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ACUTE TOXICITY TESTING OF SYNTHESIZED PYRAZOLINE DERIVATIVES IN ADULT ZEBRAFISH

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ABSTRACT: Pyrazoline are reduction product of pyrazole. They are well known nitrogen containing 5-membered heterocyclic compound with broad spectrum of biological activities such as antimicrobial, anti-inflammatory, anticancer, analgesic, anticonvulsant, anthelmintic, antioxidant and herbicidal. Pyrazoline derivatives have been synthesized from intermediate chalcone. Pyrazoles have been the recent target of numerous methodologies, mostly due to their prevalence as scaffolds in synthesis of bioactive compounds and reactions in different media. Fish are the widely used vertebrate organism in risk assessment and regulation. Out of them adult zebrafish and zebrafish embryo has a special characteristic which permits its use as a model organism in toxicological and pharmacological studies. Zebra fish known as a *Danio rerio* have risen as effective and powerful models for studying developmental processes and human disorders. Recently the zebrafish based assays have been developed for testing toxicity of drug candidates, including acute toxicity (LC_{50}), organ specific toxicity and developmental toxicity. In present article the toxicity of newly synthesized pyrazoline derivatives has been studied on adult zebrafish and zebrafish embryos according to OECD guidelines for respective models.

INTRODUCTION: Pyrazoline are nitrogen containing 5-membered heterocyclic compounds having two adjacent nitrogen atoms within the ring, and various methods have been worked out for their synthesis. Numerous pyrazoline derivatives have been found to possess considerable biological activities, which stimulated the research activity in this field.

They possess several prominent biological effects, such as antimicrobial, anti-mycobacterial, anti-fungal, anti-amoebic, anti-inflammatory, analgesic, anti-depressant and anti-cancer activities^{1, 2}. It has been considered that if a drug is effective, it will have side effects or any toxic effects after certain limit of concentration³.

The toxicity study is important to decide the maximum tolerable dose of any drug. Fish are the widely used vertebrate organism in risk assessment and regulation. Out of them adult zebrafish and zebrafish embryo has a special characteristic which permits its use as a model organism in toxicological and pharmacological studies⁴.

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Zebra fish known as a *Danio rerio* have risen as effective and powerful models for studying developmental processes and human disorders⁵. Zebra fish shares many features with the mammals. They develop most of the organs found in mammals including those of the nervous, digestive, reproductive, immune, excretory, and cardiovascular systems, immune system⁶. Zebrafish have various special focal points like its small size and low cost, include fecundity, with each female capable of laying 200 - 300 eggs per week, external fertilization and rapid development of embryos, which allows the direct observation of developing internal organs and tissues *in-vivo* situating them for rapid drug discovery and toxicity studies. They can easily absorb the compounds which are solubilized in water, make drug administration simple and feasible.

Fish generate huge number of progenies, therefore offering them to being as appropriate as *in vitro* frameworks. The maintenance cost is less expensive than for other animal models. Zebra fish develop rapidly, allowing for assays of drug toxicities on organ development^{7, 8}. In present article the toxicity of newly synthesized pyrazoline derivatives has been studied.

MATERIAL AND METHOD:

Pyrazoline Derivatives: Pyrazoline is reduction product of pyrazole. Literature survey shows that pyrazoline derivatives show promising antitumor, analgesic and anti-inflammatory activity. The pyrazoline derivatives are synthesized from the chalcone. The chalcone is synthesized from aromatic ketone and aromatic aldehyde in ethanolic NaOH⁹. Further this chalcone is used to synthesize N-acetamide and benzamide pyrazoline derivatives. The pyrazoline derivative obtained from the same institute and the compound are selected on the basis

of their results of cell line study. The compound which shows High to moderate activities in cell line study were selected for toxicity study. Acute toxicity testing of following four pyrazoline derivatives was conducted in adult zebrafish and in zebrafish embryo.

Compound 1: N-(2-(1-carbamothioyl - 5 - (4-chlorophenyl) - 4 - 5 dihydro-1-H- pyrazol - 3-yl) phenyl) acetamide:

Molecular weight: 372, Melting point : 229 ° C, Molecular formula: C₁₈H₂₇ON₄SCl Nature: Whitish powder, R_f value: 0.55 [chloroform: methanol (10:1)] UV λ_{max}: 315.

