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Original Research Article

Raw turmeric and pure curcumin: a comparison of embryonic cytotoxicity in zebrafish

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ABSTRACT

Background: Turmeric *(Curcuma longa)* is widely used in ayurveda and siddha formulations for treating various ailments. This study compares the gross effects of curcumin and natural state turmeric on zebrafish embryos.
Methods: Turmeric powder and curcumin were used in this study. Zebrafish embryos were treated with different concentration of both turmeric and curcumin to evaluate their gross effects including toxicity. Morphological variations including hatching, survival, heart rates, head width, eye width, tail width and yolk sac area were examined by stereomicroscope.
Results: Embryos treated with curcumin or raw turmeric with concentration of 27.628 and 36.83µg/ml exhibited a reduction of survival rates (15-20%) at 24, 48 and 72hpf. Turmeric treated embryos had near normal hatching rates when compared to curcumin treated embryos at 48hpf whereas decreased hatching rate was observed in turmeric treated groups at 72hpf when compared to curcumin treated group. No significant morphological anomaly in head /eye / tail width, length of yolk sac and average embryo length were witnessed among all the 3 groups. The toxicity assays revealed various morphological deformities in embryos treated with different concentrations of both turmeric and curcumin.
Conclusions: Zebrafish embryos treated with increasing concentrations of curcumin in pure form or raw turmeric exhibited similar morphometric and toxic effects indicating that raw turmeric could elicit anti cancer activity with similar potency as that of active ingredient curcumin due to their toxicity attribute. It may be beneficial to explore the negative attributes of such common natural food ingredients that may be beneficial for therapeutic interventions.

Keywords: Curcumin, Raw turmeric, Toxicity, Zebrafish

INTRODUCTION

Turmeric *(Curcuma longa)* is a rhizomatous herbaceous perennial plant of the ginger family, Zingiberaceae, native to Southern Asia, and used in India for thousands of years as a part of food preparation and many major ayurvedic and siddha drugs.[1,2] It was first used as a dye and later for its medicinal properties and is reported that its extracts have antifungal and antibacterial properties.[3] Turmeric is under evaluation for its potential effect on Alzheimer's disease and diabetes.[4,5] Turmeric is widely used in ayurveda and siddha formulations for treating biliary disorders, anorexia, cough, diabetic wounds, hepatic disorders, rheumatism and sinusitis.[6] Turmeric contains many phytochemicals like curcumin, bis-methoxy-curcumin, d-methoxy-curcumin, curcuminol, curcumol, eugenol, terta-hydro-curcumin, tri-ethyl-curcumin, turmerine, turmerols, volatile oils (turmerone, atlantone, and zingiberene), sugars, proteins, and resins.[7,8]

Curcumin (diferuloylmethane) is a nontoxic dietary polyphenol with a variety of therapeutic properties including anti-oxidant, analgesic, anti-inflammatory and antiseptic activity, and constitutes on an average 3.14% of powdered turmeric.[9] Curcumin as a purified product has a reported effect on cancer by inhibiting new blood vessel formation which is a character of cancers that aids

