Effects of Sodium Benzoate Using Zebrafish Animal Model

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The Degree of Master of Technology

By

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This thesis entitled **"Effects of Sodium Benzoate Using Zebrafish Animal Model"** by **SRINTIHI .P** is approved for the degree of Master of Technology.

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BO16MTECH11008

Abbreviations

ADHD	-	Attention deficit hyperactivity disorder
ATP	-	Adenosine triphosphate
$CaCl_2.2H_2O$	-	Calcium chloride dehydrate
cDNA	-	Complementary DNA
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribo nucleic acid
dNTP	-	Deoxyribo nucleotide triphosphate
EC_{50}	-	Effective concentration 50
FDA	-	Food and Drug Administration
FET	-	Fish Embryo Toxicity
GRAS	-	Generally recognized as safe
gDNA	-	genomic DNA
GSH	-	Glutathione (reduced)
gsr	-	Glutathione reductase
glo1	-	Glyoxalase 1
hpf	-	hours post fertilization
KCl	-	Potassium chloride
LD_{50}	-	Lethal dose 50
MDA	-	Malondialdehyde
$MgCl_2.6H_2O$	-	Magnesium chloride hexa hydrate
mRNA	-	messenger RNA
NaCl	-	Sodium chloride
NCBI	-	National Centre for Biotechnology Information
NTC	-	Negative Template Control
PCR	-	Polymerase chain reaction
ppm	-	Parts per million
RNA	-	Ribo nucleic acid
RNS	-	Reactive Nitrogen Species
RO	-	Reverse Osmosis

ROS	-	Reactive Oxygen Species
RT- PCR	-	Reverse transcription polymerase chain reaction
RT	-	Reverse transcriptase
SB	-	Sodium Benzoate
SRL	-	Sisco Research Limited
TAE	-	Tris-acetate-EDTA

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Abstract

The advent of food preservation has solved many food borne diseases and it also increased the food security over the years around the globe. From traditional boiling to modern day artificial food additives, there are numerous ways of preserving food items. The one that concerns most of the people around the globe is the addition of chemicals as preservatives in food items like jams, pickles, beverages etc. The most used chemical preservative sodium benzoate (SB) has gained more lime light due to some of its controversial effects on human health. Our study aims to investigate the toxic effects of sodium benzoate on the development, behaviour and oxidative stress in zebrafish embryos. We exposed zebrafish larvae of 5 hpf with 100 to 2000 ppm of SB and studied developmental, behavioural and gene expression changes. Our studies indicated that SB induced morphological abnormalities like pericardial edema, Yolk sac edema and tail bending. The malformations were more pronounced with the increase in dose of SB and time of exposure and also SB was found to delay the hatching process. The LC_{50} was found to be around 400 ppm at 48 hours of drug exposure. The behavioural experiment indicated increased thigmotaxis in treated larvae and there was a two fold increase in the gene expression of *gsr* (glutathione reductase) and no change in *glo1* (glyoxalase1) expression. Our study strongly supports the toxic effects on vertebrates at increasing doses. Thus, we suggest caution in the extensive use of this preservative in processed and convenience foods.

Contents

Introduction

1.1 Sodium Benzoate

Sodium benzoate is the sodium salt of benzoic acid. It is soluble in water, ethanol, methanol and ethylene glycol. Its molecular weight is 144.11 and has a melting point above 300^{o}C [1]. It is a white crystalline powder, odourless with sweetish and astringent taste [2]. FDA as classified sodium benzoate under "GRAS" (Generally Recognized as Safe), with the E number E211 [3]. It is a widely used food preservative in jams, pickles, salad dressings, carbonated beverages. Sodium benzoate is highly absorbed via dermal contact and via the food. It is converted into hippuric acid in the mitochondria and excreted by urine and bio accumulation is very less [1]. The undissociated benzoic acid has fungicidal and bacteriostatic action, by which it helps to prevent the microbial growth in foods with acidic nature [1]. The allowed limit of sodium benzoate in the food products are 0.1% by FDA.

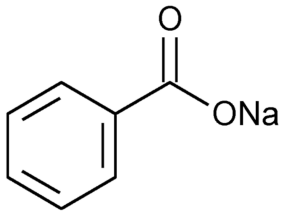

Figure: - 1. Structure of Sodium Benzoate

Figure: - 2 Food and shampoo labels indicating Sodium Benzoate

1.2 Anxiety

Anxiety is a mental state illness characterised by excessive worry about everything in one's life [4]. Occasional anxiety is experienced by all individuals but it becomes a disorder when it lasts for long time without any actual problem or fear. There are several types of anxiety disorders they are generalized anxiety disorder, panic disorder and social anxiety disorder [5]. The symptoms include restlessness, irritability, sleep problems, sweating, rapid heartbeat, muscle tension etc. [5]. Anxiety disorders can happen due to genetics, behavioural inhibition in childhood, traumatic events in the past, troubled childhood [5].

1.3 Oxidative stress

Free radicals are produced as by-products of ATP production and various other metabolic processes, molecular oxygen is one of the most widely produced free radical [6]. Theses free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) cause oxidative stress to the cell leading to cell damage. Oxidative stress can cause lipid peroxidation and the intermediate products formed by the free radicals cause damage to the cells [7]. It has been reported that oxidative stress is one of the causes for various neuro degenerative diseases like ALS, Schizophrenia, major depression etc. [7]. There is also a correlation between oxidative stress and anxiety phenotype [8]. In our

study we are using zebrafish as our model organism to study the effect of sodium benzoate in anxiety.

1.4 Zebrafish Model

Zebrafish (*Danio rerio*) is a fresh water fish and a widely used vertebrate model for research studies. They are emerging model to study the developmental toxicology because of rapid development and their fertilization occurs externally. The zebrafish embryos are transparent and their development can be seen using microscope. They produce hundreds off springs at a single time [9]. Zebrafish is also a validated model for performing behavioural experiments, since behaviour is associated with neuronal development. The structure of zebrafish brain is very much similar to the brain of humans. Thus for studying anxiety associated behaviour we have used Zebrafish model for experiment purposes [10].

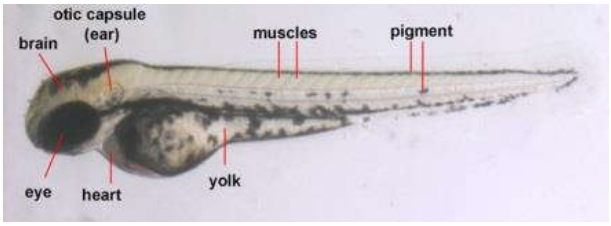

Figure: - 3. 48 hpf zebrafish larvae

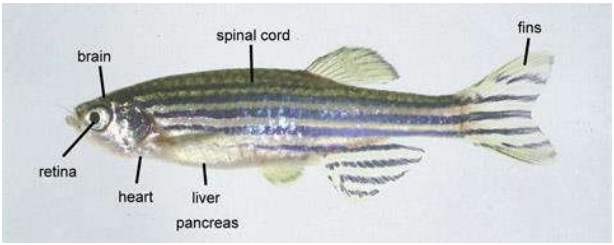

Figure: - 4. Mature Adult Zebrafish (90 days – 2years)

Review of Literature

2.1 Adverse effects of sodium Benzoate

2.1.1 In humans

There are very few clinical trials on human beings to test the adverse effects of sodium benzoate. McCann et al has reported ADHD like hyperactivity in children is seen in a community based, double blinded, placebo controlled group study. The challenge drink offered to the children consists of sodium benzoate along with one or two food additives and their hyperactivity level was measured using Global Hyperactive Aggregate. From their research the authors concluded that sodium benzoate and additives in the beverages resulted in increased hyperactivity in children [11]. Bateman et al also conducted a double blinded placebo control study where the preschool children were enrolled, in this study the children were observed under various periods like first diet devoid of preservatives and artificial colours, followed by a diet with preservatives and again diet devoid of the preservatives and artificial colours. Each period of study have measure their attention and impulsiveness through certain games and activities and also parental ratings of their children behaviour have taken into account. An increase in the impulsiveness and hyperactivity is observed in children and after the withdrawal of benzoate and other additives there is an observed decrease in their impulsiveness. From this study it is concluded that these preservatives and additives in the food cause impulsiveness in children which cannot be detected in clinical test but observed by their parents through the change of behaviour [12]. In another study by Beezhold et al it is reported that high intake of beverages with sodium benzoate may lead to ADHD like hyperactivity in college students. This study was conducted by survey questionnaire and the results were analysed using ADHD screening tool, Adult Self Report Scale. The study reported that the college going population is highly exposed to beverages with preservatives and their intake may exceed the daily allowed intake and make them a vulnerable group for clinical hyperactivity [13]. There was one study done in humans by Lennerz et al., which relates SB and glucose homeostasis. In this study 14 over weight human subjects were given SB challenge and the change in 146 metabolites were targeted out of these there were significant increase in metabolites like hippuric acid,

anthranallic acid, benzoate and hippurate, but the study concluded that GRAS doses of SB does not have acute effect on glucose metabolism [14].

2.1.2 In animal models

The adverse effects of sodium benzoate was found in animal models like Zebrafish, mouse etc. Neurotoxicity has been reported by Tsay et al and Chen et al., in zebrafish model and Noorafshan et al., has reported damage to the cerebellum region in mouse models. Tsay et al has reported that sodium benzoate causes malformations in the zebrafish larvae. The malformations include gut abnormalities, altered muscle fiber alignments, neuro muscular junction outgrowth, malformation of pronephric tube and teratogenicity [15]. Chen et al has described about the downregulation of tyrosine hydroxylase enzyme and Dopamine transporter in the neurons of ventral diencephalon, which leads to decreased locomotor activity. The decrease in the expression was reported to be dose and time dependent [16]. Another study conducted by Noorafshan et al has showed that the structure of cerebellum was decreased after treated with sodium benzoate which could possibly answer the motor impairment and impulsiveness in the rat model [17]. Yadav et al has proposed the immunomodulatory effect of sodium benzoate in female rats. Sodium benzoate was found to suppress the functional response of T and B lymphocytes by modulating the expression pattern of various activation receptors, co stimulatory molecules and regulatory cytokines [18].

2.1.3 Cell lines

The genotoxic effect of sodium benzoate was reported by Zengin et al in human lymphocytes by using chromosomal aberration, sister chromatid exchange and micronuclei analysis. Sodium benzoate increased the chromosomal aberrations significantly in human lymphocytes and decreased the mitotic index [19]. This was supported by the genotoxicity studies performed by Pongsave by using human lymphocyte cell line. This study has showed that sodium benzoate induces micronuclei induction and gap in chromosomes [20].

