Copatti et al. 2019a).

Antioxidant 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). 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. But it is not feasible to generalise that the fish was under stress because antioxidant defence is always coordinated (Bagnyukova et al. 2006). Therefore, CAT and GSH might also have a role to play in protecting Koi carps from high hardness. Biomarker evaluation is majorly dependent on many factors including experimental conditions (Bagnyukova et al. 2006). 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 show 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. It can also be deduced that soft and moderately hard waters can prove detrimental to freshwater fishes due to tissue oxidation induced elevated MDA concentrations. 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.

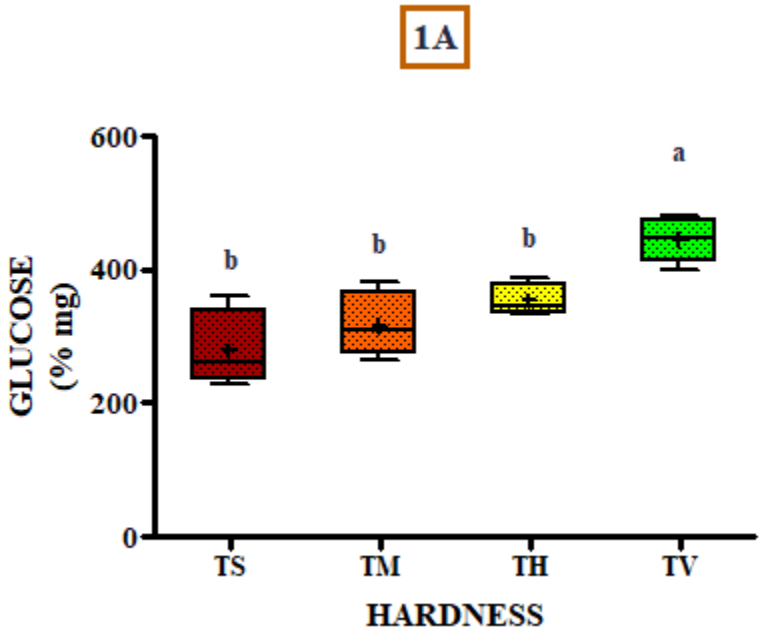
Figure 2 - MDA concentration due to Lipid Peroxidation (LPO) in **(A)** gill and **(B)** white muscle of Koi carps (n = 6) exposed to water hardness of 75 (Soft - TS), 150 (Moderate - TM), 225 (Hard - TH) and 300 (Very Hard - TV) mg $CaCO_3$/L. Different superscripts among exposure groups indicate significant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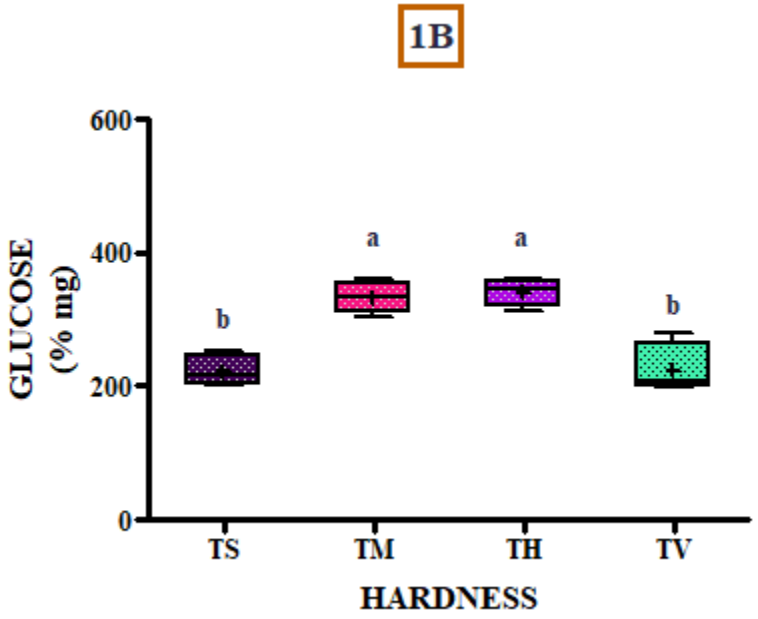
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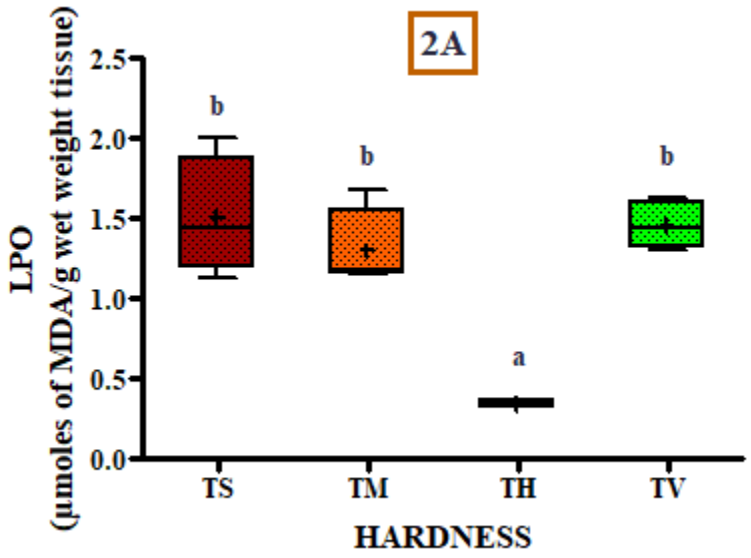
HARDNESS