metastatic spread.[10] More recently curcumin has been found to possess anti-cancer activities due to its effect on a variety of biological pathways involved in mutagenesis, oncogene expression, cell cycle regulation, apoptosis, tumorigenesis and metastasis.[11] Curcumin also possesses a potent anti-oxidant and free-radical quenching property, and plays an important role in the inhibition of progress in the initial stages of carcinogenesis.[12] Curcumin has been studied in many human cancers including melanoma, head and neck, breast, colon, pancreatic, prostate and ovarian cancers.[13-19] Epidemiological studies attribute the low incidence of colon cancer in India to the chemo-preventive and antioxidant properties of diets rich in curcumin. It has been reported that curcumin has the ability to suppress UV irradiation-induced DNA mutagenesis and induction of cellular SOS functions.[20] In addition it also has an (1) inhibitory effect on production of nitric oxide (NO), (2) ability to scavenge DNA damaging superoxide radicals, (3) effect on Phase I and II enzymes of hepatic cytochrome p450 enzyme system useful in oxidation and detoxification of toxic substances, and (4) inhibitory effect on Phase I enzymes (including cytochrome p450 iso-forms and p450 reductase) reportedly inducing response to toxin induced carcinogenic DNA adduct formation.[21] Curcumin also induces Phase II enzymes leading to detoxification of toxic metabolites (including glutathione S-transferase, glutathione peroxidase and glutathione reductase).[22] Inhibitory effect of curcumin on carcinogenesis has been demonstrated in several animal models of various tumor types including oral cancer, mammary carcinoma and intestinal tumors.[23-25] Zebrafish (Danio rerio) is fast becoming a powerful model for drug discovery and it is extensively used to screen developmental changes because of its small embryo size; large clutch size and permeability to small molecules, further it develops ex utero, offering visual access to most stages throughout development.[26,27] However detailed studies on the pharmacological activity and potency of turmeric in its unrefined natural state are sparse. This study compares the gross effects of curcumin and natural state turmeric on zebrafish embryos.

METHODS

Turmeric

Turmeric (100g) cultivated without pesticide or synthetic manures was purchased from local market in Tirunelveli city, Tamil Nadu, and stored at room temperature in the laboratory.

Curcumin

Curcumin was procured as 5g vials (Sigma Aldrich Inc) and stored as per manufacturer's catalog.

Dimethyl sulfoxide (DMSO)

Dimethyl sulfoxide (DMSO) was procured as 50ml pack (Sigma Aldrich Inc).

Maintenance of zebrafish

Adult, wild-strain, zebrafish were obtained from a local aquarium and housed in the laboratory in 50litre tanks maintained separately for male and female at 28±2°C with constant light and dark (14-10 hrs) cycles.[26] The water was continuously aerated, renewed in semi-static state and cleaned regularly to avoid infections. Fishes were fed twice a day with commercially available dry flake feed, bloodworms and live brine shrimp. The health of the fishes was checked daily.

Zebrafish breeding

Selected male, female fishes were placed in breeding tanks as 8:4 male-female ratio (2:1 ratio). The fish were left undisturbed overnight and eggs were collected next morning.[27]

Embryo collection

Embryos were carefully transferred to a 10cm diameter petri dishes using Pasteur pipette and were washed twice with chlorine free water, to remove the debris and other dust particles to avoid infection.[28] A total of ~ 1000-1200 embryo were collected. Embryos were then segregated into 6 well culture plates (20embryos/well). Water was completely replaced with embryo medium (2ml/well) and the embryo plates were kept in 28±2°C.

Curcumin and turmeric solutions

1mM stock solution of curcumin was prepared in DMSO. Naturally turmeric powder constitutes an average of 3.14% curcumin.[9] Therefore turmeric stock solution was prepared and its concentration was equalized to curcumin percentage by 5.936mg of natural turmeric powder dissolved in 10 ml of DMSO.

The test

- *Group I (Control):* Consisted of 20 embryos placed in 6 wells of a tissue culture plate at 50% epiboly stage and were incubated in embryo medium.
- *Group II (Curcumin)*: Consisted of 20 embryos placed in 6 wells of a tissue culture plate at 50% epiboly stage and were treated with curcumin at 0.3683, 1.4732, 2.2098, 7.366, 10.3124, 18.419, 27.6285 and 36.83µg respectively.
- *Group III (Turmeric)*: Consisted of 20 embryos placed in 6 wells of a tissue culture plate at 50% epiboly stage and were treated with serially diluted of turmeric stock solution as same as curcumin.

Morphological screens

Embryos of all the 3 groups were maintained in wells of culture plates at 28±2°C until 72hpf (hours post fertilization) and were inspected for survival rate, heart rate, hatching rate, eye and tail development and gross

phenotypical features at 24, 48 and 72hpf. Morphometric deformities were photographed at various time intervals.

Heart rate (HR) measurement

The zebrafish embryos (48-72 hpf) were anesthetized with 0.6mM Tricaine methane sulphonate (MS-222) prepared in embryo medium, prior to counting the HR. Each HR measurement was done by counting the contractions of either of the two chambers for at least 30secs. Heart rate was counted for 1minute and repeated for three times for each embryo and the average was taken.