2.2 Sodium benzoate and oxidative stress

Limited studies done by the researchers have showed some mixed views on the relation between sodium benzoate and oxidative stress. Yetuk et al has reported that sodium benzoate at higher concentrations induce oxidative stress and it leads to lipid

peroxidation in the erythrocytes and also leads to reduced enzymatic activity of several important antioxidant enzymes like superoxide dismutase, Glutathione reductase etc. [21] Piper et al., research suggests that in yeast cells benzoic acid and sorbic acid act as pro oxidant and they also found to be mutagenic towards the mitochondrial DNA of the yeast. The authors have discussed that frequent intake of weak acid preservatives like benzoic acid and sorbate may contribute to mitochondrial damages in humans [22]. While report from Modi et al., showed that SB is produced as a metabolite of cinnamon consumption. When cinnamon was administrated orally to Alzheimer mouse model, cinnamon broke down and released SB as metabolite, SB attenuated the oxidative stress produced in the hippocampal region and protected the neurons in the hippocampal region from damage and it was also reported to enhance the memory and learning in the mouse model [23]. Another report from Khasnavis et al., showed that SB upregulated the neuroprotective protein in the mouse brain [24] but a report from Khoshnoud et al., in mouse model suggested that consumption of SB decreased the GSH content in the mouse brain and increased MDA content in the brain. The report also suggested that short term consumption of SB lead to memory impairment and increased brain oxidative stress [25]. Further research is needed to investigate the relation between SB and oxidative stress.

2.3 Sodium benzoate and anxiety

Noorafshan et al., has conducted an experiment on Sprague- Dawley male rats, where the rats were given sodium benzoate through their diet and distilled water is given for control groups. The treated and control group animals behaviour and motor function was analysed using Elevated Pulse Maze and rotarod. From this study the authors conclude that in the sodium benzoate treated group the rats were showing motor impairment in the rotarod test and reduced performance in the Elevated Pulse Maze indicating the anxiety behaviour [26]. One study on mice model investigating oxidative stress has indicated a link between anxiety and oxidative stress by the expression of Glyoxalse1 and Glutathione reductase genes. These genes were found to be up regulated during anxiety and when these gene expression were knocked down the anxiety phenotype was found to be reduced [8]. However no such data is available in the context of SB although reports have shown individually that it may cause anxiety but the

the context of SB although reports have shown individually that it may cause anxiety but the molecular evidence was lacking and also there were controversial results for the oxidative stress induced by sodium Benzoate.

Therefore we wanted to study the developmental changes, biochemical and behavioural changes due to Sodium Benzoate in Zebrafish model.

Scope of the study

3.1 Aim

Data regarding toxicity of SB is divergent and controversial with studies reporting both harmful and beneficial effects. Therefore, we did a systematic dose dependent toxicity study of SB using zebrafish vertebrate animal model. We also investigated oxidative stress and anxiety-like behaviour in zebrafish larvae treated with SB

3.2 Objectives

- To find the effect of sodium benzoate on the mortality and hatching rate of zebrafish embryos
- To find the LD_{50} and EC_{50}
- To study the behavioural changes due to sodium benzoate
- To find the gene expression of *gsr* and *glo1* due to sodium benzoate (oxidative stress)

Materials and Methods

4.1 Zebrafish Housing

Zebrafishes were purchased from local commercial supplier and maintained in 10L and 6L rectangular tanks. The fishes were maintained in RO water with 4ml and 2ml of E3 medium which consisted of (0.0595 NaCl, 0.021 KCl, 0.039 $CaCl_2.2H_2O$ and 0.048 $MgCl_2.6H_2O$: pH 7.2, sterile) (in M) respectively. The fishes were given pellet diet twice a day. The tanks were cleaned on the alternative days. Temperature was maintained at $26^oC \pm 28^oC$ using heaters and dissolved oxygen was provided by air stone.

4.2 Zebrafish mating

In order to get embryos, the fishes were maintained in separate mating chambers. It was a rectangular box with mesh inside to collect the embryos at the bottom while the fishes were kept in the upper chamber. Two female fishes and one male fish were used for mating (2:1) ratio. The fishes were given 12 hours of dark condition and next day followed by 1 hour of light condition. The embryos were produced during the light condition and were collected using Pasteur pipette. The embryos were washed with RO water and cleaned thoroughly to remove the debris and washed twice with 1X E3 medium. The number of embryos were counted and maintained in a petri plate with 20 ml of E3 medium. The embryos were kept in the incubator at 28^oC for 5 hours before drug incubation.

4.3 Preparation of Drug solution

Sodium benzoate was purchased from SRL. 200000 ppm stock is prepared by dissolving 1g of sodium benzoate in 5ml of E3 medium in 15ml falcon tube and mixed using vortex mixer. The following table gives the preparation of the stock and respective working concentration preparation.

Table: -1 Preparation of stock

Concentration Needed (ppm)	Final Volume (µl)	Stock (ppm)	Volume to add from stock (µl)	Volume of E3 medium to add (µl)
20000	1000	200000	100	900
2000	1000	20000	100	900

Table: - 2 Preparation of working Concentration

Concentration Needed (ppm)	Final Volume (µl)	Stock (ppm)	Volume to add from stock (µl)	Volume of E3 medium to add (µl)
2000	2000	200000	20	1980
1000	2000	20000	100	1900
500	2000	20000	50	1950
200	2000	20000	20	1980
100	2000	2000	100	1900

4.4 Treatment with Sodium Benzoate

After 5 hours of incubation, the embryos were removed from the incubator. The dead ones were removed from the healthy embryos manually using Pasteur pipette. The drug exposure was carried out in a 24 well plate for a period of 96 hours according to Fish Embryo Acute Toxicity Test (FET) guidelines. Each well contained upto a maximum of 12 embryos, with 200µl of the drug solution in the following concentrations 100 ppm, 200 ppm, 500 ppm, 1000 ppm and 2000 ppm. Control embryos were maintained in E3 medium (control). Before drug treatment all E3 were removed from the well and washed with the respective drug solution and then placed for treatment. The solutions were changed after every 24 hours of treatment and the dead embryos were removed from the wells during the experiment period and recorded. Mortality and hatching rates were calculated as described in next section.

4.5 Hatching and Mortality rate

Hatching is the process where the zebrafish embryo comes out of the chorion layer. Hatching rate was recorded at various time points: 43 hours, 48 hours and 72 hours of drug exposure and was calculated using the below formula

% Hatching = No. of embryo hatched / Total number of live embryos * 100

Mortality was calculated at the end of every 24 hours for a period of 96 hours of drug exposure. Mortality rate was calculated using the below formula

% Mortality = No. of dead embryo/ Total number of embryos * 100

LC_{50} (lethal concentration, 50%), the dose required to kill 50% of the tested population after a specified test duration was obtained from the mortality curve at 48 hours post SB exposure.

4.6 Imaging the embryos

All the embryos were observed individually by placing in the glass slide under inverted bright field microscope, Olympus lX73 series in 0.5x zoom and at a resolution of 800* 600 with 4x objective. The pictures were captured using Procam HS-10 MP camera.

4.7 Motility

5 hpf zebrafish larvae were exposed to 50 ppm of SB for a period of 72 hours and larvae exposed to 1X E3 medium were taken as control. The drug solution was changed for every 24 hours. Number of larvae moved after swirling was considered as the end point of the experiment. Two different petri plate (90mm in diameter) were filled with 30 ml of 1X E3 medium; about 20 embryos were placed in each petri plate (one for control and other for treated), using Pasteur pipette. The petri plate was swirled three times and videos were recorded for a time period of 30 seconds. Videos were taken from the time of swirling. Motility rate was calculated as follows

% Motility = No. of larvae showed movement/ Total number larvae *100

A graph was plotted between % motility and concentration of SB

4.8 Thigmotaxis activity:

5 hpf zebrafish larvae were exposed to 50 ppm of SB for a period of 72 hours and larvae exposed to 1X E3 medium were taken as control. Thigmotaxis (preference of edge or wall) activity of the larvae was considered as an end point for anxiety like behaviour. Two separate well plates were used monitor the behaviour of control and Sodium benzoate treated larvae. Each well was filled with 500µl of E3 medium. One larva was dropped in one well and the video was recorded for 30 seconds with digital camera. The same procedure was repeated for both control and treated. The number larvae moved to the boundary of the well is noted and % thigmotaxis was calculated as follows

%thigmotaxis = No. of larva moved towards the wall/ Total number of larvae * 100.

A graph was plotted between % thigmotaxis and concentration of SB.

4.10 RNA Isolation

RNA isolation was done in order to study the gene expression changes due to sodium benzoate treatment. 48 hours treated larvae at 400 ppm and 50 ppm were taken for this experiment. RNA isolation was done by TRIzol® method. Total RNA was isolated from the treated cells using TRIzol® reagent (Life technologies). TRIzol® reagent is a monophasic solution of phenol, guanidine isothiocyanate and other proprietary components which facilitate the isolation of a variety of RNA species of large or small molecular size. TRIzol® reagent maintains the integrity of the RNA due to highly effective inhibition of RNase activity while disrupting cells and dissolving all cell components during homogenization.

Materials Required:

- Lab coat
- Gloves
- RNaseZap
- Ice and ice bucket
- Autoclaved Pipettes
- Sterile Barrier tips
- Autoclaved eppendorf tubes (1.5 ml, 0.5 ml)
- Hand held homogenizer (MIULAB)

- Autoclaved homogenizer tips
- TRIzol reagent
- Chloroform
- Isopropanol
- 100% ethanol
- Nuclease free water
- Heating block (genetix)
- Cooling Centrifuge with 12,300g force (Thermo scientific)
- Kim wipes

Procedure:

- The workbench, pipette handles and homogenizer tips were cleaned with RNaseZap
- The samples stored in TRIzol (-80^{o}C) were thawed in ice and proceed to homogenization. In case of live embryos remove all E3 medium and add 1ml of TRIzol and shake vigorously and proceed to homogenization.
- Homogenization was done using handheld homogenizer for 30 seconds in minimal speed. Note: Use different homogenizer tips for different samples.
- The homogenized samples were incubated for 5 minutes in ice to permit complete dissociation of the nucleoprotein complex.
- After 5 minutes of incubation the samples were centrifuged for 10 minutes at 12000g at 4^{o}C. The supernatant was taken in a fresh 1.5 ml eppendrof tube and proceed with the following steps
- 200 µl of chloroform was added to the supernatant and mixed vigorously by invert mixing for 15 seconds and incubate in ice for 5 minutes.
- The samples were centrifuged at 12000g for 15 minutes at 4 ^{o}C
- The contents of the tubes now separate into Upper aqueous phase – RNA; Interphase – DNA; Lower red phase - Protein
- The aqueous phase is carefully transferred to new microfuge tube.
- 1:1 ratio of Isopropanol was added to the transferred aqueous phase.
- The samples were mixed vigorously by invert mixing for 15 seconds and incubated in ice for 10 minutes.
- The samples were centrifuged at 12000g for 10 minutes at 4^{o}C

