Toxicity and Selective Biochemical Assessment of Quercetin, Gallic Acid, and Curcumin in Zebrafish

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In recent years, numerous research outcomes were established on various naturally occurring compounds that have been shown to have beneficial antioxidant and other biological activities. Antioxidant defence mechanism plays a vital role in combating various diseases mainly due to oxidative stress. However, various models have been utilized to identify their bioactivities using these compounds (quercetin, gallic acid and curcumin). Their toxicity level also has to be explored to determine the threshold levels on the usage of these compounds. In this study, we investigated the lethal concentration of these compounds and abnormalities, biochemical and morphological changes in zebrafish embryo (Danio rerio). Toxicity level was evaluated by calculating the LD₅₀ on the embryonic stages at 24, 48 and 72 h. Antioxidant parameters such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and biological assays such as lipid peroxidation, protein estimation were performed. Microscopic evaluations were also observed to find out morphological abnormalities. However, these naturally derived compounds are reported to have their protective and curative role in many health complications. From the above assays, we are studying the effect of the drugs in both biochemical and molecular way in the zebrafish model organism.

Key words zebralsh; Danio rerio; quercetin; gallic acid; curcumin; toxicity

INTRODUCTION

Increased interest for various plant-derived compounds such as quercetin, gallic acid, and curcumin has been seen over the last couple of decades because of the various health benefits these compounds possess. However, the toxic effect of such compounds has to be evaluated with other alternative animal models like Caenorhabditis elegans (roundworm), Drosophila melanogaster (fruit fly), Galleria mellonella (greater wax moth), and so on.²

Quercetin is derived from the Latin word quercus, which means “oak.” The name has been used since 1857 and is derived from quercetum (oak forest) after “Quercus.” Quercetin, namely 3,3',4',5,7-pentahydroxyflavone which is a flavonoid. Fruits such as apple, berries etc. and some vegetables like onion, broccoli etc. are in abundance of quercetin.²⁻⁴ Free as well as a bounded form of quercetin in combination with carbohydrates and alcohols are found to exist in plants. The main functions of quercetin include its ability to act as an antioxidant, anti-inflammatory, antibacterial, and antiviral agent found helpful in treating various conditions, obesity and cardiovascular diseases being two among them.²⁻⁷ However, a recent study states that the higher dosage of quercetin for long-term exposure has led to toxicity and carcinogenicity when administered with male and female rats.⁸ Oral administration of quercetin has shown LD₅₀ at 161 mg/kg in rats and 159 mg/kg in mouse. Subcutaneous administration showed LD₅₀ at 97 mg/kg and intravenous administration showed about 18 mg/kg in the mouse model.⁹

Gallic acid is a polyphenol compound which is chemically called as 3,4,5-trihydroxybenzoic acid. Polyphenolic compounds have been known to elucidate antioxidant properties along with anticancer, antibacterial, antiviral, antilucre, anticholesterol and various others.¹⁰⁻¹² The radical scavenging ability of this compound is of pharmacological importance.¹³ Gallic acid has shown a lot of potential as a therapeutic and preventive agent for diseases involving oxidative stress such as cardiovascular diseases, a neurodegenerative disorder, cancer, and even ageing. Gallic acid is the active component in red wine, green tea and fruits, which is responsible for reducing Coronary Arterial Disease (CAD) and arterial thrombosis.¹⁴⁻¹⁵ Gallic acid has also been reported to improve glucose tolerance, total cholesterol, triglyceride concentration and low density lipoprotein (LDL)-cholesterol in diet-induced obesity animals.¹⁶⁻¹⁷ In contrast, the dose-based abnormality was reported in F344 rats at both sexes, which includes reduction of haemoglobin concentration, hematocrit and red blood cell counts and increase in reticulocytes. All these results indicated that 5% of gallic acid was toxic which leads to haemolytic anaemia and hepatic hypertrophy.¹⁸ Intravenous and intraperitoneal administration of gallic acid has shown LD₅₀ in the mouse at 320 mg/kg and 4300 mg/kg, respectively. Oral administration showed LD₅₀ at 5 g/kg in rabbit model.¹⁹