RESULTS

Survival rate

The acute toxicity test determined the mortality in zebrafish embryos exposed to a wide range of curcumin and turmeric concentrations for 72hpf and monitored for severe malformations and death. The observed percentages of zebrafish survival for curcumin and turmeric for different concentrations are shown in Figures 1, 2 and 3.

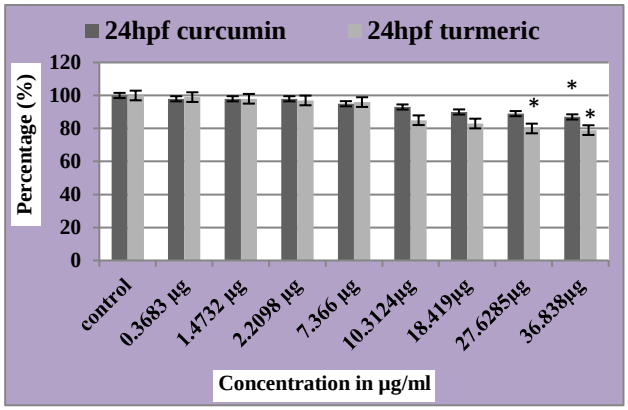

Figure 1: Survival rates and concentration of curcumin and turmeric at 24 hours post fertilization.

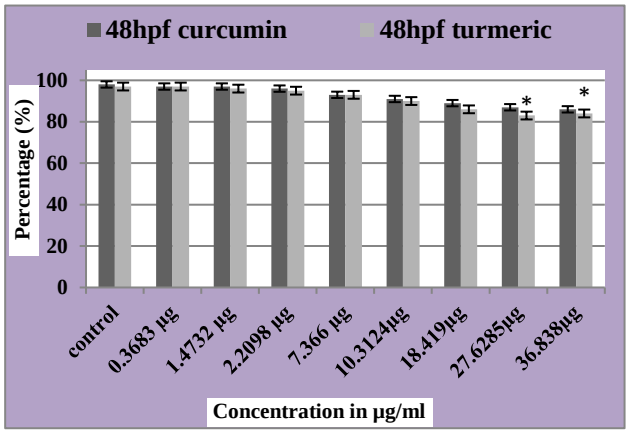

Figure 2: Survival rates and concentration of curcumin and turmeric at 48 hours post fertilization.

No significant reduction in survival rate was observed until a concentration of 18.419µg/ml curcumin was reached in both the groups. However, embryos treated with curcumin or raw turmeric with concentration of 27.628 and 36.83 µg/ml exhibited a reduction of survival rates (15-20%) at 24, 48 and 72hpf. No statistically significant difference of survival rates is inferred between curcumin and raw turmeric treated embryos. However, the survival rates when compared with the controls showed a minimal decrease in survival rates with increasing concentration of both curcumin and raw turmeric. Further, none of the zebrafish survived beyond 7 days on exposure to curcumin concentrations of 36.83µg/ml in both group II and III.

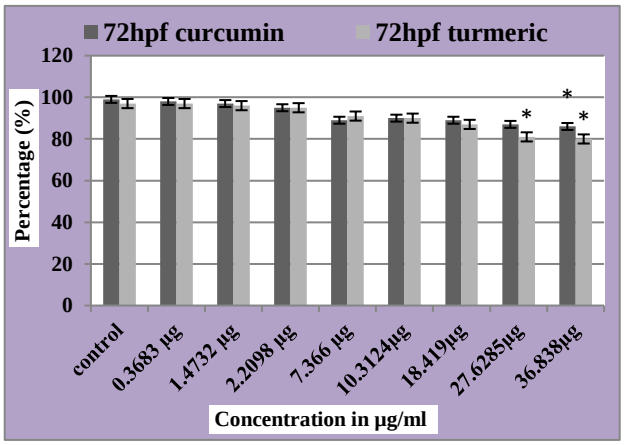

Figure 3: Survival rates and concentration of curcumin and turmeric at 72 hours post fertilization.