- The supernatant was discarded after centrifugation and the RNA pellet is obtained at the bottom of the tube.
- The pellet was washed, with 500 µl of 75 % Ethanol.
- The samples are, then centrifuged at 12000g for 5 minutes at 4^{o}C
- The pellet was again washed with 75% ethanol and centrifuge at 12000g for 5 minutes at 4^{o}C.
- After centrifugation, the ethanol was discarded and the pellet was allowed for drying. Use kim wipes to absorb the extra ethanol from the tube.
- The RNA pellet was resuspended in 30 µl nuclease free water.
- The samples were kept in the water bath at 55 ^{o}C for 5 minutes and the tubes were tapped twice in between. This was done in order to open up the isolated RNA.
- The obtained RNA was spanned for 30 s with minimum g say 1500g and then aliquoted in three tubes with 10 µl each.
- The isolated RNA needs to be quantified for purity check and for calculation of volumes to be added for cDNA conversion. Quantification is done using Nanodrop (ND TM 1000).
- 2 µl of resuspended RNA was placed in the Nanodrop and absorbance ratio at 260/280 and 260/230 are measured along with concentration of the obtained RNA. Ideal $A_{260}/A_{280} = 1.9 – 2.0$; $A_{260}/_{230} = < 1$
- The isolated RNA can be stored in -80^{o}C. Avoid repeated freeze thawing of the RNA sample as it may result in degradation.
- NOTE: 15 µg of RNA is ideally expected from 50 zebrafish embryos.

4.11 RNA Gel:

RNA gel is prepared to check the integrity of the isolated RNA.

Materials Required:

- Agarose
- SYBR Safe
- 1x TAE Buffer
- Microwave oven
- Electrophoresis unit with power supply

- 1x Gel loading dye

Procedure:

- 0.5g of agarose was measured and dissolved in 50ml of 1x TAE buffer (1%). The mixture was heated in microwave oven until the solution became transparent.
- The solution was cooled and when it was little warm 5µl of SYBR safe (photosensitive make sure to switch off the light before adding it) was added and poured in the casting tray to solidify and the gel was covered
- 5µl of isolated RNA was taken and mixed with 1µl of gel loading dye (in case of 6X dye), if you use 10X gel loading dye, take 9 µl of RNA sample mixed with 1 µl of gel loading dye.
- Once the gel was turned opaque and solidified, it was kept in the electrophoresis unit and covered with 1x TAE buffer.
- The prepared sample was loaded into the gel and the power supply was switched on
- 50V was given at the start; once the RNA crossed from the well the voltage was changed to 70V.
- Once the gel travelled 3/4th distance, power supply was switched off and gel was viewed under UV transilluminator.
- Two bands were observed indicating the 28s and 18s RNA.

4.12 cDNA preparation

The integrity is checked, if the RNA is of good quality it is taken for cDNA preparation. The cDNA preparation was done using Quantitect Reverse transcriptase kit (Qiagen).

- The cDNA kit components were thawed on ice. The genomic DNA elimination reaction is prepared according to the following table

Table: - 3 Genomic DNA elimination reaction

Components	Volume to add (µl)
gDNA Wipeout buffer 7x	2
Template RNA (1µg)	Varies according to experiment
RNase free water	Varies according to experiment
Total Volume	14

- The reaction mixture was incubated for 2 minutes at 42°C in dry bath and after incubation it is placed in ice
- The reverse transcription mixture was prepared according to the following table

Table: - 4 Reverse transcription reaction mixture

Components	Volume to add (µl)
Reverse transcriptase	1
RT buffer 5x	4
RT primer mix	1
Template RNA (from gDNA elimination reaction)	14
Total	20

- The reaction mixture was incubated for 15 minutes at 42°C
- The reaction mixture was incubated at 95°C for 3 minutes to inactivate the enzyme.
- The obtained cDNA was stored at -20°C.

4.13 Primer design

The gene specific primer sequences for *actb1*, *gsr* and *glo1* taken from previously cited works. The primer sequences were aligned with the mRNA sequences retrieved from Nucleotide database of NCBI. The mRNA sequences and the primer sequences were aligned using multiple sequence alignment in ClustalOmega server. Refer the appendix page for primer alignment results, T_m and length of the primers.

Table: - 5 List of gene specific primers

Gene	Forward primer	Reverse primer	References
actb1	CCGTGACATCAAGGATAAGCT	TCGTGGATACCGCAAGATTCC	[28]
gsr	TGAAAAGGGCAAAATTGAGTTTA	TTTCGAGAGGTAATGGCGTAATA	[29]
glo1	CCGCGTGTAAAGAGGGAAGC	GGCAGCATAGACATCCGGTAC	[30]

4.14 RT-PCR

PCR is an enzymatic DNA amplification process, mimicking to some extent in vivo replication, divided into a series of cycles. Theoretically, if optimal reaction conditions exist, every cycle of the PCR process doubles the amount of the desired DNA fragment available in the sample, resulting in exponential product accumulation.

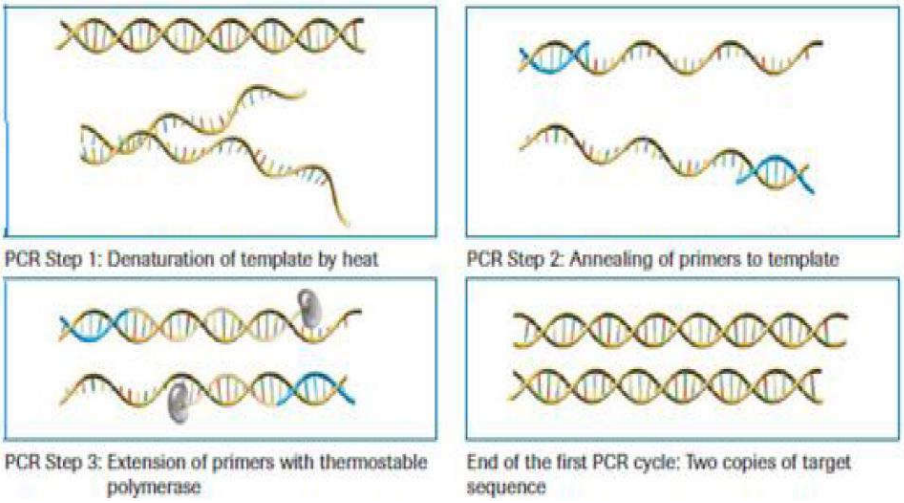

Figure: - 5 Overall PCR reaction process

Reverse Transcription Polymerase Chain Reaction (RT-PCR) is a type of PCR which is used to study the gene expression changes semi quantitatively.

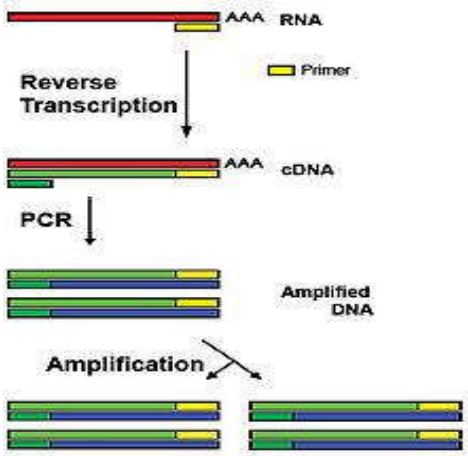

Figure: - 6 Overall process of RT-PCR

In this method the isolated RNA was subjected to first strand cDNA conversion with help of reverse transcriptase enzyme, and then the cDNA was subjected to normal PCR

reaction with gene specific primers. PCR reaction was carried out as mentioned in the table.

Prepare Master Mix according to the available samples. The master mix should be prepared excluding the template and primers. While preparing the Negative Template Control (NTC) PCR reaction mix, template cDNA should not be added.

Table: - 6 PCR Reactions

Components	Volume (µl)
HS Taq DNA Polymerase (1.25 U)	0.25
Buffer (1X)	5
DNTP's (200 µM)	4
DMSO (3%)	1.5
Forward primer (200 nM)	1
Reverse primer (200 nM)	1
cDNA template (1 µg)	1
Sterile MilliQ water	36.25
Total	50

PCR Program:

- Initial Denaturation - 94^{o}C - 3 minutes
- Denaturation - 94^{o}C - 45 seconds
- Annealing - 42^{o}C - 45 seconds
- Extension - 72^{o} C - 1 minute
- Final Extension - 72^{o} C - 10 minutes

No. of cycles: 30

4.15 Gel quantification by ImageJ

- ImageJ software was opened. The gel image (make sure the bands are horizontal) to be quantified in the ImageJ window was dragged and dropped.

- After the image was opened, select rectangular selection in the upper left corner of the tools menu bar.
- A suitable Region of Interest was selected as shown in the figure, covering the width and height of the band.

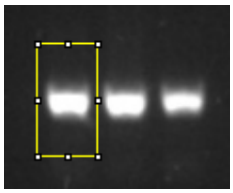

Figure: - 7a Suitable Region of Interest

- After the Region of interest was selected, press ctrl and #1 simultaneously. The band would be marked as 1.
- The same rectangular box was dragged and fitted into the next band and ctrl and #2 was pressed simultaneously. Repeat the same step for next band also. The bands would look like figure 7b

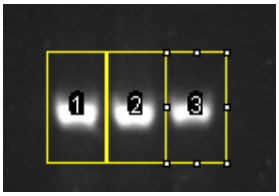

Figure: - 7b Marked bands

- After ctrl and #3 were pressed, inverted histograms would be generated which indicate the gel intensity.
- Once the histograms were generated, a line was drawn using the line option in the tool bar across the top of the histogram from where it first begins to drop steeply until where it levels out again.

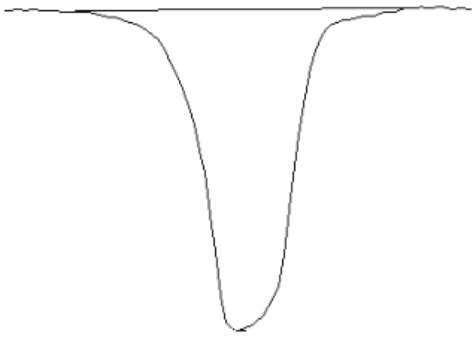

Figure: - 7c Marked histogram

- Using the magic wand symbol in the tools menu, the click anywhere inside the histogram, the selected area would be coloured in yellow and a new window would appear as "Results" which indicate the intensities of the band as a numerical value. Brighter the band higher the number.