Curcumin is a polyphenol derived from the plant Curcuma longa. Chemically it is termed as 1,6-heptadiene-3,5-dione-1,7-bis(4-hydroxy-3-methoxyphenyl)-(1E,6E).¹⁹ Curcumin exhibits...
strong antioxidant activity comparable to that of vitamins C and E. It was shown to be a potent scavenger of a variety of reactive oxygen species including superoxide anion radicals, hydroxyl radicals and nitrogen dioxide radicals, which generally deteriorate the biomolecules such as protein, lipids and nucleic acids especially DNA damage.20,22 The hydroxypyrenyl unit in curcumin has been shown to be crucial to its anti-inflammatory activity.22 Intrapitoneal administration of curcumin has shown LD50 in the mouse at 1500 mg/kg.27

Zebrafish (Danio rerio) a small-sized fresh-water fish used widely as a powerful model organism for the study of vertebrate biology, being well suited to both developmental and genetic analysis is a model organism in many fields like genetics, developmental biology, toxicology, pharmacology and so on.23 The genome of zebrafish is roughly 71% homologous with that of a human and 84% of genes associated with human diseases have a zebrafish counterpart.24 Other advantages of using zebrafish as a model are that it is small in size and easy to maintain and breed in less space, also it is optically transparent which makes it easy to observe the changes that take place. Drug chemicals can be easily delivered by addition to the water; the possibility of using large sample sizes; short generation time. National Institutes of Health bringing this model system to its full potential for the study of vertebrate biology, physiology and human disease.25,26)

This study was designed to investigate various biochemical changes on the exposure of quercetin, gallic acid and curcumin on zebrafish (Danio rerio). However, these compounds were evaluated for toxicity in other models like Wistar rats, mouse and rabbit. It is the first report of quercetin and gallic acid on the evaluation of developmental toxicity in zebrafish embryo model; as the use of zebrafish as an experimental animal model has many advantages, zebrafish possess high fecundity. It is found that the hatching of eggs and organogenesis occurs rapidly. In contrast to other mammalian models, they develop outside uterus which makes it possible to raise them in Petri dishes or in multi-well plates containing water. They can be used for larval experiments from 3rd days post fertilization (dpf). The embryos are also transparent.27 To standardize drug toxicity in this model the lethal dosage of about fifty per cent (LD50) was calculated and their role in oxidative stress by reducing the antioxidant level was estimated. Antioxidant enzymes studies by measuring the biomarkers such as superoxide dismutase (SOD), catalase (CAT), lipid peroxidase, glutathione (GSH), and biological assays such as lipid peroxidation, protein estimation were analysed.

MATERIALS AND METHODS

Chemicals Drug compounds for toxicity assessment (Quercetin, Gallic acid and Curcumin) were purchased from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) from Himedia, India. The embryonic medium (E3 medium) was prepared and toxicity approaches followed using standard methods from OECD guidelines-236.

Fish Husbandry and Embryo Collection The wild-type zebra fishes (Danio rerio) were obtained from Madurai Kamaraj University, Madurai, Tamilnadu. After acclimatization, breeding was done in our laboratory. Fish were kept for spawning in the early morning. Then all embryos were collected and completely washed with E3 media and were transferred to a petri dish where the healthy and the dead embryos were separated. The healthy embryos were selected at 6h post-fertilization (hpf) by observing them under a microscope. Feeding is not required for 7d post fertilization as the required nutrition is received from the yolk sac.27

Exposure of Zebrafish Embryo and Larvae The healthy fertilized eggs at 6h post-fertilization (hpf) were transferred to a 24-well cell culture plate. About 10 embryos were taken in each well with different concentrations of drug solution (10, 50, 100, 200, and 500 µg/mL). Treatment with each solution was done in triplicates. Embryos with drug solutions were incubated at 28 ± 1°C for different time periods (24, 48, and 72h). At each checkpoint, dead embryos were counted for calculating mortality rate (%) and live embryos/hatched out larvae were used for the further experiment.

Superoxide Dismutase Test (SOD) The SOD activity was measured by the ability of the enzyme to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT). A reaction mixture (consisting of 50 mM phosphate buffer (pH 7.8), 100 µM ethylenediaminetetraacetic acid (EDTA), 130 mM methionine, 750 µM NBT, 20 µM riboflavin, and 50 µL enzyme supernatant) were mixed up and illuminated with the help of a 4000lx fluorescent lamp for 15 min. After the illumination, the absorbance was measured at 560 nm using an UV-visible spectrophotometer.28

CAT CAT activity is determined with the help of UV-visible spectrophotometer. Phosphate buffer containing 2mM of H2O2 is added in an experimental cuvette to which enzyme extract is added and mixed thoroughly. The enzyme activity is determined by finding out the absorbance using Shimadzu UV visible spectrophotometer absorbance at 240 nm in a spectrophotometer. The absorbance decreases when hydrogen peroxide is degraded by the enzyme CAT and its activity can be determined.28