Hatching rate of zebrafish

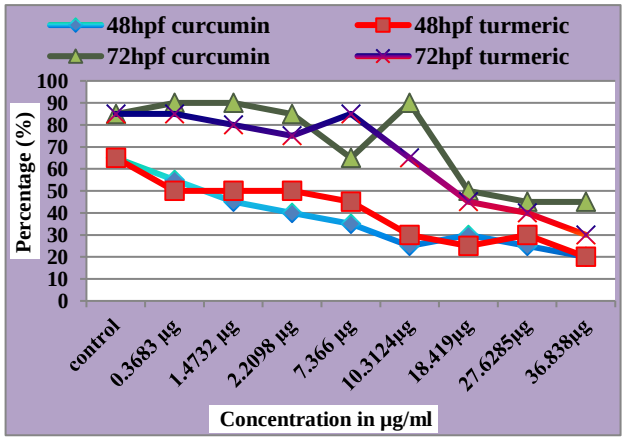

Figure 4: Hatching rates and concentration of curcumin and turmeric at 48 and 72 hours post fertilization.

In the control group 70% and 90 % of the embryos hatched out at 48hpf and 72hpf respectively. Hatching rate were considerably decreased in curcumin treated group than turmeric treated group at 48hpf. At lower concentration (0.3683 and 1.4732µg/ml) of curcumin treated embryos had 90% hatching rate whereas in turmeric treated groups it is decreased (80 to 75%) at 72hpf. This observation

showed that turmeric treated embryos had near normal hatching rates when compared to curcumin treated embryos at 48hpf whereas decreased hatching rate was observed in turmeric treated groups at 72hpf when compared to curcumin treated group (Figure 4). However, significant hatching delay was observed at higher concentration (27.6285 and 36.838µg/ml) in both the groups when compared to control at 72hpf.

Heart rate (HR)

There was no significant difference (Figure 5) in heart rate in both the treatment groups compared to control. However, curcumin treated embryos had increased HR (120bpm) at 48hpf.

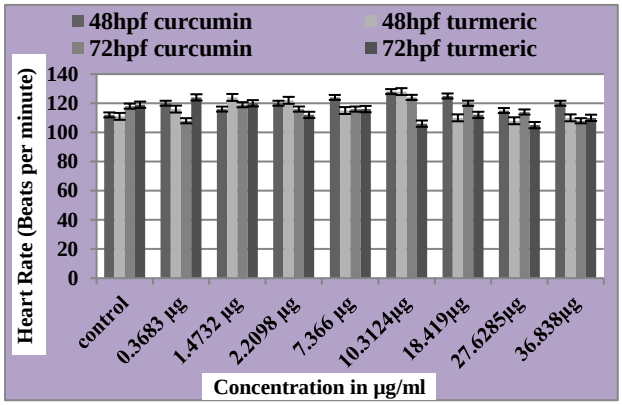

Figure 5: Heart rates and concentration of curcumin and turmeric at 48 and 72 hours post fertilization.

Morphology

No significant morphological anomaly in head /eye / tail width, length of yolk sac and average embryo length were witnessed among all the 3 groups. However, a decrease of average head and eye width were visible at 48 and 72hpf in both the treatment groups, in comparison to controls. A slight increase in yolk sac length was evident in both treatment groups.

Phenotypic abnormalities

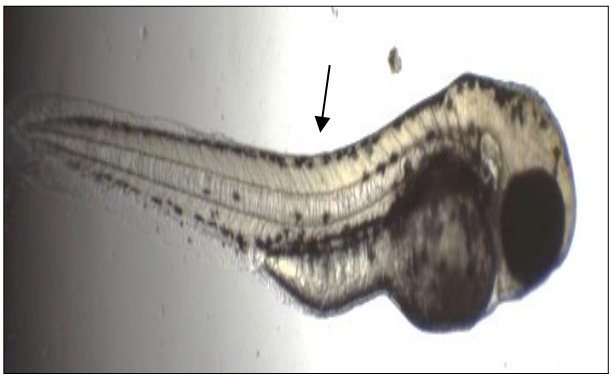

Figure 6: Embryo treated with 0.3683µg curcumin at 48hpf shows a mild whole body curvature.