Figure 2A & B


2A
LPO
(µmoles of MDA/g wet weight tissue)
2.5
2.0
1.5
1.0
0.5
0.0
b
b
a
b
TS
TM
TH
TV
HARDNESS

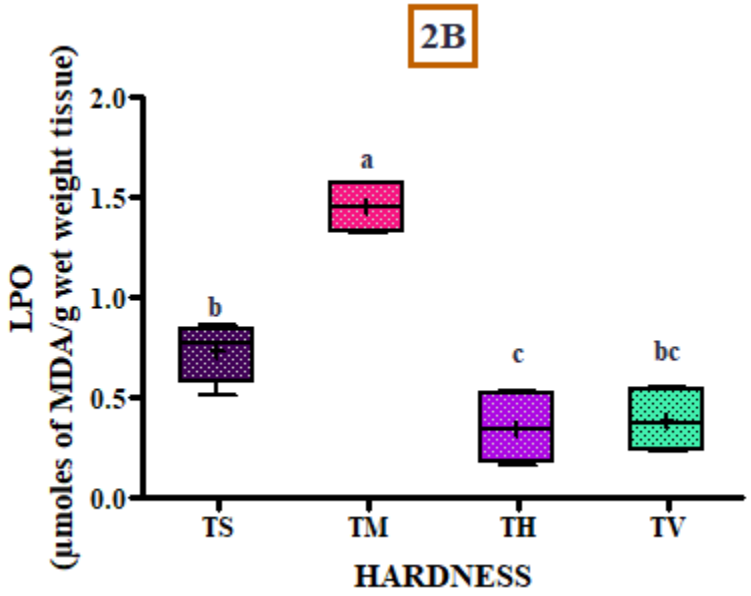
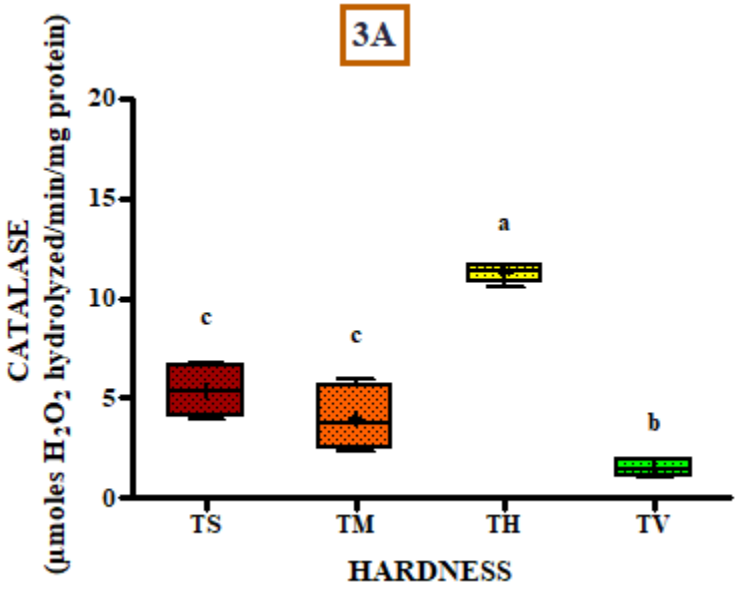