Compound 2: N-(3-(4-chlorophenyl)-5-(thiophen-2-yl)-4,5-dihydro-1-H-pyrazole - 1 - carbonothioyl) acetamide

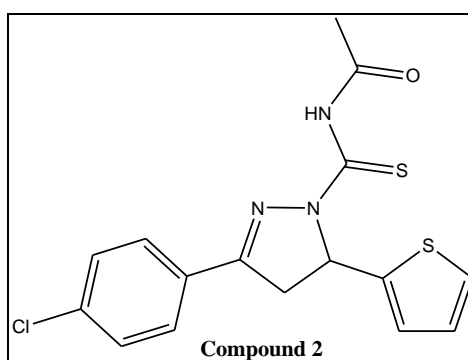
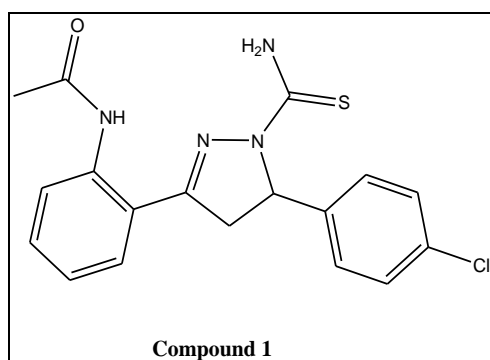
Molecular weight: 363, Melting point: 174°C, Molecular formula: C₁₆H₁₄ON₃S₂Cl Nature: Dark yellow colored crystals, R_f value: 0.67[n-Hexane: Ethyl acetate (3:1)] UV λ_{max}: 301 nm

Compound 3: N-(2-(5-(4-bromophenyl)-1-carbamothioyl - 4 - 5- dihydro - 1- H - pyrazol-3-yl) phenyl) acetamide

Molecular weight: 417, Molecular formula: C₁₈H₁₇ON₄SBr, Nature: white powder, Melting point: 231°C, R_f value: 0.55 [chloroform: methanol (10:1)] UV λ_{max}: 316 nm

Compound 4: N-[5-(4-bromophenyl)-3-phenyl-4,5 - dihydro - 1H - pyrazole - 1- carbonothioyl) benzamide

Molecular weight: 464, Melting point: 161-163 °C, Molecular formula: C₂₃H₁₈N₃OSBr Nature: Brown colored crystals, R_f value: 0.60 [n-Hexane: Ethyl acetate (2:1)], UV λ_{max}: 296 nm



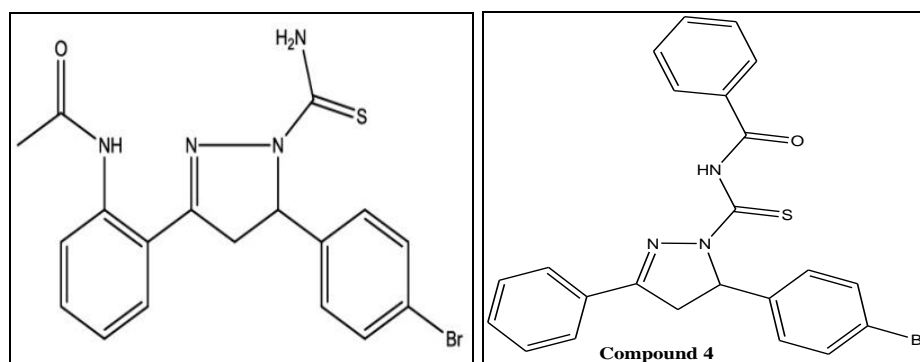


FIG. 1: PYRAZOLINE DERIVATIVES

Acute Toxicity Testing of Synthetic Pyrazoline Derivatives: Toxicity study was performed according to OECD test guideline- 203 (Fish, Acute Toxicity Test) in order to decide lethal concentration (LC_{50}) of test compound in adult zebrafish and according to OECD guideline 236 (Fish Embryo Toxicity Test) to determine acute toxicity of chemicals on embryonic stages of fish.

Procedure for Acute Toxicity in Adult Zebrafish: The wild type adult zebra fish weighing between 0.5 - 1.5 gm were procured from local supplier. They were kept into water tank and proper aeration was provided. 12 hrs light and 12 hrs dark light cycles was maintained. Fish were fed three times daily with micro pellets. They were kept for 15 days for acclimatization¹⁰. Total 119 fishes were divided in to seventeen different groups with 7 fishes per group. The four different concentration of each compound were prepared in geometric series with an increasing factor 2.2.

Group I served as a control group (1ml DMSO). Group II to group V received 0.20 mg, 0.44 mg, 0.96 mg and 2.12 mg dose of compound 1 respectively. Group VI to group IX received 0.20 mg, 0.44 mg, 0.96 mg and 2.12 mg dose of compound 2 respectively. Group X to group XIII received 0.30 mg, 0.66 mg, 1.45 mg and 3.19 mg dose of compound 3 respectively. While group XIV to group XVII received 0.20 mg, 0.44 mg, 0.96 mg and 2.12 mg dose of compound 4. The drug was dissolved in DMSO prior to addition in to respective fish tank. The fishes were exposed to

test compounds for a period of 96 hours. The mortalities were recorded at 24, 48, 72 and 96 hours and the concentration which killed 50 % of the fish (LC_{50}) was determined¹¹.

