RESEARCH ARTICLE

In-vivo Toxicity Profile of 2-Butyl-3-(3, 5-Diiodo-4-Hydroxybenzoyl) Benzofuran on Different Experimental Models

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ABSTRACT

The anti-cancer properties of 2-butyl-3-(3, 5-Diiodo-4-Hydroxybenzoyl) benzofuran have been determined in earlier *in-vitro* studies, but safety and efficacy still need to be resolved. This research investigates the acute oral toxicity of 2-butyl-3-(3, 5-diiodo-4-hydroxybenzoyl) benzofuran on female Wistar rats, zebrafish, and brine shrimp. The present study was conducted on rats to evaluate the compound acute oral toxicity, following the protocols established by the Organization for Economic Cooperation and Development (OECD) 423. After 14 days of duration, histopathological changes were observed in the liver and heart of animals that received a dosage of 2,000 mg/kg. The compound was tested at a limit test concentration of 100 mg/L in zebrafish for period of 96 hours. During this time, sub-lethal clinical signs and mortalities were observed at 24, 48, 72, and 96 hours. Brine shrimps were exposed to various concentrations for 24 hours to evaluate their cytotoxicity and calculate the percentage of mortality. Histopathological changes are primarily observed in the liver (multifocal necrosis of hepatocytes), heart (myocardial inflammation) and lung (low alveolar/interstitial inflammation). There are no recordings of mortalities at 300 and 2,000 mg/kg treated rats. Behavioral patterns remained unchanged, whereas food intake and body weight decreased significantly. The oral administration of the test chemical to rats would result in an LD_{50} greater than 2,000 mg/kg, ranking it in the fifth category of the GHS. The LC_{50} (322.96 µg/mL) for the brine shrimp lethality assay was calculated using a plotted graph. The test compound's LC_{50} value in the zebrafish model would be higher than 100 mg/L. The acute toxicity profile of 2-butyl-3-(3, 5-diiodo-4-hydroxybenzoyl) benzofuran has been demonstrated using rodents, zebrafish, and brine shrimp lethality assay. This study will guide subsequent chronic toxicological assessments.

Keywords: Acute toxicity, Brine shrimp, Rats, OECD, Zebrafish

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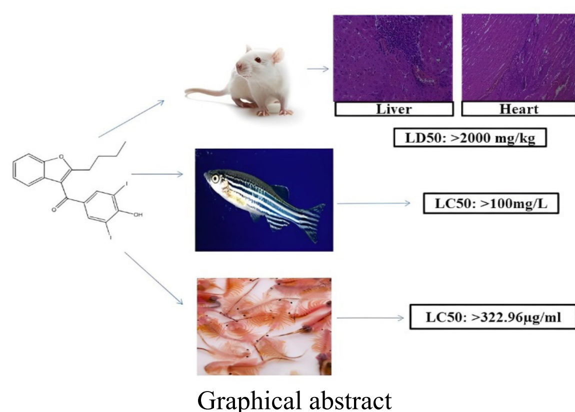

Graphical abstract

INTRODUCTION

The toxicity assessment is crucial for identifying the test material and its adverse consequences. Novel pharmaceuticals must undergo comprehensive toxicity testing before human administration.[1] Chemical risk assessment methodologies facilitate data-driven and scientific decision making and are anticipated to improve the accuracy of risk prediction." Regulatory agencies are obligated to employ modeling approaches, uncertainty factors, and default values in situations where data is inconsistent or unavailable. New methods and testing could fill data gaps, clarify inconsistencies, or reduce uncertainty. These methods can improve regulatory decision-making accuracy and scientific credibility if used properly.