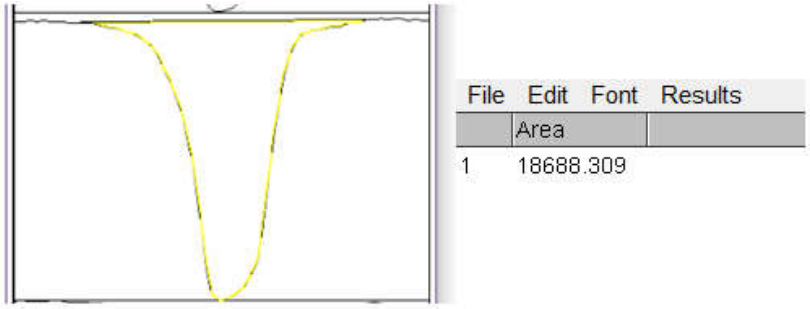

Figure: - 7d Highlighted histogram and results tab

- The results were copied and pasted in the excel sheet and the intensities of *gsr* and *glo1* were normalized to housekeeping gene *actb1*

4.16 Statistics

All the dose response experiments were performed five times independently. The statistical analysis was done by one way ANOVA followed by Dunns post-test. All the tests were performed using Graphpad prism software version 5.0. The LD_{50} values were plotted using the 48H mortality data as Mean±SEM using Hill equation in Origin software version 94E. The behaviour experiments were performed five times independently and statistical analysis is done by Mann-Witney t-test using Graph pad prism software version 5.0. The RT-PCR experiments were performed three times independently and statistical analysis is done by Mann-Whitney t-test using Graphpad prism software version 5.0, where $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$

Results

5.1 SB induced physical malformations

To study the physical malformations induced by sodium benzoate, the zebrafish embryos were exposed after 5 hpf (hours post fertilization) to the various concentrations of sodium benzoate: 100, 200, 500, 1000 and 2000 ppm. The embryos were examined for every 24 hour time interval and their images were taken. As shown in Figure 8, the larvae showed abnormalities like pericardial edema, yolk sac edema and tail bending, while control group larvae showed no such abnormalities. The abnormalities increased with time and dose.

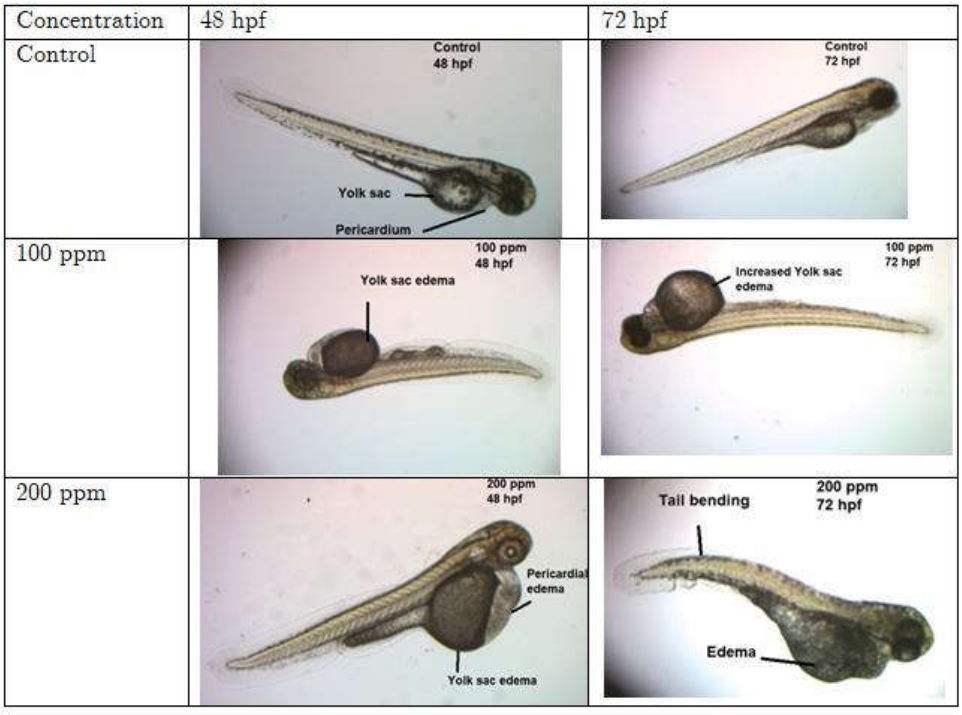

Figure: - 8 Abnormalities observed in zebrafish larvae upon SB treatment.

The first panel was the larvae from control with normal yolk sac and pericardium and with normal development. The second and third panel showed the abnormalities from 100 and 200 ppm concentration where the edema increased with time in 100 ppm and at 200 ppm the edema was severe and at the end of 72H leading to the death of the larvae.

5.2 Effect of SB on Hatching

To examine the developmental defects in the zebrafish embryos, the zebrafish embryos were exposed to Sodium benzoate after 5 hpf (hours post fertilization) and their hatching rate were monitored at various time points: 43H, 48H and 72H. As shown in Figure 9 at the end of 48H of drug exposure there was a significant decrease in the hatching rate from 100% ± 0% (N=46) to 74.89%± 15.38 (N=46), 3.33% ± 3.33% (N=47) in control, 100 ppm and 500 ppm respectively. Normally embryos hatch by 48 hpf, so we selected three time points such as 43 hours, 48 hours and 72 hours of SB exposure.

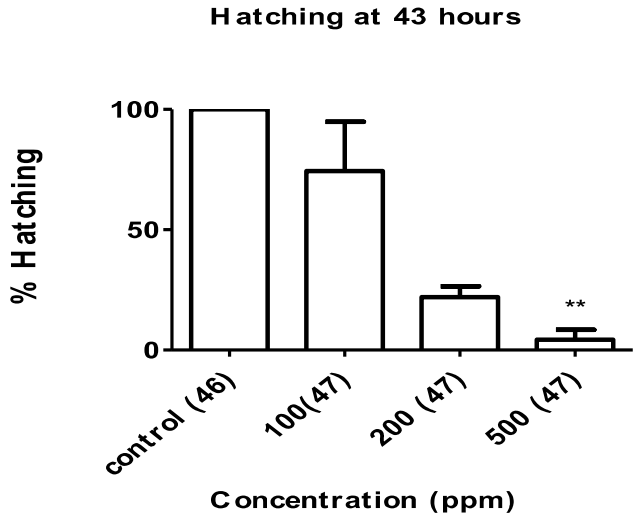

Figure: - 9a Effect of SB on hatching at 43 hours exposure

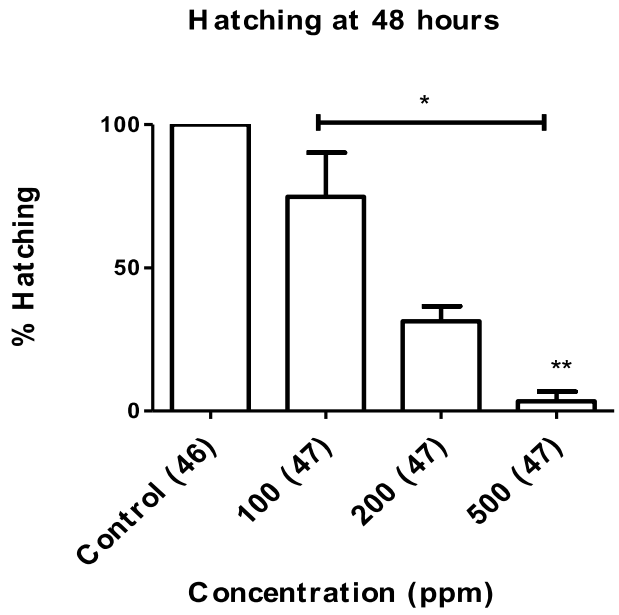

Figure: - 9b Effect of SB on hatching at 48 hours of SB exposure

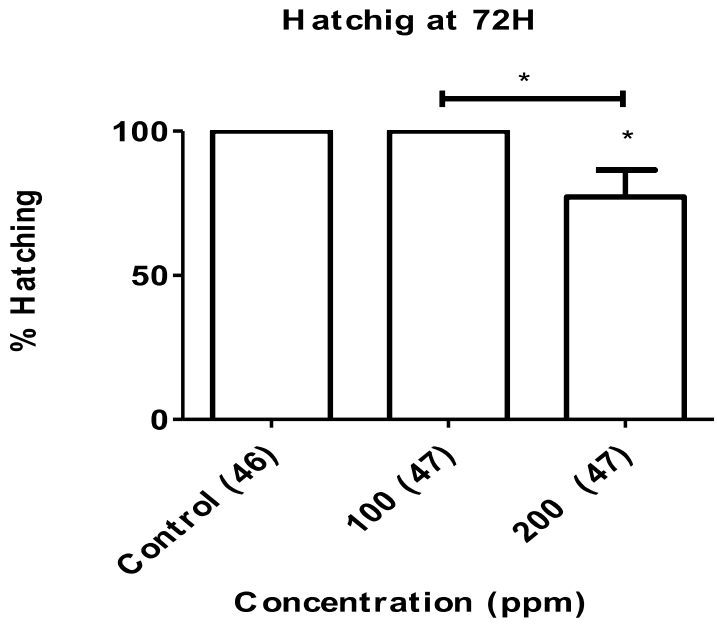

Figure: - 9c Effect of SB on hatching at 72 hours of SB exposure

Sodium benzoate causes delay in hatching process of the zebrafish embryo. The experiments were performed five times and the data was represented as mean (±SEM). The number of larvae was shown in parenthesis.

5.3 Effect of SB on Mortality

To study the effect of SB on the mortality rate, 5 hpf zebrafish larvae were exposed to the various concentration of SB: 100 ppm, 200 ppm, 500 ppm, 1000 ppm and 2000 ppm respectively. The mortality rates were observed and recorded at the end of every 24 hours of drug exposure. 100% mortality was observed in 1000 and 2000 ppm at the end of 24 hours of SB exposure. While, at the end of 48 hours the mortality rate was significantly increased from 2.5% ± 2.5%(N=47) to 3.64% ± 3.64% (N=47), 80.00% ± 12.53% (N= 47) and 100% ± 0% (N=46) and 100% ± 0% in 100 ppm, 200 ppm, 500 ppm, 1000 ppm and 2000 ppm respectively as shown in figure 10b. From the graph we could conclude that mortality rate was dose dependent. As the time increases the mortality rate also significantly increased from 27.95%±8.68% (N=47) to 66.67%±19.9% (N=47) and 100% (N=47) in 100 ppm, 200 ppm and 500 ppm concentrations of SB respectively which was shown in Figure 10c and 100% mortality was observed in all concentrations except the control at the end 96 hours as shown in figure 10d. From this we could conclude that SB induced mortality in a time dependent manner. On the whole SB induced mortality was time and dose dependent manner.