Procedure for Acute Toxicity in Zebrafish Embryos: Zebrafish embryos were procured from local supplier. Zebrafish embryos are kept in embryonic medium¹². Embryos were divided in to twenty different groups with 12 embryos per group. 96 well microtitre plates were used for this study. Each embryo was transferred in individual well plate. The five different concentration of test compound were prepared by diluting the solvent in a geometric series with an increasing factor of 2. Group I served as a control. Group II to V, Group VI to IX, Group X to XIII and Group XIV to group XVII received 10 μ g, 8 μ g and 6 μ g and 4 μ g dose of compound 1, 2, 3 and 4 respectively. The drug was dissolved in DMSO prior to addition in to respective groups. The embryos were exposed to test compounds for a period of 96 hours. Coagulation of embryos, lack of somite formation, and non-detachment of the tail, lack of heartbeat indicates mortality. The mortalities were recorded at 24, 48, 72 and 96 hrs and the toxic concentration was determined¹³.

RESULT: The maximum concentration at which the 50 % of the fish was killed was recorded. The abnormal changes and mortality of fish for every test compound was recorded after 24, 48, 72 and 96 hours given in following **Tables**.

TABLE 1: TABLE SHOWING THE RESULTS OF ACUTE TOXICITY TEST IN ADULT ZEBRAFISH

Compounds	Group	Dose	Abnormal changes		Mortality			
			Swimming	Pigmentation	24 hrs	48 hrs	72 hrs	96 hrs
Control	Group I	-	-	-	-	-	-	-
	Group II	0.2mg	-	-	-	-	1	1
Compound 1	Group III	0.44mg	-	-	1	1	2	3

Compound 2	Group IV	0.96mg	✓	✓	1	2	3	3
	Group V	2.12mg	✓	✓	2	4	7	7
	Group VI	0.2mg	-	-	-	-	1	1
	Group VII	0.44mg		✓		2	3	4
Compound 3	Group VIII	0.96mg	✓	✓	-	2	5	7
	Group IX	2.12mg	✓	✓	1	3	6	7
	Group X	0.3mg	-	✓	-	-	1	2
	Group XI	0.66mg	✓	✓	-	2	3	6
Compound 4	Group XII	1.45mg	✓	✓	-	3	6	7
	Group XIII	3.19mg	✓	✓	4	6	7	7
	Group XIV	0.2mg	-	-	-	1	2	2
	Group XV	0.44mg	-	✓	-	1	4	5
	Group XVI	0.96mg	✓	✓	1	2	5	7
	Group XVII	2.12mg	✓	✓	2	7	7	7

TABLE 2: TABLE SHOWING THE RESULTS OF ACUTE TOXICITY TEST IN ZEBRAFISH EMBRYO

Compounds	Group	Dose (µg)	Mortality			
			24 hrs	48hrs	72hrs	96hrs
Control	Group I	-	-	-	-	-
Compound 1	Group II	10	8	10	12	12
	Group III	8	6	8	10	12
	Group IV	6	4	7	8	8
	Group V	4	4	6	7	7
Compound 2	Group VI	10	8	12	12	12
	Group VII	8	7	10	12	12
	Group VIII	6	6	8	10	10
	Group IX	4	6	7	8	10
Compound 3	Group X	10	11	9	7	5
	Group XI	8	11	9	7	4
	Group XII	6	5	7	9	10
	Group XIII	4	4	5	8	9
Compound 4	Group XIV	10	9	12	12	12
	Group XV	8	6	8	8	10
	Group XVI	6	5	6	6	7
	Group XVII	4	3	4	4	5

DISCUSSION: The present study was undertaken to determine the toxic dose for the each test compounds. OECD test guideline- 203 (Fish, Acute Toxicity Test) was performed in order to decide lethal concentration (LC₅₀) of test compound in adult zebrafish. No abnormal changes in swimming behavior, pigmentation and survival were observed in the control group fishes. Changes in swimming behavior and pigmentation of zebrafish treated with test compound were observed. From the result the LD₅₀ was found to be 2.5 mg for compound 1; 1.9 mg for compound 2; 1.25 mg for compound 3 and 1.47mg for compound 4. OECD guideline 236 (Fish Embryo Toxicity Test) for acute toxicity of chemicals was performed to decide the lethal concentration of test compounds. The LD₅₀ was found to be 4.31 mg for compound 1; 3.18 mg for compound 2; 4.86 mg for compound 3 and 5.85 mg for compound 4.

CONCLUSION: This present study predicts that zebrafish in test compounds shows toxicity at lower dose when expose to longer time. The pyrazoline derivatives are highly toxic in lower concentration. From this study zebrafish was demonstrated as a viable model for screening compounds toxicity. On the basis of this toxicity study the compounds were further used in performing the regenerational and developmental angiogenesis assay in adult zebrafish.

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CONFLICT OF INTEREST: Nil

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