Reduced GSH GSH is assayed by the procedure of Moron et al.29 The conversion of oxidized GSH to reduced GSH is catalyzed by GSH reductase with the help of reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a substrate where the amount of NADPH represents enzyme activity. To proceed with the experiment, GSH standard of different concentration (100, 200, 500, and 1000 µg/mL) were taken to which phosphate buffered saline (PBS) and 5,5’-dithiobis-2-nitrobenzoic acid (DTNB) were added to make the total volume 3 mL. After incubation for 10 min, the absorbance was taken using spectrophotometer at 412 nm. A standard curve was plotted using the obtained absorbance of the GSH standards and using this curve the GSH present in the sample was determined in the GraphPad Prism version 5.01 software.29

Lipid Peroxidation Assay The lipid peroxidation is the biological assay in which the concentration of malondialdehyde (MDA) is measured. MDA is the end product formed from the lipids. This MDA activity can be determined with the help of UV-visible spectrophotometer. After reaching 72 h few samples of the embryos were lysed in the lysis buffer, and it was added with TCA to the lysed homogenate and then centrifugation at 2500 rpm for 10 min. The supernatant was collected to which the thiobarbituric acid (TBA) was added and kept in a water bath at 95°C for 10 min. The butanol was added to the supernatant followed by centrifugation. The butanol layer was separated and the OD was measured at
**Protein Estimation**  The quantity of protein in the 72h drug-treated embryos were estimated by using the Bradford method. About 20µL of drug-treated lysed embryo homogenerate was added with 80µL of distilled water and 900µL of Bradford reagent was added into it and was incubated for 5min in dark and was checked for absorbance at 595nm. The estimation was determined using standard slop with the bovine serum albumin was taken as the standard protein ranging from 0, 5, 10, 25, 50, and 100µg/mL.

**Statistical Analysis**  All the data were done in triplicates and the percentage of mortality was calculated using Graph pad prism version 5.01. Data are mentioned here as a mean ± standard deviation (S.D.). One-way ANOVA was carried out for statistical significance using Dunnett’s multiple comparison test with \( p < 0.05 \).

**RESULTS AND DISCUSSION**

**Toxic Effect of Selective Compounds on Zebrafish Embryo**  Curcumin was reported to have the embryotoxic and teratogenic effects on the development of zebrafish. From the exposure of the curcumin for the period of 24h, \( \text{LD}_{50} \) values were reported as 7.5 mM for embryos and 5 mM for larvae respectively.\(^{32}\) In the case of gallic acid, it was earlier reported as the \( \text{LD}_{50} \) on adult zebrafish were showed at the dose of 100mg/L.\(^{33}\) This study is for assessment of mortality using quercetin, gallic acid and curcumin on zebrafish embryo to determine its \( \text{LD}_{50} \) values. The embryos treatment started from 6 till 72hpf. In our study, the results were graphically presented in Fig. 1 as a, b and c for quercetin, gallic acid and curcumin respectively. The \( \text{LD}_{50} \) values for Quercetin, Gallic acid, and curcumin are 484, 304, and 135µg/mL, respectively. The earlier study comparably showed the toxic effect of curcumin on zebrafish even at the lesser concentration.\(^{32}\) From this, we can see that quercetin has the least toxicity and curcumin more toxic of the three compounds.

**Antioxidant Parameters**  Antioxidants play a major role in defending against oxidative stress and free radicals such as superoxides (\( \text{O}_2^- \)), hydroxyl (OH·) and hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) radicals.\(^{30}\) The endogenous antioxidants are catalysing these free radicals into a non-toxic form.

**SOD**  Superoxide radicals are the preliminary oxidizing agent which was catalysed by the enzyme SOD. The conversion of yellow colour NBT reagent was transformed into formazan crystals by reacting with superoxide radicals.\(^{34}\) SOD will inhibit the conversion of NBT, hence we found the less crystal formation in quercetin (10, 50, and 100µg/mL), though gallic acid moderately less toxic it triggers superoxide formation. So, the intensity is higher in 10 to 200µg/mL of gallic acid whereas in curcumin it is very sensitive in inducing toxicity and the formazan formation is also high in all the concentration. The significant increase in the absorbance was observed in quercetin and gallic acid treatment. Whereas a significant decrease was observed in curcumin treatment with zebrafish represented in Figs. 2a, b, and c.