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Most toxicity research was conducted on experimental animals, revealing test compounds' risks and adverse reactions. The continued use of animals in toxicity testing is expected to endure in the foreseeable future, owing to their inherent benefits in the examination of functional organisms.[2] The investigation and evaluation of a drug's potentially harmful properties typically begin with evaluating the drug's acute oral toxicity. Acute toxicity determines the dose for further research and guides additional chronic toxicological testing.[3]

In our previous investigations, we discovered that a compound called 2-butyl-3-(3,5-diiodo-4-hydroxybenzoyl) benzofuran showed strong anti-cancer effects against HT-29 (human colon cancer) and A549 (human lung cancer) cells.[3] This molecule obeys Lipinski's rule five, molecular weight is 546, partition coefficient between octanol and water (log P) is 5.49, hydrogen bond donor is one, and hydrogen bond acceptor 3. DataWarrior v04.04.04 software predicts that this substance does not pose significant risks of toxicity, such as mutagenicity, tumorigenicity, gonadal toxicity, and irritant effects.[4] In comparison to EGFR, ER, and anaphylactic lymphoma kinase, this exhibits a high affinity for tubulin protein binding, as determined by the *in-silico* method. Using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay, this compound demonstrated a more potent cytotoxic effect against A549, MCF-7, HT-29, and DU145 cancer cells (IC_{50}: 10.66, 6.05, 5.17, 7.02 µg/mL) in comparison to the standard doxorubicin effect (IC50: 14.54, 5.96, 2.4, 6.3 µg/mL).compared to the standard doxorubicin effect (IC_{50}: 14.54, 5.96, 2.4, 6.3 µg/mL)

In growing cancer cells, this substance demonstrated significant anti-proliferative properties in a dose-dependent manner. The HT-29 cell death rate was 80% in the early cell cycle stage and 50% of cells arrested in the G0/G1 phase at IC_{50} (5.17 µg/mL). This compound induces programmed cell death by altering the ratio of Bax/Bcl-2 proteins and enhancing the activation of caspase-3. Enzymes such as DNase become active following caspase-3 activation, leading to DNA fragmentation and apoptosis. The level of DNA fragmentation was 6.5-fold greater in treated cells compared to untreated cells.[4]

As per *in-silico* toxicity studies, 2-butyl-3- (3, 5-diiodo-4-hydroxybenzoyl) benzofuran proved as safe that is free from mutagenicity, tumorigenicity, gonadal toxicity, and irritant effect.[4] There is no previously reported *in-vivo* toxicity of the profile of this selected compound. It was necessary to utilize *in-vivo* models to confirm that this particular compound was also safe. If this compound is determined to be safe using *in-vivo* models, we can proceed further anti-cancer research work. The main aim of this study is to evaluate the acute toxic effects of orally ingesting 2-butyl-3-(3, 5-diiodo-4-hydroxybenzoyl) benzofuran using three different experimental models.

Organization for Economic Cooperation and Development (OECD) recommends testing a drug on animals to assess its median oral toxicity LD_{50} and effective/therapeutic dose.[5] The LD_{50} is a method used to assess the acute toxicity, or the potential for short-term poisoning, of a substance. The oral toxicity of drugs has been assessed using rats and mice. Determination of acute oral toxicity is helpful to do further chronic toxicological evaluations.[6] The assessment of fish toxicity is a critical component of many regulations worldwide that assess environmental risks and hazards.[7] The zebrafish has evolved as a crucial toxicological model organism since 1950. Zebrafish (*Danio rerio*) are superior alternative models due to their small size, quick external growth, optical transparency during early development, permeability to tiny chemicals, genetic closeness to humans, and high fertility.[8] The OECD test guideline 203 (TG 203) helps evaluate chemical compounds' acute fish toxicity. Zebrafish have distinct advantages over other vertebrate models that simulate human diseases, particularly for assessing developmental toxicity and other biomedical research applications.[9] The brine shrimp cytotoxicity test (BSCT) assesses the lethal effects of a substance on a significant zoological organism, such as the shrimp species *Artemia* salina.[10] The brine shrimp bioassay is a trustworthy, practical, and economical method for discovering synthetic and plant-based bioactivities.[11]

