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Acute And Chronic Toxicity Effects Of 2, 6-Bis (2, 5-Dimethoxybenzy-Lidene)Cyclohexanone (Bdmc33) In Zebrafish (*Daniorerio*) Tested On Adulthood Life Stage

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Abstract: The toxicity effects of 2,6-bis(2,5-dimethoxybenzy-lidene)cyclohexanone (BDMC33)on adult zebrafish (>6 months old) was investigated in this study. The acute and subchronic toxicity tests by exposing adult zebrafish (>6 months old) to various concentrations of BDMC33 for 48 hrs and 14 days, respectively, were conducted. The 48 hrs LC_{50} value was determined, mean weight, malondialdehyde (MDA) and myeloperoxidase (MPO) activities, and the pathological changes in the intestine and liver of treated zebrafish were evaluated after 14 days of exposure. Similar analyzes on aspirin (positive control) and 3-(2-Fluoro-benzylidene)-5-(2-fluorocyclohexylmethylene)-piperidin-4-one (EF-24)(check control) were also examined for comparison. The 48 hrs LC_{50} values were 8 µM (aspirin), 10 µM (EF-24), and 20 µM (BDMC33). The average weights at 14 days were 0.87 mg (aspirin), 1.89 mg (EF-24), and 2.01mg (BDMC33). The BDMC33 yielded low level of MDA and MPO, EF-24 gave similar value, and aspirin produced higher malondialdehyde (MDA) level and myeloperoxidase (MPO) activities during the period of exposure. In aspirin-treated fish, the epithelial/mucosal wall and liver hepatic injuries were observed. No pathological changes were noticed on the intestine and liver of zebrafish exposed to BDMC33 (20 µM) and EF-24 (10 µM) at 14 days of treatments. These findings demonstrated that BDMC33 is better than EF-24 and aspirin. Therefore, BDMC33 could be a leading feature, anti-inflammatory agent.

Keywords: Acute toxicity; BDMC3; chronic toxicity; curcumin analogues; zebrafish

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I. INTRODUCTION

In fish, the most commonly estimated acute toxicity tests of chemical compounds being investigated are performed through a short-term test, predominantly on juvenile or adult zebrafish[1,2]. The exposure of chemical compounds to early or adulthood life stage of zebrafish (*Daniorerio*) is one of the highly sensitive screening tests established to investigate the toxicity of chemical compounds on humans and animals prior consumption[3,4]. The results of the toxicity test with zebrafish can be transformed and used for the toxicity test in humans and animals. Zebrafishare freshwater tropical species, native to India and Pakistan, and a member of the Cyprinidae family, it is small in size, robust, easy to maintain, short time generation, highly reproducible and has about 80% correlation to high animal models. Zebrafish has almost the same number of chromosomes with humans (25 vs. 23 pairs), respectively, and that about 71.4% of human genes have at least one zebrafish orthologue[5, 6]. These unique properties have made it be the most important toxicological and biomedical models as a whole vertebrate organism use to assess the systemic toxicity and complex mechanism[6].

Curcumin is a hydrophobic polyphenol compound derived from the rhizomes of *Curcuma longa* Linn. It has high potentials of biological and pharmacological activities[7]. Curcumin biological potencies in oxidative and inflammatory disorders, cancer, Alzheimer's and cardiovascular diseases, and neurological disorders have been documented[8]. The pharmacological safety and efficacy of curcumin has made it a high therapeutic agent against a wide variety of human ailments[8]. But, the pharmacological efficacy and therapeutic index of curcumin has been hampered as a result of its poor systemic availability outside the gastrointestinal tract. Curcumin structural instability and rapid metabolism in the system, which was due to the presence of active methylene groups and a β-diketone moiety, are responsible for its limited bioavailability outside the gut[9,10]. This has deprived curcumin not been accepted as a therapeutic agent[11]. In clinical studies, the excretion of curcumin and its metabolites via both feces and urine after oral doses in humans have been documented[12, 13].

In animal study, about 75% of compounds similar to curcumin were detected in feces whereas none or little amounts were found in the urine after oral dose (1 g/kg) of a dietary [9]. In efforts to increase the bioavailability, biological activity or tissue targeting ability and maintain the low toxicity of curcumin, chemical synthesis of curcumin analogue; 3-(2-fluoro-benzylidene)-5-(2-fluorocyclohexylmethylene)-piperidin-4-one(EF-24) was adopted and proved to be better than curcumin[14, 15, 16].Despite all these promises exhibited by EF-24, its development as the anti-inflammatory agent has been prevented due to its poor aqueous solubility. Previously, our research group has synthesized a curcumin analogue, which is 2,6-bis(2,5-dimethoxybenzy-lidene)cyclohexanone (BDMC33) (Figure 1B) with high solubility and anti-inflammatory activities than curcumin[10]. Potent antioxidant and anti-inflammatory activities of BDMC33 have been documented and proved to inhibit inducible nitric oxide synthase (iNOS), enhanced PGE_2 synthesis and COX expressionin anti-inflammatory assay than curcumin[10, 17, 18].