Zebrafish embryos treated with curcumin and raw turmeric generated a series of phenotypic variants with whole body curvature, pericardial edema, and abnormal elongation of yolk sac.

Figure 7: Embryo treated with 2.2098µg curcumin at 48hpf shows a mild pericardial edema.

Figure 8: Embryo treated with 4.4196µg curcumin at 48hpf shows enlargement of yolk sac.

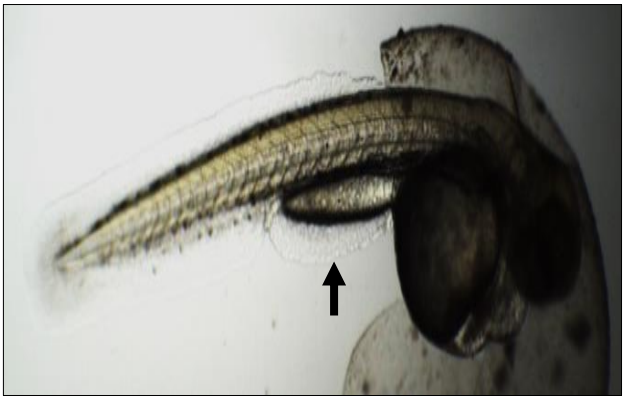

Figure 9: Embryo treated with 5.1562µg curcumin at 48hpf shows pericardial edema, whole body curvature and enlargement of yolk sac.

Whole body curvature was the most marked abnormal phenotype observed in both curcumin and turmeric treated groups. Group treated with 0.3683µg/ml curcumin

possessed mild body curvature and 5.1562µg/ml showed that yolk sac enlargement, pericardial edema and whole body curvature at 48hpf (Figure 6 to Figure 13).

Figure 10: Embryo treated with 3.683µg turmeric at 48hpf shows a mild whole body curvature.

Figure 11: Embryo treated with 2.2098µg turmeric at 48hpf shows a severe pericardial edema.

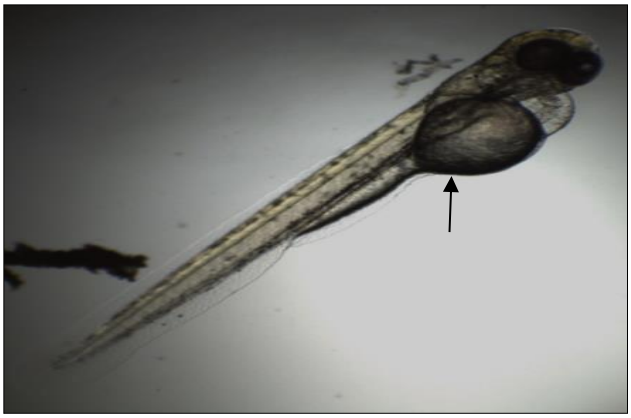

Figure 12: Embryo treated with 1.4732µg turmeric at 48hpf shows a enlargement of yolk sac.

However, curcumin treated group (2.9464µg/ml) exhibited yolk sac enlargement and split in yolk sac at 72hpf. At 72hpf turmeric treated group (0.3683µg/ml) showed mild pericardial edema and 2.2098µg/ml treated group exhibited yolk sac enlargement and severe pericardial edema.

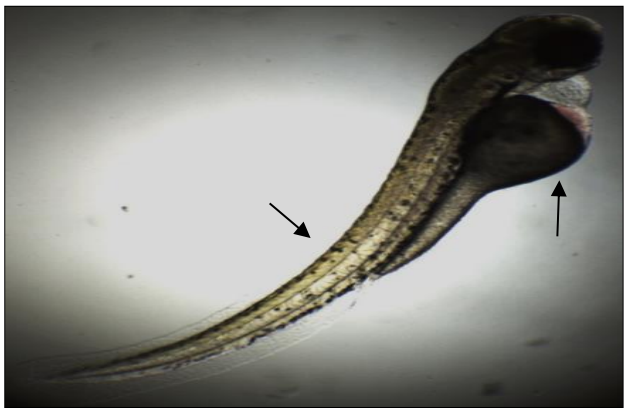

Figure 13: Embryo treated with 5.1562µg turmeric at 48hpf shows severe pericardial edema, whole body curvature and severe enlargement of yolk sac.