MATERIALS AND METHODS

Acute Oral Toxicity Tests

This sighting study aimed to evaluate the safety of synthetic chemical compounds. Animals fasted for 12 hours before the treatment, but the water could not be withheld. Test compound delivered in single doses following the OECD flow chart in Figure 1. Three animals were administered the compound at a starting dose of 300 mg/kg body weight *via* oral gavage for animal welfare considerations due to a lack of knowledge about the desired compound. As depicted in Figure 1, this study utilized three female Sprague Dawley rats per stage based on the animal's mortality and moribund status. Individual animal observations were conducted after administration of a single dose within the first 30 minutes, intermittently during the first 24 hours, with a particular focus on the first 4 hours, and then daily for 14 days. At least 24 hours will elapse between each animal's dosages; they will observe for 14 days and measure their food and water intake daily. Monitor and record any modifications in the skin and fur, eyes and mucous membranes, respiratory system, behavior, salivation, tear production, bowel movements, energy levels, responsiveness, mood, sleep patterns, mental state, and mortality over a 14-day period. Perform histopathological examinations on the animals by sacrificing them on the 15th day.[12]

Histopathological Evaluations

All visceral organs of rats given acute oral exposure to the compound at a dose of 2000 mg/kg were histopathologically examined for defects in tissue architecture. After sacrificing the animals, clean tissues with ice-cold normal saline (0.9%) to eliminate debris. After fixation, Yorco automatic tissue processors implanted the tissues in paraffin blocks. Leica RM2135RTS cuts 3 mm ribbons from paraffin blocks and places them on microscope slides. After H and E staining, the tissue was examined under 4X, 10X, and 40 X magnifications with a Labomed microscope for histological changes.[13]

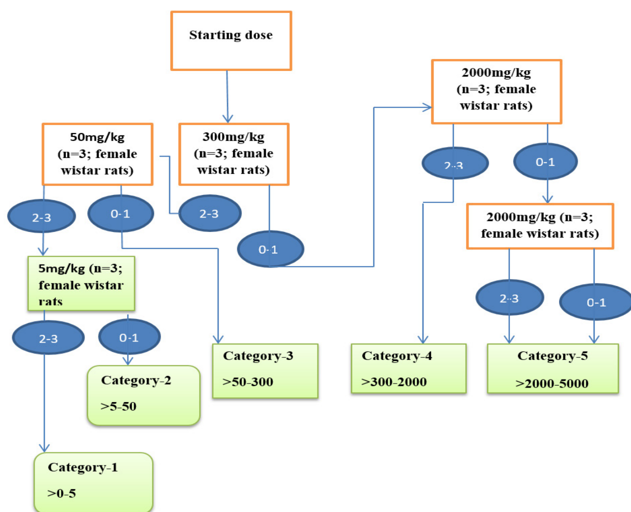

Figure 1: OECD Guidelines-423 (starting dose from 300mg/kg) for the testing of chemicals

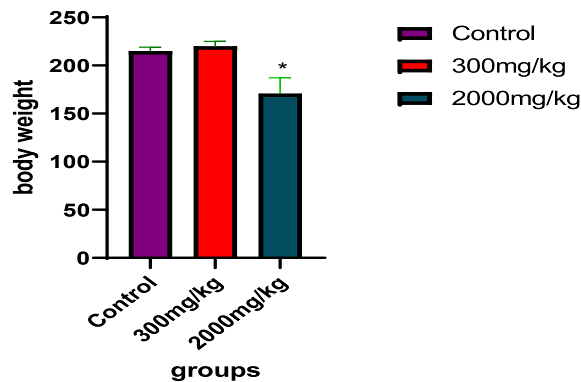

Figure 2: Body weight changes of experimental rats during 14 days of experimental period.