**CAT**  After the processing of SOD, the hydrogen peroxide radicals will be more. It has to be converted into a non-toxic form of \( \text{H}_2\text{O} \) and \( \text{O}_2 \) by the endogenous enzyme CAT. Here the graphs infer that there is more enzyme activity in the control compared to the test samples for quercetin, gallic acid and curcumin. So, by performing this antioxidant assay it clearly states that the enzyme CAT level significantly increased in quercetin and gallic acid exposure whereas in curcumin the CAT significantly increased also decreased it is because of the deterioration of antioxidant defence against curcumin (Figs.
Reduced GSH

GSH is the one type of antioxidant assay in which the enzyme GSH reductase catalyses the conversion of GSH disulphide to reduced GSH. Reduced GSH protects from the oxidative stress. And the enzyme GSH peroxidase and GSH reductase utilize GSH to maintain the normal homeostasis. The earlier report using silver nanoparticles (AgNPs) mediated liver toxicity in adult zebrafish showed as the concentration of the AgNPs (120 mg/L) increases, the GSH level increases to control the oxidative stress. It was comparatively similar in case of quercetin and gallic acid. Whereas in the case of curcumin has more toxicity even at the lower dose, the GSH content seems to be higher and significantly decreases as the concentration of curcumin increases. It was due to the inadequate endogenous GSH content as the oxidative stress raises. After the incubation period, the GSH content was estimated along with the control. The quercetin significantly increases the GSH concentration when compared to other compounds (Fig. 3).

Biochemical Tests

Lipid Peroxidation Test

MDA is one of the lipid peroxide formed when treated with compounds; quercetin, gallic acid and curcumin at different concentration. The results indicated, as the concentration of compounds when gradually increased from (10 to 500) the zebrafish accumulated the compounds and produced higher concentrations of MDA. The significant increase in MDA content after the exposure of quercetin, gallic acid and curcumin were observed and represented in Fig. 4.

The MDA content is a widely used biomarker for lipid peroxidation of fatty acids because of its reaction with TBA. The TBA test is based upon the reaction of TBA towards MDA to yield fluorescent red colour and it was previously used by food chemists to evaluate antioxidative degradation of fats and oils. In our experiment, curcumin was more toxic than the...
other compounds and the observed MDA content on a higher dose of curcumin showed 187.39 ± 0.57 ng/mL.

**Protein Estimation**

Protein content had been estimated to characterize the effect of each drug separately. With an increase in the concentration of the drug, there was a gradual decline in the protein content, which described the varying effect of each drug on zebrafish larvae under different concentrations.

Protein estimation by Bradford method is the highly used experiment on protein estimation because of its sensitivity and...
more accurate than Lowry’s method.\(^{38}\) In support to the above all experiment, protein content also was significantly reduced as the concentration increases (Fig. 5).

**Morphological Deformation** After performing toxicity assessment with different concentrations, morphological abnormalities and anatomical variations were observed at
72hpf. Among the three compounds, curcumin has elevated more structural deformity (Fig. 6) even at lower concentration (5 µg/mL) and the abnormalities namely spinal curvature (SC) and pericardial edema (PE) relatively increased at the concentration of 100 µg/mL. The reason behind the curcumin toxicity was previously reported as; it induced caspase-3 dependent apoptosis in Human Jurkat cells. 70 Gallic acid shows PE as well as delayed hatching (Dh) in treatment at 200 µg/mL whereas quercetin showed pericardial abnormalities at higher concentration (500 µg/mL).

In the previous report, curcumin showed the developmental defects such as bent or hook-like tails, spinal column curving, edema in the pericardial sac, retarded yolk sac resorption, and shorter body length. 71 Quercetin was reported to show topological changes including curvature of the body axis leading to cause decreased locomotor performance. It was also stated that quercetin at higher concentration affected the ATP production. Thus, larval behaviour was affected to cause locomotion. 72

In our study, the acute toxicity of these compounds for this model has been previously evaluated. It also helps in preventing angiogenesis by inhibiting VEGFR2 mediated network. 73, 74 Hence, it could be a promising substance for anti-cancer activity. Gallic acid, a major substance present in fruits and vegetable the catechin is one of the derivatives.

CONCLUSION

From the three drugs used for estimating the toxic activity of zebrafish larvae, we conclude that quercetin is less toxic than the other two drugs and it was confirmed by using the biochemical assays, which gave lower lipid peroxidation activity and higher protein content with an LD₅₀ value, which gave higher survival rate for quercetin drug.

Conflict of Interest The authors declare no conflict of interest.

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