Therefore, this study aims to evaluate the acute and subchronic toxicity effects of BDMC33 on *Daniorerio* at adulthood life stage to ascertain the future use of the curcumin analogue (BDMC33). Similar analyzes were conducted on EF-24 (check control) and aspirin (positive control) (Figure 1A and C), respectively, for comparison.

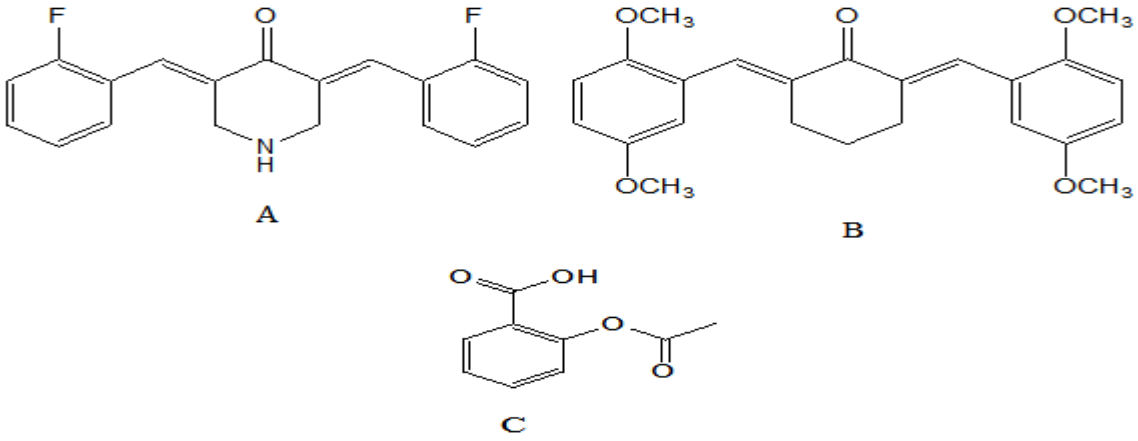

Figure 1: Chemical structures of (A) EF-24: 3-(2-Fluoro-benzylidene)-5-(2-fluorocyclohexylmethylene)-piperidin-4-one (MW: 311.31 g/mol). (B) BDMC33: The 2, 6-bis (2, 5-dimethoxybenzylidene) cyclohexanone (MW: 394.46 g/mol), and (C) aspirin (MW: 180.157 g/mol).

II. MATERIALS AND METHODS

Chemicals and reagents

The following chemicals and reagents: dimethyl sulphoxide (DMSO) and EF-24, were purchased from Sigma Chemical CO. (St. Louis, MO, USA). Genesis (to remove chlorine from tap water), Ammonia test solution, Super battle bacteria (to combat bacteria from aquarium water) was purchased from a local pet shop, Aquatics international SDN. BHD. 260A, Jalan 1A, KampungBaruSubang, 40150 Shah Alam, Malaysia). All chemicals used were of analytical grade.

Chemical synthesis of 2,6-bis(2,5-dimethoxylidene)cyclohexanone (BDMC33)

The compound; 2,6-bis(2,5-dimethoxybenzylidene)cyclohexanone (BDMC33) was chemically synthesized at the Institute of Bioscience, Universiti Putra Malaysia (Figure 2). The synthesis was done by reacting the appropriate aromatic aldehydewith cyclohexanone, under base-catalyzed aldol condensation, using the ratio of ketone: aldehyde (1:2) [10].

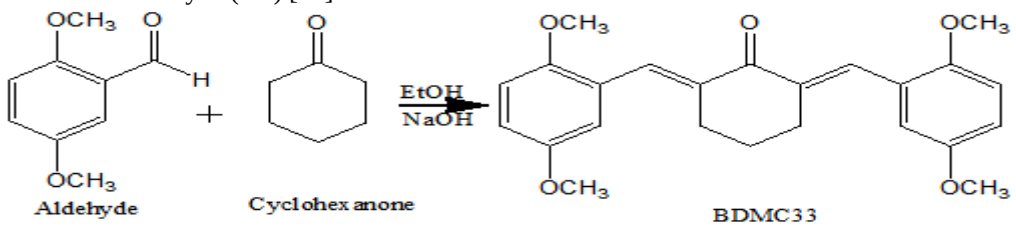

Figure 2: Chemical synthesis scheme of BDMC33; 2, 6-bis(2,5-dimethoxybenzylidene) cyclohexanone (MW: 394.49 g/mol).

The preparation and dilution of 2,6-bis(2,5-dimethoxybenzylidene) cyclohexanone (BDMC33)

Each of the test compound; BDMC33, EF-24, and aspirin (Figure 1A, B, and C), respectively, was appropriately measured, separately, in 1 mL of 100 % DMSO as a stock solution at 50 mM/L. The working concentrations of each compound were prepared by accurately measured from the stock solution in 1.5 L of dechlorinated tap water (fish medium) to obtain final concentrations (0.6 µM – 30 µM) at 7 concentrations and the control, and the final concentration of DMSO was 1%.