2B
LPO
(µmoles of MDA/g wet weight tissue)
2.0
1.5
1.0
0.5
0.0
b
a
c
bc
TS
TM
TH
TV
HARDNESS

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

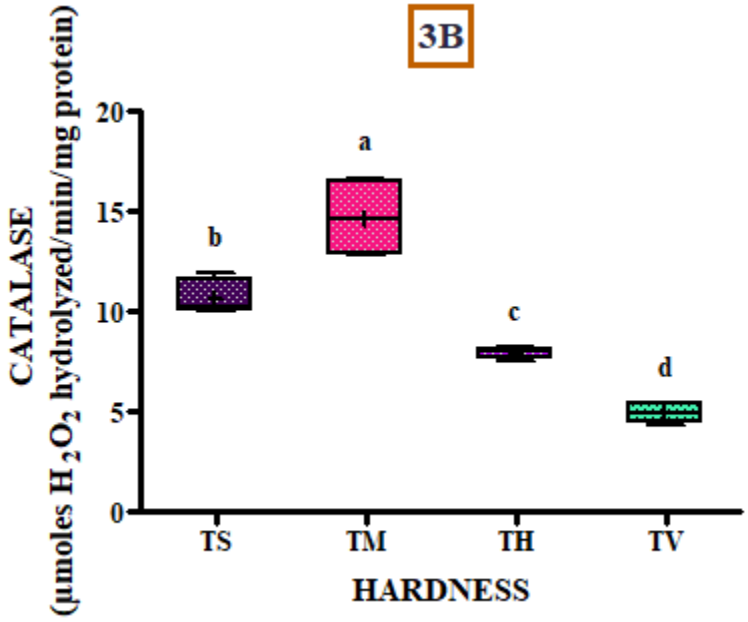
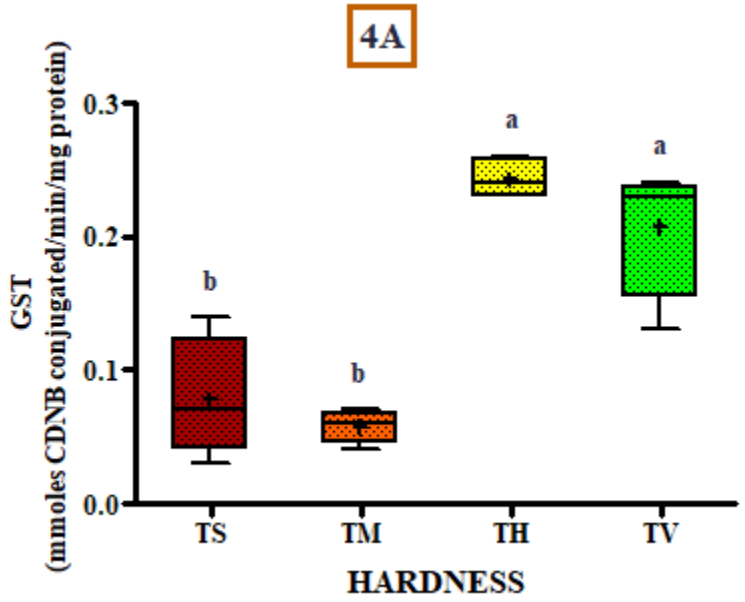

3B
CATALASE
(µmoles H_2O_2 hydrolyzed/min/mg protein)
20
15
10
5
0
b
a
c
d
TS
TM
TH
TV
HARDNESS