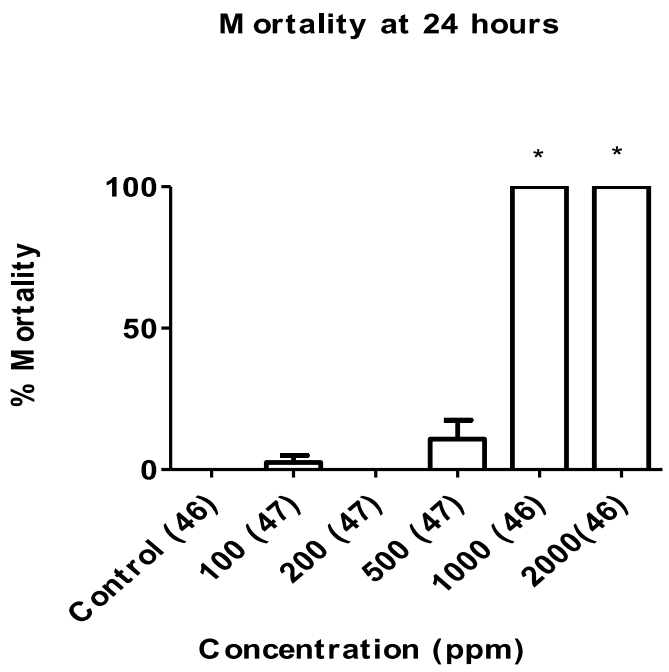

Figure: - 10a Effect of SB on mortality at 24 hours of exposure

The experiments were performed five times and the data was represented as mean (±SEM). The number of larvae was shown in parenthesis.

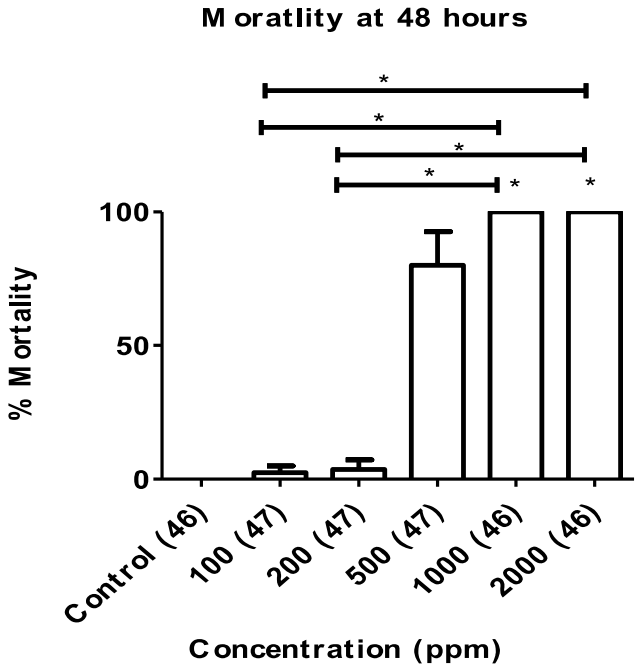

Figure: - 10b Mortality rate is dose dependent.

The experiments were performed five times and the data was represented as mean (±SEM). The number of larvae was shown in parenthesis.

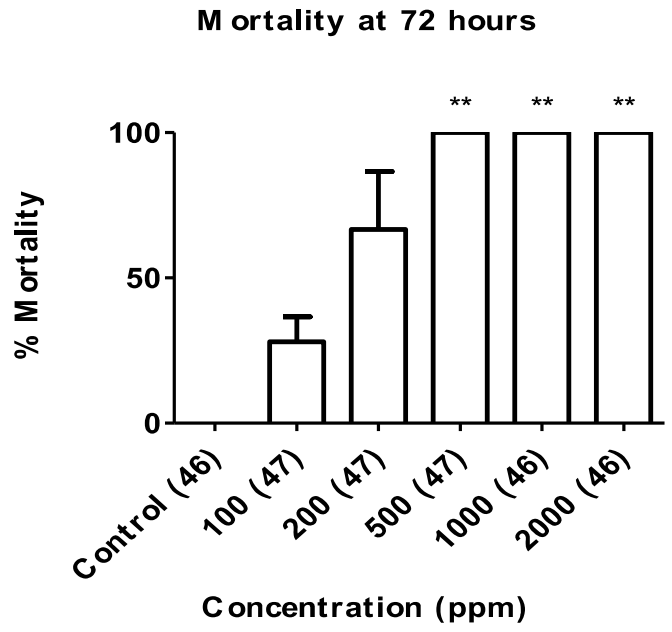

Figure: 10c Mortality rate is time dependant.

The experiments were performed five times and the data was represented as mean (±SEM). The number of larvae was shown in parenthesis.

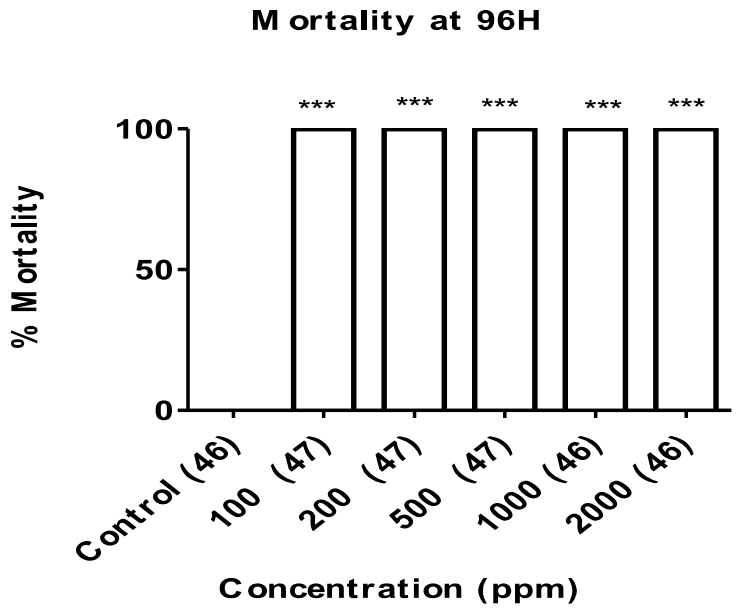

Figure: - 10d Mortality at 96 hours of SB exposure

The experiments were performed five times and the data was represented as mean (±SEM). The number of larvae was shown in parenthesis.

From the cumulative mortality, LD_{50} was calculated. LD_{50} is one of the parameter to measure the toxicity potential of any drug/chemical compound. In order to find out the LD_{50} value of SB, dose response curve was plotted for 48 hours' time point and the nonlinear curve was fitted using hill's equation using origin software as shown in Figure 11. We obtained the LD_{50} as 400 ppm.

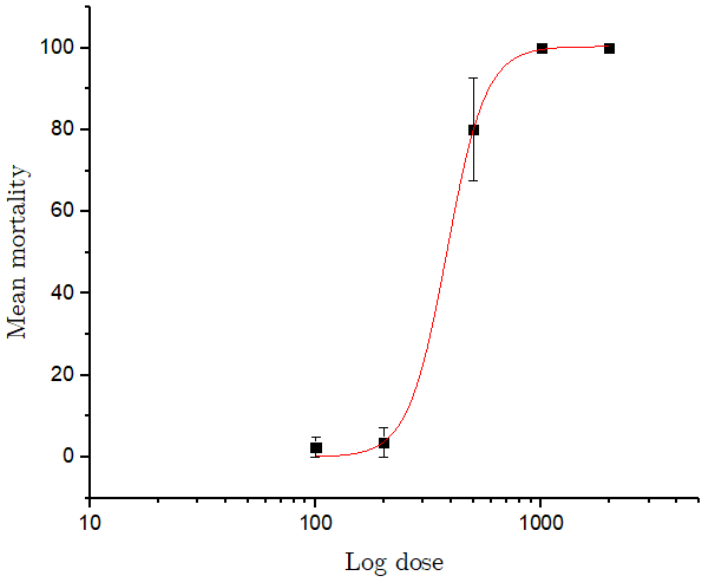

Figure: 11 Dose Response curve.

The curve was fitted with Hill's equation. The data represented as mean (±SEM).

5.5 Treatment with SB leads to reduced motility

Next, to investigate whether SB leads to any locomotor abnormalities in zebrafish larvae, 5 hpf zebrafish embryos were treated with 50 ppm sodium benzoate. After 72H of drug exposure the larvae were subjected to motility experiment. Movement of the larvae after swirling in the petri plate was considered as the end point. From the figure 12 it was shown that larvae (N=81) treated with sodium benzoate showed reduced motility of 11.01% ± 2.32% compared to control (N=80) 14.88% ± 1.24%. From this we conclude that treatment with Sb reduced the motility in zebrafish larvae.

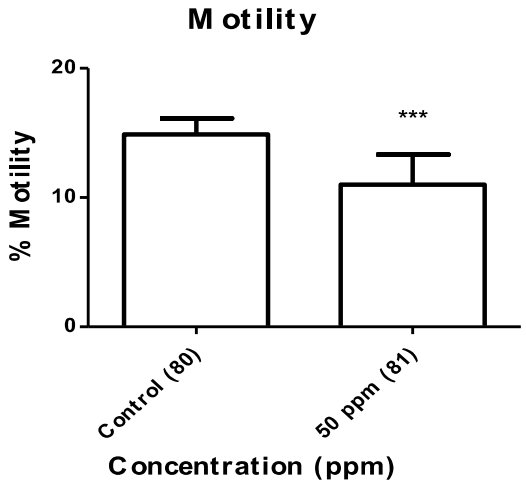

Figure: - 12 Effect of SB on Motility.

Motility of the Zebrafish larvae is reduced upon SB treatment. The experiments were performed three times and the data is plotted as mean (±SEM)

5.6 Treatment with Sodium benzoate leads to anxiety in zebrafish larvae

Next, to investigate whether sodium benzoate induces anxiety in zebrafish larvae, 5 hpf zebrafish embryos were treated with 50 ppm sodium benzoate. After 72H of drug exposure the larvae were subjected to behavioural experiment. Thigmotaxis was used as the parameter to measure the anxiety in zebrafish larvae. From figure 13, it was shown that larvae (N=40) treated with sodium benzoate showed increased thigmotaxis activity of 33.04% ± 4.47% compared to control (N= 40) 23.21% ± 1.78 %. From this we could conclude that treatment with SB leads to anxiety in zebrafish larvae. For this experiment larvae showing physical abnormalities were eliminated from the study to avoid the overlap of motility abnormalities with thigmotaxis.