Animals (Fish) maintenance

Adult, wild-types, zebrafish (>6 months old) used were purchased from a local pet shop (Aquatics international SDN. BHD. 260A, Jalan 1A, KampungBaruSubang, 40150 Shah Alam, Malaysia) and transported to the aquarium laboratory within 40 min The procedure for zebrafish maintenance and use were followed in accordance with OECD Fish Embryo Test Draft Guideline of 2006 and approved by Universiti Putra Malaysia Institutional Animal Care and use Committee (UPM/IACUC/AUP No.: R024/2014). Briefly, about three hundred (500) adult male zebrafish (>6 months old) were kept and acclimated in 550 L aquarium tank containing 500 L of dechlorinated tap water, and well equipped with Bio-Foam Filter and thermometer. The fishes were fed with brine shrimp salt (Artemia) in the morning and dried flake food in the afternoon, daily for 7 days. The zebrafish were maintained at 28 °C and pH 7.0 with the light and dark cycle at 14:10 hours[1].

Acute toxicity test

The wild-type, adult male zebrafish (>6 months old) were selected from maintenance aquarium tanks for this experiment. The criteria of Good Laboratory Practice were maintained when animal experiments were conducted in the laboratory, and the procedure for the test on zebrafish were followed in accordance with OECD 203[3] and approved by Universiti Putra Malaysia Institutional Animal Care and use Committee (UPM/IACUC/AUP No.: R024/2014). Briefly, the static method of exposure in 10 L aquarium tanks for 48 hrs was adopted. The toxicity tests were performed by exposing 6 individuals of fish/treatment to test concentrations (30, 25, 20, 15, 10, 08 and 06 µM) of each compound in 6 L of dechlorinated tap water containing 0.1% DMSO. The same volume of dechlorinated tap water containing 0.1% DMSO without test concentration was set up as the control. All treatment and control groups were maintained at 25 °C and pH 7.0. Each treatment group and control were replicated three times, with 6 fishes per replicate. At every 12 h of exposure, the number of dead fish was recorded and removed immediately from the tanks. Then, at 48 hrs of exposure, the cumulative mortalities, and the 48 hrs lethal concentrations (LC) values for each test compound was determined. All fish were not fed during the period of the experiment.

Subchronic toxicity test

About 72 healthy, wild-type, adult male zebrafish (>6 months old) were selected for this experiment. The semi-static method of exposure for 14 days was adopted following the protocol of OECD [1]. The concentration of each test compound used in this experiment was selected after the acute toxicity test. The zebrafish were randomly divided into 4 groups (control, and treatment 1, 2, and 3) with 5 individual zebrafish per treatment. Treatment 1, 2, and 3 were exposed to BDMC33 (20 µM), EF-24 (10 µM), and aspirin (8 µM), respectively, in 5 L of dechlorinated tap water containing 0.1% of DMSO, whereas the control group was exposed to an equal volume of dechlorinated tap water containing only 0.1% DMSO. Each group was replicated three times with 6 zebrafish per replicate to have independent reliable results. The feeding was one time daily, and the excess food and feces were removed one hour after feeding to ensure optimal medium quality. The fish medium condition was maintained at 28 °C and pH 7.0 and cleaning of the tanks and changing of the fish medium was every 24 hrs. The mean average weight of fish in each group was taken prior and after the experiment [19]. At the end of the experiments, fish in each group were euthanized in ice water for 20 seconds, killed, and 3 fish from each group were used for the histopathological examination of intestine and liver. The remained 3 fish from each group were used for the assay of malondialdehyde (MDA) and myeloperoxidase (MPO) activities at 7 and 14 days of treatment.

Histopathology

In this experiment, each fish was cut transversely on the abdominal site with a sharp blade and fixed in Davison's solution for 24 hrs. The fixed samples were then transferred into 10% neutral buffered formalin, dehydrated in a series of ethanol, and subsequently embedded in paraffin wax. Approximately, 5 µm thick sections were cut from each zebrafish, mounted on glass slides, and allowed to dry for 24 hrs. All the mounted slides were. Then, at once, both intestine and liver on the sections were observed under an optical microscope (ZEISS Axio Scope A1) at 40 × magnifications.

Enzymatic malondialdehyde (MDA) and myeloperoxidase (MPO) of zebrafish extracts

Crude enzyme extract procedure for the determination of MDA and MPO activities were prepared according to the method of Nayyar and Gupt [20] with some modification. Zebrafish lysates of control and treatment groups were separately prepared in 10 ml ice-cold lysis buffer containing 1 M of NaCl, 1 M of Triton X-100, and 1 M of EDTA at pH 7.4, homogenized and transferred into pre-cold centrifuge tubes (15 ml) and centrifuged at 10,000 x g for 30 min at 4 $^{\circ}$C in a Microcentrifuge to separate the supernatant from the cell debris. Supernatants were separately transferred into fresh tubes and stored at -80 $^{\circ}$C for the analysis of MDA and MPO activities.