DISCUSSION

Curcumin the bioactive phytochemical of the turmeric tuber, has been used in purified form as a research material for a wide variety of clinical conditions and diseases. In India turmeric in the raw impure form is part of the regular daily diet and hence ingested by most Indians. The question of whether turmeric is a better or lesser alternative to purified curcumin and whether the other phytochemicals present in the raw turmeric alter or interfere with the function of curcumin needs documentation. This study evaluates the effects of raw turmeric and curcumin in pure form on zebrafish embryos. The embryos treated with supra concentrations revealed decline in survival rate compared to control ($P \leq 0.05$) (Student's-Newman-Keul's test) and on extended exposure for longer period lead to complete mortality. No significant difference in the mortality at lower concentrations (0.3683, 1.4732, 2.2098 and 7.366µg/ml) signify that these compounds are beneficial effects. The reduced fitness and growth of fish occurs at sub-lethal levels depending on the exposure time, toxicity, and concentration of the chemical substances involved. Our data showed that exposure to both curcumin and turmeric caused a dose dependent embryonic toxicity (e.g., hatchability and mortality) of zebrafish. In this study, the 72h LC_{50} for both drugs (curcumin and turmeric) was found to be 36.838µg/ml. According to Zhang Z et al, the reproductive ability and early life stages of fish, such as eggs and larvae, are particularly sensitive to contaminants.[29] Thus, this study clearly demonstrates that the mortality of the embryos escalate with increasing concentration and exposure duration (time) of both curcumin and turmeric.

Reduction in survival rate resulted because of the induction of acute toxicity unveiled by supramolar levels of the bioactive component curcumin both at crude and

purified form. Similarly, treatments exhibited significant defects in growth, body length etc., of zebrafish further highlighting the toxic nature of these bioactive essential component- curcumin in the dietary food of common man.

We have shown distinct and dose-dependent morphometric effects of curcumin and turmeric on zebrafish embryos. These effects include significant acute HR lowering and eventual complete heart cessation. Although the existing body of literature involving studies with natural state of turmeric and its actions is quite complex, we have initiated on the use of zebrafish embryos as an in vivo model system for exploring the effects of turmeric and curcumin. This study reports novel data on dose-dependent effects of natural state of turmeric and purified compound curcumin on HR and explores its actions by simple observations using stereo microscope and digital camera. The curcumin concentrations tested in this study has caused an immediate drop in HR, followed by a period of relative stability, finally resulting in complete cessation of the heart. Together all our observations clearly demonstrates the curcumin or turmeric could act as toxic agents at supramolar levels and this attribute of the bioactive principle in the crude form could be envisaged for the treatment of disorders like cancer.

CONCLUSION

Zebrafish embryos treated with increasing concentrations of curcumin in pure form or raw turmeric, do not sustain and there were no statistically significant differences in embryo length, head / eye / tail width, yolk sac length or spinal curvature. This proves that raw turmeric has a similar potency as pure curcumin. Furthermore, natural state turmeric is a toxic substance in higher concentration as proved on developing zebrafish embryos in this study and such properties needs further exploration. This indicates that raw turmeric could partake anticancer activity due to its relative toxic properties exhibited in developing zebrafish embryos. Turmeric is an essential part of Indian ethnic cuisine, and it is consumed on a daily basis by millions of people in this country. It has been renowned for its numerous therapeutic properties that can be attributed to its active ingredient curcumin. The only limitation of curcumin is its low bio-availablity, which scientists have solved through the addition of other components as supplements. With growing interest in healthy living and natural supplements, there is a growing perception that anything natural is healthy. Thus, it may be beneficial to explore such attributes of food ingredients that may be beneficial.

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Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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