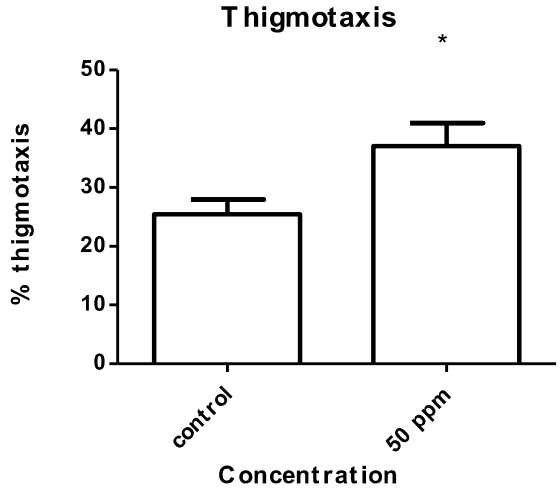

Figure: - 13 Thigmotaxis in SB treated larvae.

The experiments are performed five times and the data was represented as mean (±SEM).

5.7 Gene expression of gsr and glo1 upon SB treatment

Next, to examine the genotypic changes induced by the treatment of sodium benzoate, 5 hpf larvae was exposed to 400 ppm and 50 ppm of Sodium benzoate and the gene expression of glutathione reductase and glyoxalase 1 were studied using semi quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) at the end of 48H of drug exposure this time point was selected from the dose response curve. Glutathione reductase is one of the important anti-oxidant defense mechanism which catalyses the reduction of GSSG (glutathione disulphide) to GSH (glutathione) which reacts with free radical and reduces oxidative stress, while Glyoxalase 1 eliminates the toxic methylglyoxal formed as a by-product during glycolysis by eliminating the toxic by-products glo1 prevents the cell from oxidative stress. The band intensities were quantified and normalized to housekeeping gene *actb1* and plotted as shown in Figure 14a and Figure 14b

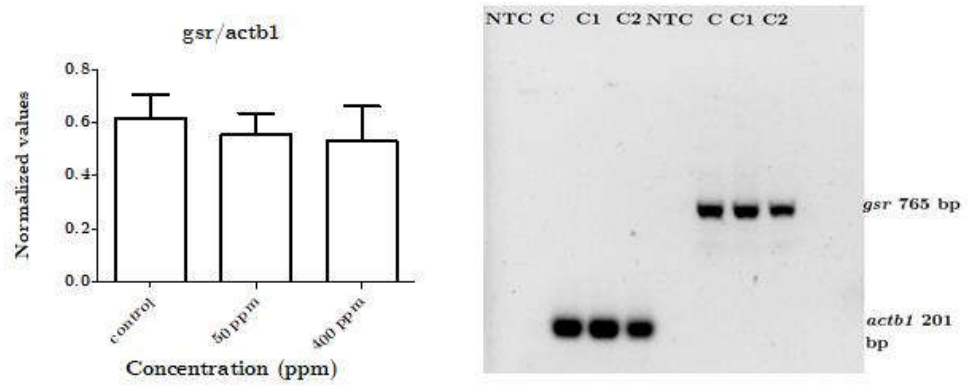

Figure: - 14a Gene expression of gsr upon SB treatment.

RT-PCR is used to study the gene expression changes in *gsr*. The PCR products were separated using 1% agarose gel. NTC – Negative Template Control; C1- 400 ppm; C2 – 50 ppm; *actb1* - 201 bp; *gsr* – 765 bp; Band intensities of *gsr* normalized to housekeeping gene *actb1*. The experiments were performed three times and the data was represented as mean (±SEM)

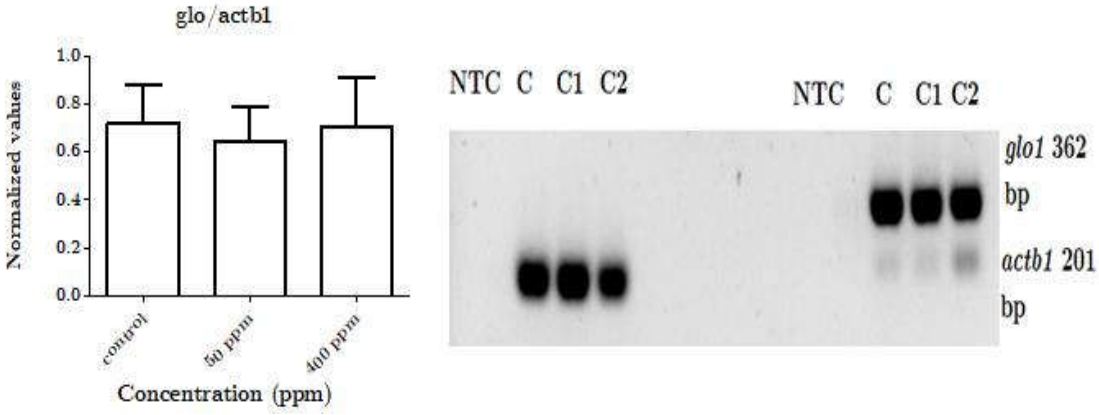

Figure: - 14b Gene expression of glo1 upon SB treatment.

RT-PCR is used to study the gene expression changes in *glo1*. The PCR products were separated using 1% agarose gel. NTC – Negative Template Control; C1- 400 ppm; C2 – 50 ppm; *actb1* - 201 bp; *glo1* – 362 bp; Band intensities of *glo1* normalized to housekeeping gene *actb1*. The experiments were performed three times and the data was represented as mean (±SEM).

5.8 Gene expression of gsr and glo1 upon SB treatment analysed by qPCR

Our RT-PCR results at two concentrations of SB exposure; 50 ppm (concentration same as behaviour experiments) and 400 ppm (rounded off LC_{50}), did not shown any changes in the expression of both gsr and glo1 genes as described above. However in our lab using qPCR approach (which is more sensitive than RT-PCR), we found that expression of gsr was found to be 2-fold upregulated in the SB treated group with no change in glo1 gene expression [30]. These qPCR experiments were part of work done by a Ph.D. student in our lab. qPCR experiments were performed at 400 ppm (rounded off LC_{50}) to maximize any changes in the gene expression that may be happening due to treatment with SB.

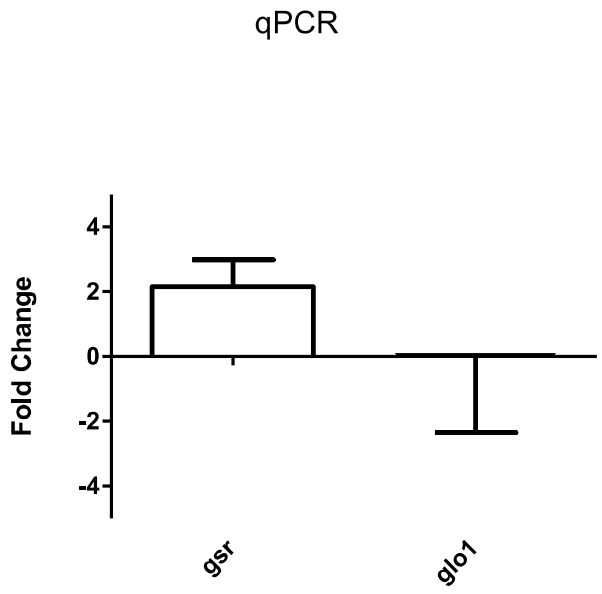

Figure: - 15 qPCR gene expression data

The qPCR data showed fold change as compared to control. The experiments were performed two times (in replicates) and the data is plotted as mean ± SEM.

Discussions

The use of preservatives like sodium benzoate in the food has prevented food borne diseases all over world and increased food security over a period of time. Nowadays preservatives are part and parcel of our daily life, from food to cosmetics everywhere we can find them. Growing need, made these preservatives for extensive usage. Even though FDA has placed Sodium Benzoate in GRAS category, the usage of these preservative in our daily routine has exceeded the safety limit and therefore there is a raising need to examine the toxicity effects of Sodium benzoate and other preservatives. Our study has examined the toxic effects of sodium benzoate in Zebrafish model. In our study the effects of sodium benzoate in hatching rate and mortality rate are studied for a time of 96 hours as per Fish Embryo Acute Toxicity Test (FET) guidelines.

Physical malformations in zebrafish embryos upon sodium benzoate has been reported by Tsay et al., 2007, in agreement to their study, we also found similar malformations in the zebrafish embryos like pericardial edema, yolk sac edema and tail bending. The malformations were found to be progressed with the increase in dose of SB and time of exposure.

Normal zebrafish embryos hatch by 48 hpf (Kimmel et al., 1995) but in our experiments it is shown that in sodium benzoate treated embryos there is a delay in hatching process. We found that sodium benzoate does not make the larvae to hatch prematurely but the hatching process is delayed in a dose dependent manner.

According to the best of our knowledge, SB's effect on hatching has not been reported, so this makes our study the first to report the effect SB on the hatching rate of zebrafish embryos. In relation to humans delayed hatching in zebrafish larvae could be translated to developmental defects.

We also studied the effect of SB on the mortality rate of the zebrafish embryos as we did for hatching studies, Mortality rate is found to increase in dose and time dependent which is found to be in agreement with the previously reported work of Tsay et al., and Chen et al., 2009. But there is variation with the LD_{50} value, earlier report by Tsay et al., 2007 suggested 1400-1500 ppm range as the LD_{50} value but in our study we got around 400 ppm. This variation in LD_{50} might be due to the difference in the study design, as Tsay et al., 2007 exposed the larvae after 48 hpf we started our study from 5 hpf zebrafish embryo.

Next we moved on to study whether SB causes any behaviour changes in the zebrafish larvae. Since SB induces anxiety like behaviour in rodent model has been done by Noorafshan et al., 2014, we wanted to study this effect of SB in zebrafish model. Thigmotaxis activity which represents the preference of edge behaviour is a parameter to measure anxiety like behaviour in the zebrafish larvae model. If the larvae show increased thigmotaxis activity then it is more anxious. We found that larvae treated with SB showed more thigmotaxis activity compared to control larvae. From this we have concluded that SB induced anxiety like behaviour in zebrafish larvae.

Next we wanted to examine the genotypic changes caused by SB, Hovatta et al., 2006, have reported a relation between oxidative stress and anxiety. They found the upregulation of *gsr* and *glo1* in the mouse models which have anxiety, so we wanted to see the expression of these two genes upon SB treatment in the zebrafish larvae through Semi quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR). However our RT-PCR was not able to pick up small changes in the gene expression, but qPCR (Real Time PCR) data from our lab showed that there was an upregulation in the gene expression *gsr* but *glo1* showed no change in expression.

From this we might speculate that SB induces oxidative through the selective upregulation of glutathione reductase (*gsr*) and we can also speculate that anxiety like behaviour is also an implication of neurotoxicity caused by SB. Further investigations are required to find the link between SB induced anxiety like behaviour by modulating oxidative stress.