Determination of malondialdehyde (MDA)

The index of oxidative damage by examining the quantity of LPO was determined via the measurement of thiobarbituric acid reactive substances in the experimental sample following a method described by Menozzi et al.[21] with some modifications. About 1 mL of each sample crude protein extracted was added to a mixture of 0.1 mL of KCl (0.15 M) and 0.5 mL of 1 M chloroacetic acid, and then allowed to stand for 15 min for protein to be precipitated. The mixture was then centrifuged at 500 $\times g$ for 15 min. To 1.5 mL of supernatant, 1 mL of 0.6% thiobarbituric acid (125 mg of thiobarbituric acid in 3 mL of 1 M NaOH and 30 mL of distilled H_2O), 3 mL of 1% phosphoric acid, and 10 mL of 2% solution of 2,6-ditert-butyl-4-methylphenol in 95% ethanol was added. The mixtures were then heated at 60 $^{\circ}$C for 30 min, then ice-cooled, and centrifuged at 1,200 $\times g$ for 10 min. The absorbance was then measured at 530 nm in 96-well Microplates by a Microplate reader (SpectraMax Plus, Molecular Device Inc. Sunnyvale, CA, USA). The experiments were performed in three replicates and the average absorbance of the replicates was calculated. Results were obtained from the standard graph and expressed as µmoles of malondialdehyde (MDA)/mg sample.

Malondialdehyde (MDA) Standard curve

Standard solutions of MDA were prepared by appropriately measuring 25 µL of 1, 1, 3, 3-Tetraethoxypropane (TEP) (Sigma Co., USA) in 100 mL of distilled H_2O to have 1 mM of stock solution. Working concentrations were prepared by hydrolyzing 1 mL of TEP stock solution in 40 µM of 1% H_2SO_4 (1 mL H_2SO_4 in 99 mL H_2O) to have 40, 20, 10, and 5 µM/mL through serial dilutions. The MDA standard curve was prepared by mixing 100 µL of each working standard concentration with 100 µM 2, 4-Dinitrophynyhydrazine (DNPH) (Sigma Co., USA) solutions and incubated for 15 min at 37 $^{\circ}$C. Absorbance was read at 530 nm using a microplate reader (SpectraMax Plus, Molecular Device Inc. Sunnyvale, CA, USA). The average value for each triplicate of wells was calculated, and the average absorbance of the control was subtracted from each average value. A graph of the standard curve was plotted as absorbance (y-axis) against their concentration (x-axis).

Determination of myeloperoxidase (MPO)

The myeloperoxidase (MPO) activity indicated a quantitative index of epithelial injury and was determined using the method of Menozzi et al. [21] with some modification. About 1 mL of reaction mixture, which contained 500 µL of crude enzyme extract (fish lysate), 500 µL hexadecyltrimethylammonium bromide (HTAB) buffer (50 µM of 0.5% phosphate buffer, pH 6.0) was prepared, and then centrifuged at 15,000 × g for 15 min at 4 $^{\circ}$C. Then, 1 mL of supernatant was added to 200 µL of o-dianisidine dihydrochloride (0.167 mg/mL) and 0.0005% of hydrogen peroxide in 50 mM of phosphate buffer at pH 6.0. The oxidation of the O-dianisidine dihydrochloride was monitored at 450 nm in 96-well Microplates by a Microplate reader (SpectraMax Plus, Molecular Device Inc. Sunnyvale, CA, USA), and the difference in absorbance was calculated. MPO activity was expressed as U/min/mg and 1 unit of MPO activity is the amount of enzyme that consumes 1 µmol H_2O_2/min). Data were presented as units of MPO/min/mg of sample. The experiments were performed on ice water and in three replicates.

Statistical analysis

The statistical significance of differences between the experimental groups and the control group was analyzed using a one-way analysis of variance (ANOVA) by Dunnett's Multiple Comparison Test (Graph Pad prism 5). Differences between the means at the 5 % ($p<0.05$) level were considered significant. All values were reported as the mean of three replicates ± SD.

III. RESULTS

Acute toxicity effects of curcumin analogue in adult male zebrafish

Acute toxicity of increasing concentrations (6 – 30 µM) of BDMC33, EF-24, and aspirin, separately, were tested in adult male zebrafish (>6 months old) for 48 hrs and the results of cumulative mortality (%) are presented (Figure 3). Mortality of zebrafish (>6 months old) was not observed in the control groups throughout

the period of incubation (Figure 3). The mean cumulative mortality (n=3) of adult male zebrafish subjected to BDMC33, EF-24, and aspirin was 0%, 5%, and 31% at 6 µM, respectively, to 100% at 30 µM (BDMC33), 25 µM (EF-24), and 15 µM (aspirin) after 48 hrs of treatments. The mean mortality (n=3) of zebrafish observed in BDMC33 (6 - 20 µM), EF-24 (6 - 10 µM), and aspirin (6 - 8 µM) for 48 hrs were <50%. But at the concentrations >20 µM for BDMC33, >10 µM for EF-24, and >8 µM for aspirin, the mean (n=3) mortality of adult male zebrafish observed were >50% at 48 hrs of treatment. Thus, these results have demonstrated that the 48 hrs LC_{50} values for BDMC33, EF-24, and aspirin on adult male zebrafish were 20 µM, 10 µM, and 8 µM, respectively (Figure 3).