Acute Toxicity Sudies on Zebrafish

OECD guidelines 203

This study was carried out in a laboratory that has been approved by the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals). The laboratory has a registration number of 1567/PO/RE/S/11/CPCSEA and adheres to animal care regulations. The Institutional Animal Ethics Committee (IAEC) (MLRIP/CPCSEA/IAEC/2022/01) has approved this investigation. A limit test (100 mg/L) was conducted with seven adult zebrafish per test group and control group (1-mL/L of DMSO) using the Static method in 5L glass containers for 96 hours. Mortalities are recorded at 24, 48, 72, and 96 hours according to acute fish toxicity testing guidelines.[14]

Brine shrimp lethality assay

- Brine shrimp hatching
- Weigh approximately 27 grams of table salt and add it to the beaker filled with 3 liters of water.
- Mix water with a spatula.
- Maintain aeration by inserting an air pump airline into the jar.
- At the top of the beaker, add 15 g of brine shrimp eggs and stir them with water.
- Activate a light bulb with a power rating of 60 to 100 watts. Placing it at a distance of a few inches from the jar.

After a period of 20 to 24 hours, the nauplii will emerge from their eggs; the hatched nauplii can be easily distinguished from the unhatched eggs. Utilize a Pasteur pipette to transfer a total of ten nauplii into a test tube. Subject the nauplii to different concentrations of compound and assess the percentage of mortality after a 24-hour period.[15]

- *Preparation of samples*

The stock solution originated by dissolving 10 mg of 2-butyl-3-(3, 5-diiodo-4-hydroxybenzofuran; 1-mL solubility) in 9 mL of water. The stock solution was serially diluted to yield 50, 100, 200, 400, and 800 µg/mL. There were 15 test tubes labeled in total, with three replicates for each concentration. Introduce the prepared solution into the test tubes containing ten nauplii, and count the number of died nauplii after 24 hours. Determine the lethality percentage by employing a mathematical formula.[16]

$$\%Death = \frac{\text{Number of dead nauplii}}{\text{Number of dead nauplii + number of live nauplii}} \times 100$$

Draw the graph from the concentration of sample versus % death and calculate the LC_50 from the graph.

RESULTS

Acute Oral Toxicity Study on Gats

Rats given 300 mg/kg did not exhibit any mortality symptoms. Clinical signs did not observed in either group at the beginning of the initial 15 minutes of monitoring. Clinical symptoms, such as dullness and paw licking, were found after 30 minutes, and they persisted for the remaining 2 hours of the observation period, returning all three animals to normal. Water intake, food intake, and weight did not differ significantly from the control group. The three animals' skin, fur, eyes, diarrhea, or behavior from day one did not exhibit any toxicity-related symptoms.

Behavioral patterns of animals treated with 2-butyl-3-(3, 5-diiodo-4-hydroxybenzoyl) benzofuran (2000 mg/kg) were studied for the first four hr and then again for 14 days. The clinical signs, such as paw licking and dullness observed after 30 minutes, normalized the animals after 3 hours. Figures 2 and 3 represent feeding patterns and body weight- significant changes in daily food intake may be a reason for significantly (p <0.05) reducing body weight. There are no notable alterations in the pigmentation of the skin, fur, eyes, mucous membranes, behavioral patterns, salivation, and sleeping patterns. Following a 14-day experimental period, three animals were euthanized and their cranial, thoracic, abdominal, and pelvic body cavities were dissected and inspected.

There were no signs of lesions in any of the three animals' organs. Figure 4 represents the histopathological observation of

Table 1: Various parameters were recorded during 96-hours on zebrafish.

Test day/observation	*Day-0, 2-3hrs*	*Day-0, 5-6 hrs*	*Day 1 FN*	*Day 1 AN*	*Day 2 FN*	*Day 2AN*	*Day 3 FN*	*Day 3 AN*	*Day 4*
Approximate observ ation time from start (hours)	2.5	5.5	24	30	48	54	72	78	96
Mortality	NO	NO	NO	NO	NO	NO	NO	NO	NO
Swimming behaviour	Normal	Excessively active	Excessively active	Excessively active	Normal	Normal	Normal	Normal	Normal
Reactive to stimulus	Normal	Excessively active	Excessively active	Excessively active	Normal	Normal	Normal	Normal	Normal
Skin pigmentation	None	None	None	None	None	None	None	None	None
Visible abnormalities	None	None	None	None	None	None	None	None	None
Abnormal ventilator function	None	None	None	None	None	None	None	None	None