Overall, in the light of controversial data regarding oxidative stress and neuroprotective effects, this study provides important data about the potential toxic effects of SB and also provides data regarding the dose dependent effects of SB. We conclude that SB overuse can have potential toxic effects and suggest caution in its extended use as a preservative agent.

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Appendix

4.12 Primer design

Primer sequences were aligned with the mRNA sequence of the genes using ClustalOmega server. The following are the primer alignment files for *actb1*, *gsr* and *glo1*

```
CLUSTAL O(1.2.4) multiple sequence alignment


fp          ------------------------------------------------------------      0
actb1       GGCACGAGAGATCTTCACTCCCCTTGTTCACAATAACCTACTAATACACAGCCATGGATG      60
rp          ------------------------------------------------------------      0


fp          ------------------------------------------------------------      0
actb1       AGGAAATCGCTGCCCTGGTCGTTGACAACGGCTCCGGTATGTGCAAAGCCGGTTTTGCTG     120
rp          ------------------------------------------------------------      0


fp          ------------------------------------------------------------      0
actb1       GAGATGAGCCCCTCGTGCTGTTTTCCCCTCCATTGTTGGACGACCCAGACATCAGGGAGT     180
rp          ------------------------------------------------------------      0


fp          ------------------------------------------------------------      0
actb1       GATGGTTGGCATGGGACAGAAAGACTCCTATGTGGGAGATGAGGCTCAGAGCAAGAGAGG     240
rp          ------------------------------------------------------------      0


fp          ------------------------------------------------------------      0
actb1       TATCCTGACCCTCAATACCCCATTGAGCACGGTATTGTGACCAACTGGGATGACATGGAG     300
rp          ------------------------------------------------------------      0


fp          ------------------------------------------------------------      0
actb1       AAGATCTGGCATCACACCTTCTACAATGAGCTCCGTGTTGCCCCTGAGGAGCACCCTGTC     360
rp          ------------------------------------------------------------      0
```

```
fp        ------------------------------------------------------------      0
actbl     GTGCTCACTGAGGCTCCCCTGATCCCAAAGCCAACAGAGAGAAGATGACACAGATCATGT      420
rp        ------------------------------------------------------------      0

fp        ------------------------------------------------------------      0
actbl     TCGAGACCTTCAACACCCCTGCCATGTATGTGGCCATCCAGGCTGTGCTCTCTCTGTACG      480
rp        ------------------------------------------------------------      0

fp        ------------------------------------------------------------      0
actbl     CTTCTGGTCGTACTACTGGTATTGTGATGACTCTGGTGATGGTGTGACCCACACCGTGCC      540
rp        ------------------------------------------------------------      0

fp        ------------------------------------------------------------      0
actbl     CATCTATGAGGGTTACGCTCTTCCCCATGCCATCCTGCGTCTGGATCTAGCTGGTCGTGA      600
rp        ------------------------------------------------------------      0

fp        ------------------------------------------------------------      0
actbl     CCTGACAGACTACCTGATGAAGATCCTGACCGAGCTGGCTACAGCTTCACCACCACAGCC      660
rp        ------------------------------------------------------------      0

fp        ----------CCGTGACATCAAGGATAAGCT----------------------------      21
actbl     GAAAGAGAAATTGTCCGTGACATCAAGGAGAAGCTGTGCTATGTGGCCCTGGACTTCGAG      720
rp        ------------------------------------------------------------      0


fp        ------------------------------------------------------------      21
actbl     CAGGAGATGGGAACCGCTGCCTCTTCTTCCTCCCTGGAGAAGGCTATGAGCTGCCTGACG      780
rp        ------------------------------------------------------------      0

fp        ------------------------------------------------------------      21
actbl     GTCAGGTCATCACCATCGGCAATGAGCGTTTCCGTTGCCCCGAGGCTCTCTTCCAGCCTT      840
rp        ------------------------------------------------------------      0

fp        ------------------------------------------------------------      21
actbl     CCTTCCTGGGTATGGAATCTTGCGGTATCCACGAGACCACCTTCAACTCATCATGAAGTG      900
rp        ----------GGAATCTTGCGGTATCCACGA--------------------------      21

fp        ------------------------------------------------------------      21
actbl     CGACGTGGACATCCGTAAGGACCTGTATGCCAACACAGTGCTGTCTGGAGGTACCACCAT      960
rp        ------------------------------------------------------------      21

fp        ------------------------------------------------------------      21
actbl     GTACCCTGGCATTGCTGACCGTATGCAGAAGGAAATCACCTCTCTTGCTCCTTCCACATG      1020
rp        ------------------------------------------------------------      21

fp        ------------------------------------------------------------      21
actbl     AAGATCAAGATCATTGCTCCCCCTGAGCGCAAATACTCCGTCTGGATCGGTGGCTCCATC      1080
rp        ------------------------------------------------------------      21

fp        ------------------------------------------------------------      21
actbl     TTGGCCTCCCTGTCCACCTTCCAGCAGATGTGGATCAGCAAGCAGGAGTACGATGAGTCT      1140
rp        ------------------------------------------------------------      21
```

```
fp          ------------------------------------------------------------      21
actb1       GGCCATCCATCGTTCACAGGAAGTGCTTCTAAACAGAACTGTTGCCACCTTAAATGGCCT      1200
rp          ------------------------------------------------------------      21

fp          ------------------------------------------------------------      21
actb1       AGCAATGAGATTCAAACGAACGACCAACCTAAACTCTCGAACAGAACAAGATGACATCAG      1260
rp          ------------------------------------------------------------      21

fp          ------------------------------------------------------------      21
actb1       CATGGCTTCTCTCTGTATGGCGCATTGACTCAGGATGCGGAAACTGGCAAAGGGAGGTAG      1320
rp          ------------------------------------------------------------      21

fp          ------------------------------------------------------------      21
actb1       TTGTCTAACAGGGGAGAGCTTTCCCCGAGAGGACAACAATGTACATTTCTTTTAGTCATT      1380
rp          ------------------------------------------------------------      21

fp          ------------------------------------------------------------      21
actb1       CCAGAAGCGTTTACCACTTGCCCTCCTCACAATGGGCGTCCATGACCTTTTTGTTATAGT      1440
rp          ------------------------------------------------------------      21


fp          ------------------------------------------------------------      21
actb1       GTTTTATGTAAATTATGTACTCGATACATTGTTTTTCTTTTTGTACTTCAGCCTTAAACT      1500
rp          ------------------------------------------------------------      21

fp          ------------------------------------------------------------      21
actb1       TGGCCCAGTTTGTTATTGTTGCAAGAGGGGAAAGCTTTACCTTTTAAAAAGTGAAGATCT      1560
rp          ------------------------------------------------------------      21

fp          ------------------------------------------------------------      21
actb1       TGCAGGACTTCCCTAGGGTATGTGAATAAGGGATGTCCCTTGAAAATGTAAGCCAGGGTG      1620
rp          ------------------------------------------------------------      21

fp          ------------------------------------------------------------      21
actb1       TCTCTGTACACTGACAAGTCAACCCAAATAAACGTGCACATGTAAAACCAAAAAAAAAAA      1680
rp          ------------------------------------------------------------      21

fp          -------     21
actb1       AAAAAAA     1687
rp          -------     21
```