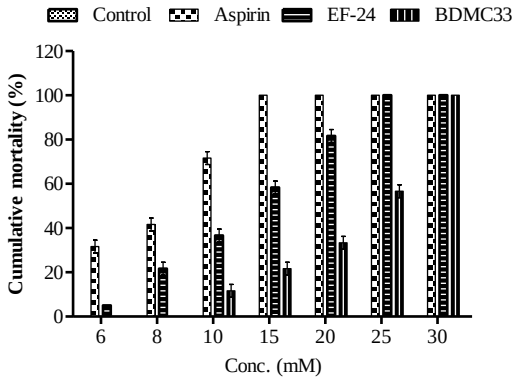

Figure 3: Effects of increasing concentrations of curcumin analogues on the cumulative mortality of adult zebrafish (>6 months old). Values are presented as % mean ± SD (n = 3). LC_{50} values for BDMC33 was 20 µM while that of EF-24 was 10 µM, and for the aspirin was 8 µM.

The average weight of zebrafish

The average weight (n=3) of adult male zebrafish exposed to 20 µM of BDMC33 and 10 µM of EF-24, and 8 µM of aspirin for 14 days were determined and the results are shown in Figure 4. It was revealed that the mean weight (n=3) of zebrafish (>6 months old) exposed to aspirin (8 µM) reduced significantly ($p<0.001$) with 1.72 ± 0.01mg compared to the untreated group (2.52 ± 0.02 mg).

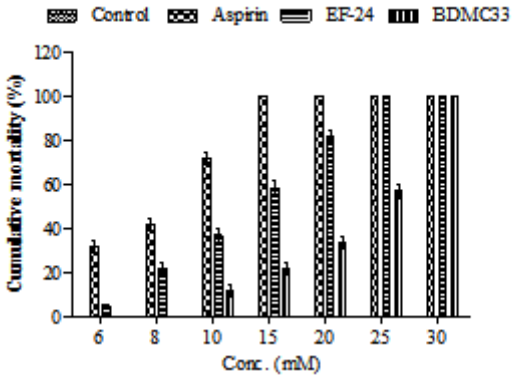

Figure 4: Average weight of adult male zebrafish (>6 months old) after 14 days of exposure. Values are presented as % mean ± SD (n = 3). ***p<0.001 was statistically different against the control group.

However, those exposed to BDMC33 (20 µM) and EF-24 (10 µM), had their mean weight (n=3) ranged from 2.47 – 2.49 mg, which were not altered significantly ($p<0.05$), compared to untreated groups (control) with 2.52 mg at 14 days (Figure 4).

Histomorphology of intestine and liver

Histopathological examinations of intestine and liver in healthy adult male zebrafish exposed to BDMC33 (20 µM), EF-24 (10 µM), and aspirin (8 µM), separately, are shown in Figure 5 and 6, respectively. In untreated groups (control), normal mucosal architecture with clear villi covered by absorptive enterocytes, goblet cells, crypts and mucosal epithelial cells were observed (Figure 5A).

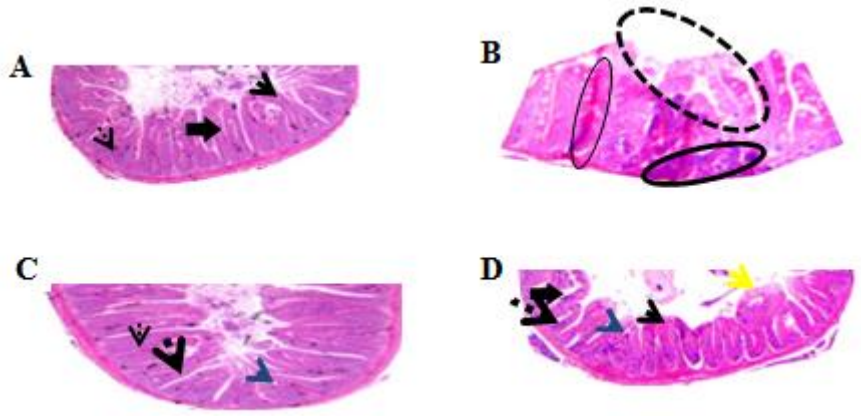

Figure 5: Histopathological assessment of intestine in healthy adult male zebrafish after 14 days of exposure (100×). **(A)** Control group, **(B)** Aspirin (8 µM)-treated group, **(C)** BDMC33 (20 µM)-treated group, **(D)** EF-24 (10 µM)-treated group. **Thick black arrow:** Intestinal crypts, **Small black arrow:** Villi with absorptive cells, **Thick dots arrow:** Sub epithelial space, **Yellow arrow:** Distorted villi, **Thin dots arrow:** Epithelial mucosal/mucosal architecture, **Blue arrow:** Intestinal goblets. **Dots outline:** Distorted villi, **Thin outline:** Red blood cells caused by bleeding, **Thick outline:** Nuclear debris.

However, in the intestines of healthy adult male zebrafish (>6 months old) exposed to aspirin (8.0 µM), red blood cells caused by bleeding as a result of mucosal injury, distorted villi and mucosal epithelial cells were observed at 14 days of treatment (Figure 5B). Interestingly, the intestine of healthy adult male zebrafish exposed to BDMC33 (20 µM) displayed normal mucosal architecture (Figure 5C), whereas slight distortion of villi andsubepithelial space (reversible lesions) were observed in the intestines of healthy zebrafish exposed to 10 µM of EF-24 (Figure 5D) at 14 days of treatments.