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

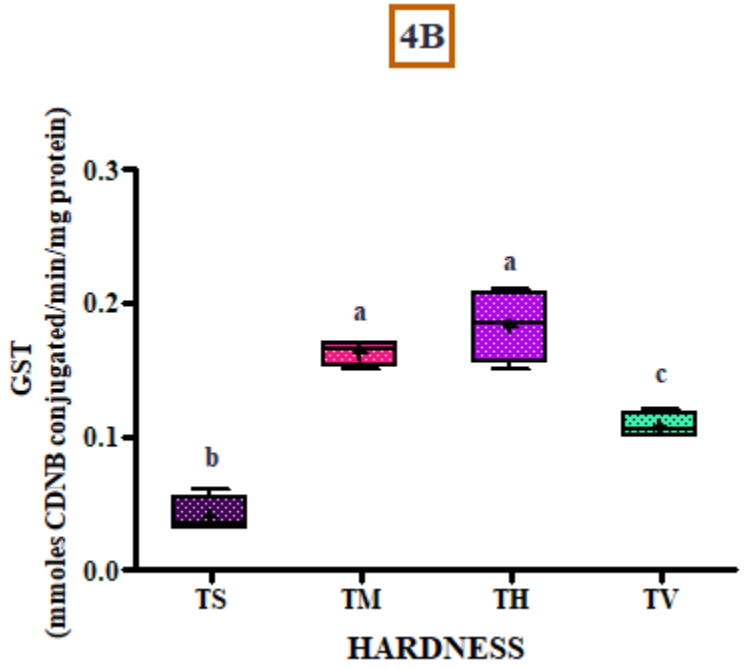
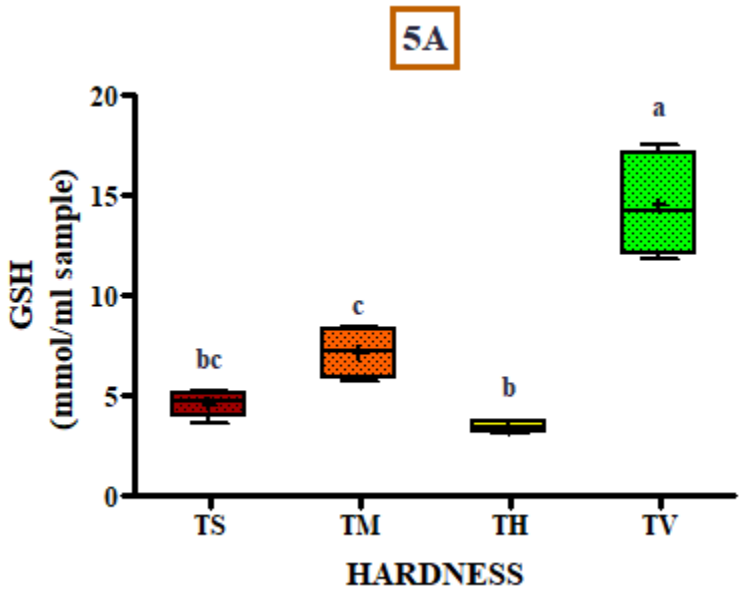
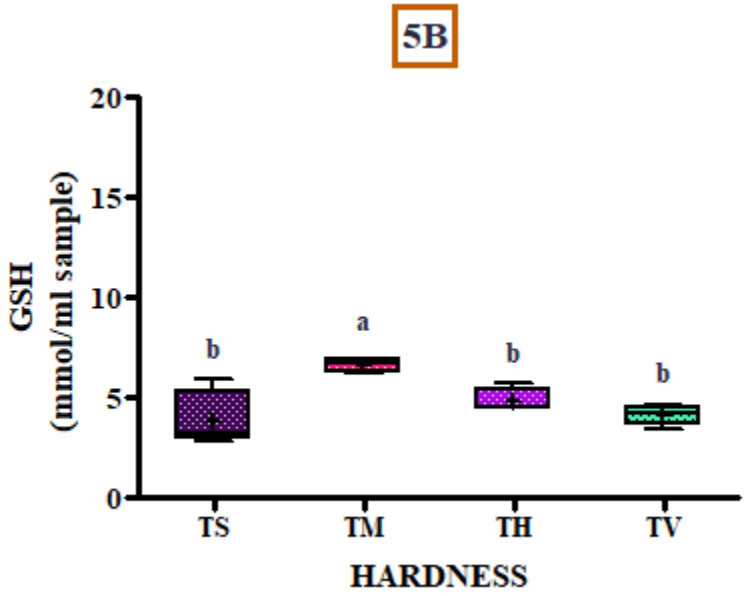

4B
GST
(mmoles CDNB conjugated/min/mg protein)
0.3
0.2
0.1
0.0
b
a
a
c
TS
TM
TH
TV
HARDNESS

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