Figure: - 15a Multiple Sequence alignment of *actb1* mRNA sequence with forward primer and reverse primer

```
CLUSTAL O(1.2.4) multiple sequence alignment

rp      ------------------------------------------------------------    0
gsr     ATAAACAAAACCGAAGATGGGCACCATAGCTAACCCTAAAGAGCTCATATCGTAACACCT    60
fp      ------------------------------------------------------------    0

rp      ------------------------------------------------------------    0
gsr     CTAAATAATGATACTCCATTTACTTCAAGGATGTTAATAATATAAGTTACAGCAAACGGC    120
fp      ------------------------------------------------------------    0

rp      ------------------------------------------------------------    0
gsr     TTTCGGCTACAGGTTTAAGCTGCACTCCACCGTCTCGGACGCTCGCTGTCATCTTCCTGC    180
fp      ------------------------------------------------------------    0

rp      ------------------------------------------------------------    0
gsr     AAACTCCTTGGTCGCAGCATGGCTTCTGGATCCGTCTCGCGCTTTGATTTTCTGGTGGTC    240
fp      ------------------------------------------------------------    0

rp      ------------------------------------------------------------    0
gsr     GGCGGAGGATCCGGTGGGCTGGCCGGTGCGAGGAGAGCGGCTGAACTCGGTGCCACCACT    300
fp      ------------------------------------------------------------    0

rp      ------------------------------------------------------------    0
gsr     GCCGTGATCGAAAGTCACAGACTCGGAGGTACCTGCGTCAATGTTGGATGTGTTCCTAAA    360
fp      ------------------------------------------------------------    0

rp      ------------------------------------------------------------    0
gsr     AAGGTTATGTGGAACACATCCACTCATGCAGAGTATCTCCATGATCATGAAGACTATGGA    420
fp      ------------------------------------------------------------    0

rp      ------------------------------------------------------------    0
gsr     TTTGAGGGAGCAAAAGCACATTTCAGCTGGCAAATCATAAAACACAAAAGGGATGCTTAC    480
fp      ------------------------------------------------------------    0

rp      ------------------------------------------------------------    0
gsr     GTGAGTCGCCTGAATCAGATTTACAGGAGCAACCTTGAAAAGGGCAAAATTGAGTTTATT    540
fp      ----------------------------------TGAAAAGGGCAAAATTGAGTTTA---    23

rp      ------------------------------------------------------------    0
gsr     CATGGCTATGCAAGGTTCACAGATGACCCTGAACCCACAGTTGAAGTCAATGGGAAGAAA    600
fp      ------------------------------------------------------------    23

rp      ------------------------------------------------------------    0
gsr     TACACAGCAACCCATATCTTAATCTCCACTGGCGGCCATCCATCCACAGTCAGTGAGGAT    660
fp      ------------------------------------------------------------    23

rp      ------------------------------------------------------------    0
gsr     GATGTGCCAGGATCCAGTTTAGGCATCACCTGTGATGGGTTCTTTGAACTTGAGTCTTGC    720
fp      ------------------------------------------------------------    23
```

```
rp      ------------------------------------------------------------  0
gsr     CCTAAACGTAGTGTTATAGTTGGAGCAGGCTATATTGCTGTGGAAATGGCTGGTATTCTT  780
fp      ------------------------------------------------------------  23

rp      ------------------------------------------------------------  0
gsr     TCCACTCTTGGGTCTAAAACGTCCATCATCATACGACAAGGAGGGGTGCTGAGGAACTTC  840
fp      ------------------------------------------------------------  23

rp      ------------------------------------------------------------  0
gsr     GATGCCTTGATAAGCTCCAATTGCACCAAAGAATTGCAAAATAATGGTATTGACTTACGG  900
fp      ------------------------------------------------------------  23

rp      ------------------------------------------------------------  0
gsr     AAAAATACTCAGGTGAAGTCAGTGAAGAAGAATGGCAAAGGCCTCTCTATAACACTGGTT  960
fp      ------------------------------------------------------------  23

rp      ------------------------------------------------------------  0
gsr     ACAAAAGACCCTGATGACAAGGATTCACAGGAGAAGTTTGACACTATTAATGATGTAGAC  1020
fp      ------------------------------------------------------------  23

rp      ------------------------------------------------------------  0
gsr     TGTCTGCTGTGGGCCATTGGCAGAGAACCCAACACCGCCGGCCTCAACCTCAGTCAAATA  1080
fp      ------------------------------------------------------------  23

rp      ------------------------------------------------------------  0
gsr     GGTGTGAAACTTGATGAACGGGGTCATATCGTGGTGGATGAGTTCCAGAACACCTCTCGT  1140
fp      ------------------------------------------------------------  23

rp      ------------------------------------------------------------  0
gsr     CCAGGCGTCTATGCAGTCGGGGATGTTTGCGGACGAGCCCTTCTGACACCTGATGAAGCA  1200
fp      ------------------------------------------------------------  23

rp      ------------------------------------------------------------TAT  3
gsr     GTTAAGACGTATGGAAAAGACAAGGTGAAAGTTTACACCACTTCTTTCACCCCCATGTAT  1260
fp      ------------------------------------------------------------  23

rp      TACGCCATTACCTCTCGAAA----------------------------------------  23
gsr     TACGCCATTACCTCTCGAAAGAGTCAGTGCATCATGAAGTTGGTGTGCGCTGGTGAAAAT  1320
fp      ------------------------------------------------------------  23

rp      ------------------------------------------------------------  23
gsr     GAAAAGGTGGTCGGTCTCCACATGCAGGGTTTTGGCTGTGATGAGATGCTTCAGGGTTTT  1380
fp      ------------------------------------------------------------  23

rp      ------------------------------------------------------------  23
gsr     GCCGTAGCCGTTAACATGGGGGCGACTAAAGCAGACTTTGACAGAACCATTGCCATCCAC  1440
fp      ------------------------------------------------------------  23
```

```
rp      ------------------------------------------------------------        23
gsr     CCAACGTCCTCAGAGGAGCTAGTAACACTGCGCTAATTAGTGCCTTTTCATTACATCTCC        1500
fp      ------------------------------------------------------------        23


rp      ------------------------------------------------------------        23
gsr     ACTGCAATCCAAAGAGTGTAAATGTAAACAAATGTAATTCCCTGGACTATTGTTCCATCT        1560
fp      ------------------------------------------------------------        23


rp      ------------------------------------------------------------        23
gsr     ACAGAACTAACAGTGTAACACCACAAGCATATGTTTGATATTGGTTGTGTAGAAGTTGCA        1620
fp      ------------------------------------------------------------        23


rp      ------------------------------------------------------------        23
gsr     CACAGTACAACCATTTATCAGGCTGCGTCTCTTGTACAGTACCTCAGATTTTTCACAGGT        1680
fp      ------------------------------------------------------------        23


rp      ------------------------------------------------------------        23
gsr     TTTATTGTCTGCTTGGAACGGAGTCAGGTAAAGGCTTGATTATGTTATAGGGTAAAATTA        1740
fp      ------------------------------------------------------------        23


rp      ------------------------------------------------------------        23
gsr     AATCTGTTTAAAGTCAAACACTGTTGCTTTTCTCTTATATTTCCAGAATATTATATTCAG        1800
fp      ------------------------------------------------------------        23


rp      ------------------------------------------------------------        23
gsr     AGTTGATTTCAATAGTAGCGGGTAGTGAGTAGAGCTTAATATGTGTAACAAATTGTGAAT        1860
fp      ------------------------------------------------------------        23


rp      ------------------------------------------------------------        23
gsr     CAGAATACATTTATTAAATAGATTTGTGTCTCTGTGTGGTTAATGGATCATTTCTTGATT        1920
fp      ------------------------------------------------------------        23


rp      ------------------------------------------------------------        23
gsr     TATGTATTGGCGAGGTCAAAATGCTTTTAATAAATGTCAATTTTCAGAAAAACTTAAAAA        1980
fp      ------------------------------------------------------------        23


rp      ---------------------  23
gsr     AAAAAAAAAAAAAAAAAAAA  2000
fp      ---------------------  23
```

Figure: - 15b Multiple sequence alignment of *gsr* mRNA sequence with forward and reverse primer

```
CLUSTAL O(1.2.4) multiple sequence alignment


rp        ------------------------------------------------------------      0
glo1      TGTCTGTCAGCCGCCAGGTGACCATCAGGCCGAAACACACCTAAAAATGACCGACCAAGG      60
fp        ------------------------------------------------------------      0


rp        ------------------------------------------------------------      0
glo1      GGTGACAAACGAGGCTGCAGCCGCCGCGTGTAAAGAGGGAAGCTCCATCACTAAAGACTT      120
fp        ----------------------CCGCGTGTAAAGAGGGAAGC----------------      20


rp        ------------------------------------------------------------      0
glo1      CATGATGCAGCAGACAATGCTGCGGGTGAAGGATCCGGTTAAATCCCTGGATTTCTACAC      180
fp        ------------------------------------------------------------      20


rp        ------------------------------------------------------------      0
glo1      ACGGATCCTGGGAATGACGCTGTTACAGAAGTTTGATTTCCCCTCGATGCGCTTCACCCT      240
fp        ------------------------------------------------------------      20


rp        ------------------------------------------------------------      0
glo1      CTACTTTCTGGGTTACGAAGATAAGAAAGAGATCCCTGCAGATGTGAAGGAGAGGACGGC      300
fp        ------------------------------------------------------------      20


rp        ------------------------------------------------------------      0
glo1      CTGGACGTTCTCCCGTAGAGCCACTATAGAGCTCACTCATAACTGGGGCTCAGAAACGGA      360
fp        ------------------------------------------------------------      20


rp        ------------------------------------------------------------      0
glo1      TGACAGCCAGTCTTACCACAACGGCAACTCAGACCCAAGAGGCTTTGGCCATATTGGAAT      420
fp        ------------------------------------------------------------      20


rp        ----GTACCGGATGTCTATGCTGCC-------------------------------------      21
glo1      TGCAGTACCGGATGTCTATGCTGCCTGCAAGCTATTTGAAGAGAATGGAGTGACCTTTGT      480
fp        ------------------------------------------------------------      20


rp        ------------------------------------------------------------      21
glo1      AAAAAAGCCTGATGAGGGTAAAATGAAGGGTCTGGCCTTTATCCAGGATCCTGACGGTTA      540
fp        ------------------------------------------------------------      20


rp        ------------------------------------------------------------      21
glo1      CTGGATTGAGATTCTCAGCCCCAATAACATGGTGTCCATCACTTCGTAAAGATCATTGTT      600
fp        ------------------------------------------------------------      20


rp        ------------------------------------------------------------      21
glo1      ATGCTGTGACCTGATCCGAAAAAACTAGCTATATAAATAAAGGACATATGGGTGATTTGA      660
fp        ------------------------------------------------------------      20


rp        ------------------------------------------------------------      21
glo1      GGAAGGGTGGTTGCTCTCTGCAAAGCAGTGCAGTGAAGACTAACAGTGTTTACATCTAAC      720
fp        ------------------------------------------------------------      20
```

```
rp      ------------------------------------------------------------  21
glo1    AATATAATGCAACAATGTCATCAGATAGTCACTTTTTGAATGAATGCATTAGAGACCCTC  780
fp      ------------------------------------------------------------  20

rp      ------------------------------------------------------------  21
glo1    ATTCAGTCAGCTCAAAGGGATAGTTCACTCAAAAATTAAACTATTTACTCACATTTAAGT  840
fp      ------------------------------------------------------------  20

rp      ------------------------------------------------------------  21
glo1    AGTTCTACACTGTTAAACACAAAAGAAGATATTTTGAAGAATGTTGGGGGGAAAATCAGT  900
fp      ------------------------------------------------------------  20

rp      ------------------------------------------------------------  21
glo1    CATTGATATCTGTAGAACACATTAAAAAAGATGATTTTGCGCTTTAAAATGAGCTGAAAC  960
fp      ------------------------------------------------------------  20

rp      ------------------------------------------------------------  21
glo1    TACATAACTTTTGCGTTTTTTGGGGGGACAACTTAATTGTTTTATGTTCAGTCTACTAAA  1020
fp      ------------------------------------------------------------  20

rp      ------------------------------------------------------------  21
glo1    ATTTGTTAAGTAACTTAATTGATTTAACATAAACCACTGAATTGTGTGGAATCTTGCTTT  1080
fp      ------------------------------------------------------------  20

rp      ------------------------------------------------------------  21
glo1    TTTTTTTTTACAGTGTATGAACACAAAATACTAAGACATAAATCAATGTCTGTTCATTAT  1140
fp      ------------------------------------------------------------  20

rp      ------------------------------------------------------------  21
glo1    CAACATTTTCTTTTTATCTTTGTGTTCAACAGAGGAAAGAAACTCAAAGGAGTAAATGAT  1200
fp      ------------------------------------------------------------  20

rp      ------------------------------------------------------------  21
glo1    AGCAGCATTTACAGTTTTGAGTGTACTGTCTCTTTAAATTACACTTTGTTAAAATAGGAA  1260
fp      ------------------------------------------------------------  20

rp      ------------------------------------------------------------  21
glo1    TATATTTGAAAGGATGTCATTCTCCTCCTAAAGCTTCTACTTTGTCAAAATAGAGTATTT  1320
fp      ------------------------------------------------------------  20

rp      ------------------------------------------------------------  21
glo1    TTTAAGTTAATACAATACATCCTTCATATCTGACCCTAAAACCCTGCCTATATTTGTTCA  1380
fp      ------------------------------------------------------------  20

rp      ------------------------------------------------------------  21
glo1    AATAAACCAAGGAAAGTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  1440
fp      ------------------------------------------------------------  20

rp      ----------------------------------------  21
glo1    AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  1480
fp      ----------------------------------------  20
```

Figure: - 15c Multiple sequence alignment of *glo1* mRNA sequence with forward and reverse primer

Table: - 7 T_m and length of primers

Gene	Primers	Length	T_m °C
actb1	Forward	21	57
	Reverse	21	59
gsr	Forward	23	52
	Reverse	23	55
glo1	Forward	20	61
	Reverse	21	60