Similarly, normal hepatocytes and sinusoid of the liver in healthy adult male zebrafish (>6 months old) without treatment (control) were observed (Fig 6A), whereas cytoplasmic vacuoles with dense vascular congestions, nuclear debris, dilated hepatocyte and sinusoid were observed in aspirin (8 µM) treatment group at 14 days of exposure (Figure 6B). Interestingly, no adverse effects were detected on the hepatocytes and sinusoid of the liver in adult male zebrafish treated with BDMC33 (20 µM) and EF-24 (10 µM) (Figure 6C and D).

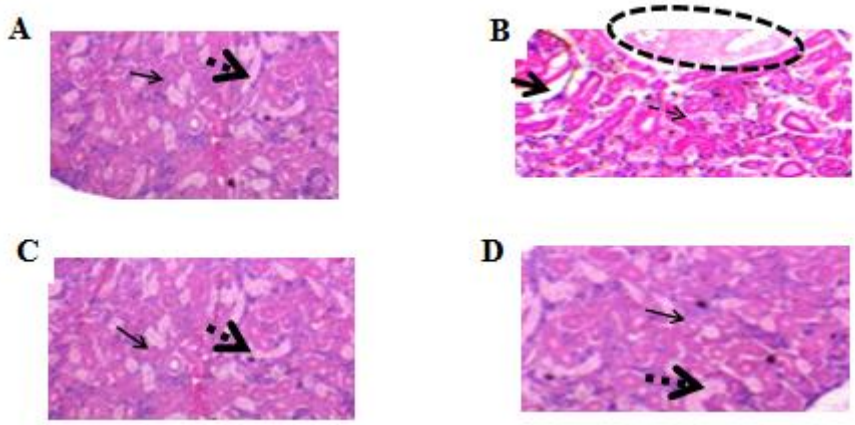

Figure 6:Histopathological assessment of liver in healthy adult male zebrafish after 14 days of exposure (100×). **(A)** Control group, **(B)** Aspirin (10 µM)-treated group, **(C)** BDMC33 (20 µM)-treated group, **(D)** EF-24 (10 µM)-treated group. **Thick dots arrow:** Sinusoid, **Thin arrow:** Hepatocyte, **Bold arrow:** Dilated sinusoid, **Thin dots arrow:** Dilated hepatocyte, **Dots outline:**Cytoplasmatic vacuole with dense vascular congestions in the liver hepatocyte and nuclear debris.

Effect on malondialdehyde (MDA) levels in adult male zebrafish

The results of the mean (n=3) of MDA levels in adult zebrafish treated with BDMC33 (20 µM), EF-24 (10 µM), and aspirin (8 µM), separately, at 14 days of treatment are presented (Figure 7). It has revealed that the mean (n=3) of MDA levels in adult zebrafish exposed to aspirin (8 µM) were 0.046 ± 0.02 µmol/mg, which is similar compared to control (0.043 ± 0.01) at 7 days of exposure. But at 14 days of treatments, the mean (n=3) of MDA levels were increased to 0.048 ± 0.12 µmol/mg, which differ significantly ($p<0.01$), compared to

control (Figure 7). Whereas, in EF-24 (10 µM) treatment group, the levels of lipid peroxidation in experimental zebrafish were maintained at 7and 14days of exposure, as demonstrated by no significant difference of MDA levels with respect to control (Figure 7). However, in BDMC33 (20 µM)-treated zebrafish the reduced levels of lipid peroxidation (0.034 ± 0.02 and 0.023 ±0.01µmol/mg), as indicated by the significant decrease of MDA levels at 7 ($p<0.01$) and 14 ($p<0.001$) days of experiments were observed, respectively, compared to the control (0.043 ± 0.001 µmol/mg) (Figure 7).

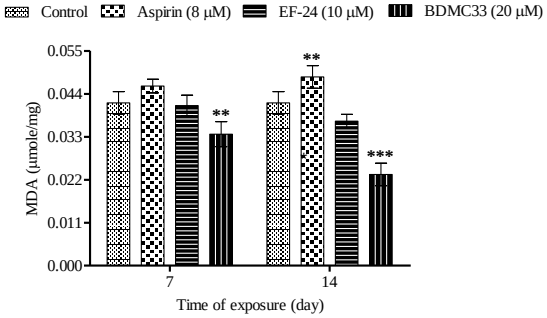

Figure 7: Malondialdehyde (MDA) levels in adult male zebrafish (>6 months old). Values are presented as means ± standard deviation of three independent experiments. **p<0.01, ***p<0.001 were statistically different against the control group.