Table 2: Brine shrimp lethality assay

Concent ration	*LogC*	*Num of shrimp taken*	*Death*	*Live*	*Perce ntage*	*LC50*
Control	-	10	0	10	0	
50µg/ml	1.69	10	1	9	10	
100µg/ml	2	10	3	7	30	
200µg/ml	2.3	10	4.8	5.2	48	
400µg/ml	2.6	10	7.3	2.67	73.3	322.96µg/ ml
800µg/ml	2.9	10	8.4	1.7	83	

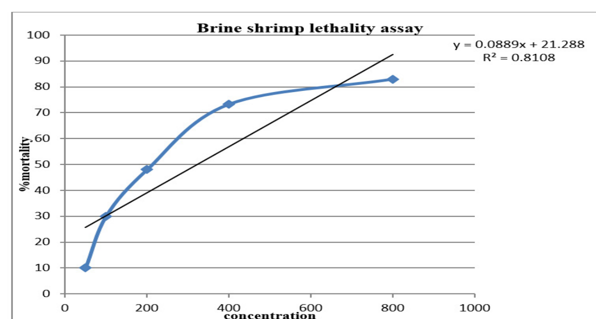

Figure 4: Brine shrimp lethality assay of compound by using graph

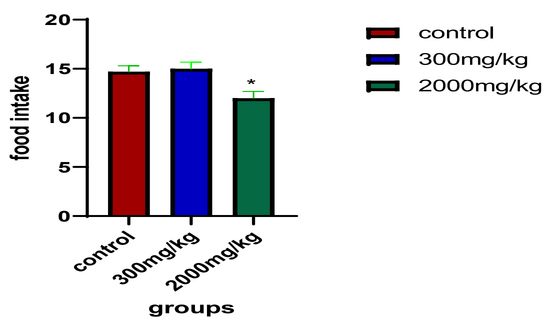

Figure 3: Feeding pattern (water intake) of rodents over a 14-days of observation period.

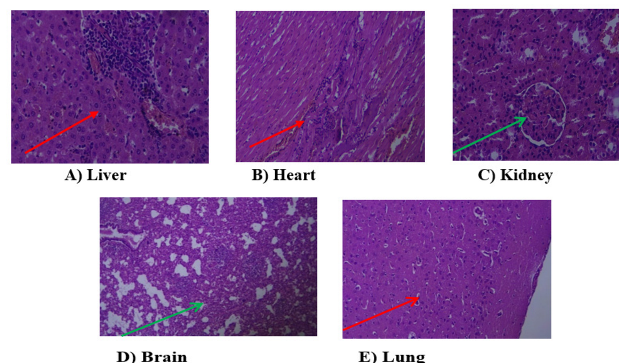

Figure 5: Histopathological View of Experimental rat vital organs after 14-days.

vital organs treated with 2000 mg/kg – a red arrow in Figure 5(a). The liver exhibits multifocal necrosis of hepatocytes in both the centrilobular and periportal regions, accompanied by infiltration of inflammatory cells. A red arrow in Figure 5(b) denotes presence of many foci of myocardial inflammation and inflammatory cell infiltration. A green arrow in Figure 5(c) shows renal tubules and glomeruli in their typical morphological configurations. A green arrow in Figure 5(d) depicts the cerebral cortex's normal morphology and no abnormalities in the brain. Figure 5(e) shows a low level of interstitial/alveolar inflammation and inflammatory cell infiltration.

Zebra-fish Acute Oral Toxicity

In the present investigation, various parameters of treated fishes compared with control group fishes. No mortality, physical, behavioral, or visible abnormalities were recorded in the control group fishes. In contrast, Table 1 revealed the various observed parameters during the exposure period. There was no observed mortality in the treated and control group of fish in the entire 96 hours of the exposure period. The fish exhibited a strong response to the stimulus, displaying heightened swimming activity after being exposed for 3 hours. We did not observe skin pigmentation, visible abnormalities, and abnormal respiratory function in treated fish during exposure.