Effect on myeloperoxidase (MPO) in adult zebrafish

The mean (n=3) results of MPO levels in adult zebrafish (>6 months old) treated with BDMC33 (20 µM), EF-24 (10 µM), and aspirin (8 µM), separately, at 7 and 14 days of treatment are presented (Figure 8). The results have revealed that the MPO activities in adult zebrafish exposed to aspirin (8 µM) were not altered significantly ($p<0.05$) both at 7 and 14 days of treatment, with 0.029 ± 0.11 µmol/min/mg compared with control group (0.027 ± 0.01 µmol/min/mg). Similarly, those exposed to 10 µM of EF-24 had the average (n=3) of MPO activities, not differ significantly ($p<0.05$), compared to the untreated group both at 7 and 14 days of treatments (Figure 8). On the contrary, zebrafish exposed to BDMC33 (20 µM) showed their MPO activities reduced significantly ($p<0.01$) with 0.019 ± 0.01 µmol/min/mg compared to control group (0.027 ± 0.01 µmol/min/mg) after 7 days of treatment.

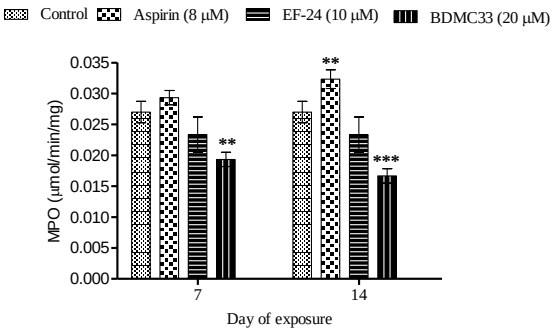

Figure 8: Myeloperoxidase (MPO) activities in adult zebrafish (>6 months old). Values are presented as means ± standard deviation of three independent experiments. **p<0.01, ***p<0.001 were statistically different against the control group.

Also, at 14 days of exposure, the MPO activities in zebrafish exposed to BDMC33 (20 µM) were further reduced, significantly ($p<0.001$), with 0.016 ± 0.01 µmol/min/mg compared to control (0.027 ± 0.01 µmol/min/mg) (Figure 8).

IV. DISCUSSIONS

The safety of the chemical compound and plant products for human consumption has to be determined using toxicological evaluation in experimental animals to ascertain the optimal dose in humans [22]. Acute toxicity experiments with increasing concentrations have to be performed to identify accurate concentration for sub-chronic toxicity experiments.Therefore, the acute and subchronic assessment of various concentrations in experimental animals could be very important in evaluating the toxicity of chemical compounds [22].

Thus, the acute toxicity results have demonstrated that the 48 hrs LC_{50} values for BDMC33 on adult male zebrafish (>6 months old) was 20 µM, whereas those of aspirin and EF-24 were 8 and 10 µM, respectively (Figure 3). This finding has suggested that BDMC33 is relatively less toxic compared to EF-24 and aspirin. It was demonstrated in our laboratory that the 5 days LC_{50} value for BDMC33 and curcumin in zebrafish embryos

and larvae were 6.25 and 5.0 µM (data not shown). Therefore, 20 µM of BDMC33 could be the optimal concentration at which adult zebrafish can be incubated for 48 hrs without or with lower related toxic effects. In addition, the mean weight of zebrafish (>6 months old) exposed to aspirin (8 µM), EF-24 (10 µM), and BDMC33 (20 µM) was examined after 14 days of subchronic toxicity tests (Figure 4).

The current study has suggested that the main weight of zebrafish (>6 months old) exposed to 8 µM of aspirin had reduced significantly, compared to the untreated (control) group. This experienced reduction in the main weight of adult zebrafish may be associated with, possibly, the ability of aspirin at 8 µM to induce oxidative stress, which might prevent the fish from feeding during the period of the experiment. According to Yakubu et al. [23], the decreased intake and total weight loss in rats could result from the oxidative stress generated from excess intake of the toxic compound and the abnormal food consumption. In the previous study, Eriksson et al.[24] reported that toxicity effects are associated with excess intake of toxic compounds.

On the contrary, EF-24 (10 µM) and BDMC33 (20 µM) treatments did not affect the mean weight of zebrafish compared to untreated groups (control) at 14 days of treatments. This result may be signified that at these concentrations the feeding pattern of the fish during the period of the experiment was not altered, which could be as a result of no oxidative stress generated in the fish during the periods of experiments. This might be associated with high antioxidant activities exhibited by BDMC33 and EF-24, which serve as the extracellular free radical scavenger. Similarly, BDMC33 and EF-24 have been reported to possess high antioxidant properties against the markers of oxidative stress in activated macrophages [10, 17, 25, 26], and nude mice [25].

In addition, the histopathology of the intestine in adult zebrafish (>6 months old) exposed to aspirin (8 µM), EF-24 (10 µM), and BDMC33 (20 µM), separately, were investigated after 14 days of treatments (Figure 5). It was demonstrated in this study that the intestines of healthy adult zebrafish (>6 months old) exposed to aspirin (8 µM) for 14 days of experiment displayed epithelial mucosal damages, which might reflect the damages resulted from aspirin-induced oxidative stress. The production of reactive oxygen species (ROS), lipid peroxidase and infiltration of the neutrophil are the symptoms of ulcers induced by aspirin [27]. Aspirin overdose in the animal model produced reactive oxygen metabolites that linked with the mucosal injury, due to the mucosal prostaglandin E_2 (PGE_2) and PGI_2 inhibition [27]. Inhibition of PGE_2 biosynthesis that causes vasoconstriction and low mucin content could be a mechanism behind the aspirin-induced gastric mucosal damage [28]. The mechanism of aspirin-induced mucosal epithelial damage could also be explained by hydrogen ions back diffusion, which causes acidosis, decreased pH in the tissue, and mitochondrial oxidative phosphorylation to generate free radicals, inflammation production and neutrophil infiltration [28]. Intact (normal) mucosal architecture (intestinal villi, goblet cells, crypt) displayed by zebrafish exposed to BDMC33 (20 µM) as compared to that of normal zebrafish could reflect it higher biological activities. It could be also explained that BDMC33 (20 µM) could be the optimal concentrations that can be tolerated by adult zebrafish for 14 days of treatments, as indicated earlier in this study. Another possible explanation for this may be probable that the excess accumulations of this compound in the body of the fish were not up to a level of causing damage to the stomach and liver tissues during the period of exposure. All these might reflect the higher biological benefits of BDMC33. Anti-inflammatory activities of BDMC33 on iNOS blockage, PGE_2 and COX-1 production and COX-2 depression *in vitro* have been reported [10, 17, 18].Conversely, the reversible mucosal lesions (subepithelial space) observed in the intestine of zebrafish exposed to EF-24 (10 µM) may be associated with the lowest toxicity effects generated in zebrafish, which could reflect its highest biological benefits. High biological potentials of EF-24 *in vitro* studies have been reported [25]. Previous studies have reported the anti-inflammatory activities of EF-24 in animal models [14].Similarly, this study has demonstrated that cytoplasmic vacuoles with dense vascular congestions, nuclear debris, dilated hepatocyte and sinusoid were observed in the liver of zebrafish exposed to 8 µM of aspirin at 14 days of the experiment.These abnormal hepatocytes produced could reflect the aspirin-induced oxidative stress due to reactive oxygen species generated during the period of the experiment. According to Raza et al. [29], doses of aspirin (5 and 10 µmol/ml) for 24 and 48 hrs, respectively, increased the oxidative stress in human hepatocyte HepG2 cells. In HepG2 cells treated with aspirin (5 and 10 µmol/ml), increased in ROS, lipid peroxidation, and reduced GSH pool, which are indications of oxidative stress production were observed[29].Interestingly, no adverse effects detected on the hepatocytes and sinusoid of the liver in adult male zebrafish treated with BDMC33 (20 µM) and EF-24 (10 µM) may be associated with their potent antioxidant and anti-inflammatory activities. Anti-inflammatory activities of BDMC33 and EF-24 on iNOS blockage, PGE_2 and COX-1 production and COX-2 depression *in vitro* [10, 17, 18] and in nude mice [14] have been reported.Lee et al.[18] has suggested that BDMC33 prevents the production of nitric oxide (NO), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) which are the major pro-inflammatory mediators in activated macrophages and microglial cells. Subsequently, a similar level of MDA and MPO activity observed at 7 days of exposure may be reflected that aspirin (8 µM) did not induce ROS and biomolecule such as lipid peroxidation that would lead to tissue damages at this period of exposure. This finding is supported by the report of Goldsmith et al. [27] that short-term use of aspirin at the recommended dose suppressed reactive oxygen species (ROS) and lipid peroxidation in the epithelial lining

membrane. Likewise, increased levels of MDA and MPO observed at 14 days of exposure to 8 µM may be due to the long-term frequent use of aspirin that could generate some advert effects. According to Goldsmith et al. [27], long-term frequent use of aspirin causes ROS production, lipid peroxidation and infiltration of the neutrophil in mucosal walls. Furthermore, no alteration of epithelial damage and lipid peroxidation as demonstrated in this study may associate with the potent biological activity of EF-24. EF-24 directly inactivates gastric lesions by inhibiting the production of ROS that may likely generate lipid peroxidation and thiol reduction [15].Decreased MDA contents and MPO activities in zebrafish subjected to BDMC33 (20 µM) as demonstrated in this study may be associated with potential biological benefits of BDMC33. Recently, several studies have suggested the potent antioxidant and anti-inflammatory activities exhibited by BDMC33 [10, 17, 18]. According to Lee et al., [10] and Lee et al. [17], BDMC33 exhibited potent biological activity against the markers of oxidative stress and promising nitric oxide inhibitory activities in activated macrophages.

V. CONCLUSION

This study has demonstrated that 20 µM and 10 µM of BDMC33 and EF-24, respectively, are the optimal concentrations in which adult male zebrafish (>6 months old) could be incubated for 48 hrs without or less toxicity. Also, the histomorphology of the intestine and liver for BDMC33 (20 µM) and EF-24 (10 µM) treatment groups showed similar intestinal and hepatic architectures compared to normal zebrafish. In addition, the MDA levels and MPO activities in BDMC33 (20 µM) treated zebrafish were dropped significantly, but in EF-24 treated fish, the levels of both MDA and MPO were not affected compared to that of normal fish at both 7 and 14 days of treatment. The information gathered above demonstrates that BDMC33 is better than EF-24. Therefore, it is with the hope that BDMC33 could be a promising anti-inflammatory agent and perhaps will perform some roles in the therapy and prevention of cancer related diseases.

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