No mortalities were observed at the limit test concentration, indicating that the LC_{50} concentration is greater than 100 mg/L.

Cytotoxic Assay-Brine Shrimp

We calculated the brine shrimp's percentage lethality and LC_{50} values after 24 hour of exposure to different concentrations of test compound (50, 100, 200, 400, and 800 µg/mL) The lethality percentage increased in direct proportion to the concentration of the test compound. Table 2 displays the lethality of various test sample concentrations of brine shrimp nauplii. The mortality rate was lowest at a concentration of 50 µg/mL, while it has shown to be the significantly highest at a concentration of 800 µg/mL. The LC_{50} of the compound has been found to be 322.96 g/mL based on a plotted graph of (Figure 5) concentration versus percentage of lethality.

DISCUSSION

Toxicology is generally the initial step in studying a substance's biological characteristics.[17] The oral acute toxic class approach is used as an alternative to the oral LD_{50} test to assess oral toxicity in rats. Examine the animals for 14 days after administering a single oral dosage of 2-butyl-3-(3, 5-diiodo-4-hydroxybenzoyl) benzofuran at 300 or 2000 mg/kg. Drug toxicity can be determined by observing rats appearance and behavioral characteristics. Animals administered 300 and 2,000 mg/kg exhibited no signs of mortality, but at 2000 mg/kg treated animals showed histopathological changes, primarily in the liver (multifocal necrosis of hepatocytes), heart (myocardial inflammation) and lung (low alveolar/ interstitial inflammation), that may indicate risk of organ damage or showing adverse drug reaction on heart, liver and lung. Further chronic toxicological evaluations are required to determine the reason for these inflammatory effects on the liver, heart, and lungs. According to earlier findings, the LD_{50} value for the compound was found to be greater than 2,000 mg/kg, considered acute oral toxicity (Globally Harmonized System) category 5 under OECD standards 423.

To assess the safety of manufactured chemicals, including plant products and industrial chemicals, acute oral toxicity studies are required in many countries.[18] The OECD test guideline 203 is the most commonly used acute toxicity guideline for zebrafish in prospective assessments around the globe. There are many chances to apply the 3Rs - reduction, replacement and refinement of animals.[19] In order to decrease the number of test groups, experiments are carried out using a single concentration (100 mg/L) of 2-butyl-3-(3, 5-diiodo-4-hydroxybenzoyl) benzofuran on seven zebrafish. The experiments are conducted using the static method for a duration of 96 hours. There is no evidence of death in both the control and treated groups of fish. According to this model, the LC_{50} compound has a concentration above 100 mg/L.

The brine shrimp cytotoxicity test (BSCT) is a way to see how harmful a test sample is to a simple animal like the shrimp (*Artemia salina*). The brine shrimp bioassay is a safe, convenient, and affordable tool for assessing synthetic and plant product bioactivities.[20] In the preliminary research on cytotoxicity, the Hatched nauplii stand out as being very important. A large number of researchers advocate the adoption of this methodology.[21] Despite being a great option for basic toxicity investigations, the brine shrimp lethality bioassay was found to exhibit dose-dependent concentrations of the compound.

CONCLUSION

This compound would be initially safe using three experimental models in this study. LD_{50} of this compound calculated by acute toxic class method would be greater than 2000 mg/kg because of no mortality at 300 and 2000 mg/kg. In the zebrafish model, there were no signs of mortality at the limit test concentration (100 mg/L) of 2-butyl-3- (3, 5-diiodo-4- hydroxybenzoyl) benzofuran, so the LC_{50} value for this compound would be greater than 100 mg/L. We observed dose-dependent lethality in hatched nauplii and estimated the LC_{50} value by plotting concentration vs mortality percentage. The LC_{50} value of this compound was 322.96 µg/mL. This study concluded that 2-butyl-3- (3, 5-diiodo-4-hydroxybenzoyl) benzofuran doesn't have acute toxicity, but it will provide a basis for future chronic.

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