The role of zebrafish larvae for studying anxiety-like behaviour

Muniandy, Y.

Chapter 5

Indication of developmental toxicity in zebrafish embryos and larvae after treatment with synthetic and herbal-based psychotropic drugs

Y. Muniandy[1,2], H. M. I. Kerkkamp[1], E. Nühn[1], N. van Duijvenvoorde[1], M. K. Richardson[1*]

[1]Animal Sciences & Health, Institute of Biology Leiden, Sylvius Laboratory, Sylviusweg 72, 2333 BE, Leiden, the Netherlands.

[2]Plant Sciences & Natural Products, Institute of Biology Leiden, Faculty of Mathematics and Natural Sciences, Sylviusweg 72, 2333 BE, Leiden, the Netherlands.

*Author for correspondence. m.richardson@biology.leidenuniv.nl

Manuscript in preparation

Abstract

Several studies have suggested that synthetic and herbal psychotropic drugs are commonly used during pregnancy. It is therefore important to have a good understanding of the potential adverse effects of these drugs on development. One way to assess developmental toxicity is by using animal models including zebrafish embryos and larvae. Here, we have assessed the developmental toxicity of eight psychotropic drugs by recording lethality (LC_{50}), and the incidence of 16 morphological abnormalities, in developing zebrafish. Exposure was done at 1-day post fertilisation (dpf) and the readout was at 2 and 5 dpf. We tested four synthetic drugs (amitriptyline, buspirone, diazepam, and fluoxetine) and four herbal extracts popularly used as psychotropic drugs (*Hypericum perforatum*, *Passiflora incarnata*, *Valeriana officinalis*, and *Withania somnifera*). All drugs and extracts tested showed concentration-dependent lethality. However, the synthetic drugs showed higher lethality (lower LC_{50}) and were associated with a higher incidence of abnormalities compared to the herbal extracts. Among the synthetic drugs, amitriptyline had the lowest LC_{50} and produced numerous abnormalities. *Hypericum perforatum* was associated with a much higher lethality than the other three extracts. Although *Valeriana officinalis* had a relatively low lethality it produced a pattern of multiple abnormalities comparable with the synthetic drugs. Circulatory-related defects were the commonest category of abnormality observed in larvae when embryos treated with amitriptyline, buspirone, and diazepam. We conclude that assays using zebrafish embryos and larvae have good predictivity for the developmental toxicity of synthetic and herbal psychotropic drugs. Given the popularity of the plant-based drugs and their easy availability without prescription, it might be useful to further characterise their pharmacology.

Introduction

Anxiety disorders are characterized by severe and sustained feelings of fear[1], often accompanied by adverse physiological symptoms including fatigue, dizziness, chest pain, and sleeping problems [2]. Anxiety disorders cause significant disability across the life span in different areas of life such as health, income, education, and interpersonal relationships [3].

Several studies have shown an increased incidence of anxiety-related disorders among pregnant women. Moreover, a significant association between antenatal anxiety and postnatal depression have also been reported [4-6]. The prevalence of anxiety and anxiety-related disorders during pregnancy in developed and developing countries are 10% and 25% respectively [7-9]. Some common synthetic drugs used during pregnancy include anxiolytics such as benzodiazepines and antidepressants such as tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), serotonin-norepinephrine reuptake inhibitors (SNRIs) and selective serotonin reuptake inhibitors (SSRIs).

However, drug management during pregnancy poses diverse risks for women afflicted with anxiety and related disorders [10]. The risks include immediate reactions such as spontaneous abortion or premature labour [10]. Moreover, synthetic anxiolytics and antidepressants have been in the past reported to cause major adverse effects such as congenital abnormalities, withdrawal symptoms to the foetus, and birth defects (morphological teratogenicity) [10].

Due to the adverse effects of some synthetic drugs, some pregnant women, especially in developing countries, use plant-based medicines (herbalism) to treat anxiety [11, 12]. Many herbal medicines in the form of tinctures, herbal teas, and essential oils are available as over the counter medicines for various mood disorders. Although plant medicines have the reputation of being safer than synthetic drugs, their potential toxicity and teratogenicity have not been investigated thoroughly, if at all. It might, therefore, be valuable to be able to screen plant drugs for developmental toxicity (including teratogenicity).

The Federal Drug Administration (FDA) in the USA and the European Medicines Agency (EMA) require developmental toxicity of drugs to be screened thoroughly as part of the drug discovery process [13]. Often, pregnant animals (of two different species: usually rodents and rabbits) will be exposed to the candidate drugs during the ‘critical period’ of development when many organ systems are being specified (i.e. the period of organogenesis) [14]. The resultant offspring are monitored for different parameters including mortality rate, morphological abnormalities, and changes in growth rate [15].

While mammalian models have the advantage of being closely related to humans, they have some disadvantages. Work on mammals can be time-consuming, labour-intensive, expensive and typically requires the sacrifice of the mother. The latter can lead to heightened ethical concerns. With the thalidomide disaster, it became apparent that multiple species are necessary for detecting risk to humans. This is because thalidomide was screened on rodents and guinea pigs and was found not to produce malformations in the offspring [15]. The thalidomide disaster is one of the factors that led to animal experimentations being criticized for their lack of consistency in predicting developmental toxicity in humans [16, 17]. Zebrafish are increasingly being used as an alternative or complementary model for drug screening [18, 19] and have been shown to be sensitive to thalidomide and treatment during early development impaired proper development of embryonic fins [20].

Zebrafish (*Danio rerio*) is a small, tropical fresh-water teleost fish whose eggs are fertilised and develop externally, and are optically transparent throughout early development [21, 22]. At 5 days post-fertilization (dpf), the zebrafish larva shows complex multiple behavioural repertoires [23-27] with distinct tissues and organs [28, 29]. These include the brain, heart, liver, intestines, muscle and the nervous system [28, 29]. These organ and tissue systems show many homologies at the physiological and molecular levels with mammals, including humans [28, 30]. Furthermore, 70% of all human genes have counterparts in the zebrafish genome [31]. Despite having a discrete organ and tissue systems, the larva is nonetheless by no means complete in its development. Thus, although larval development starts at around 3 dpf it does not finish until around 45 dpf [32].

The zebrafish is increasingly being used in different areas of toxicological analyses, such as environmental, predictive and reproductive (developmental). The main reasons behind its use in these include its small size, rapid development, and crucially, its consumption of much smaller quantities of test compound than models [33, 34]. Previous studies have exploited different life stages of the zebrafish of to screen toxic effects of various compounds. For example, adults were used to screen lead[35], malathion[36], and metronidazole[37]; juveniles for testing agricultural biocides[38]; embryos and larvae for screening different types of small molecules and nanoparticles [19, 26, 39].

Earlier studies done in our laboratory and elsewhere have shown that zebrafish larvae can be used to screen different types of chemical compounds for developmental toxicity. We have reported on the developmental toxicity and teratogenicity of different classes of water-soluble compounds in this model [40]. The compounds included alkaloids, alcohols, amides, carboxylic acids, and glycosides. Moreover, we demonstrated the presence of phenotypes that resemble foetal alcohol syndrome (craniofacial abnormalities, microphthalmia, and growth retardation) in zebrafish embryos after acute ethanol exposure [41]. In addition, Bugel and colleagues compared the developmental toxicity of various flavonoids using 5 dpf zebrafish larvae [42]. Zebrafish embryos and larvae were also used to assess developmental neurotoxicity of several compounds, including atrazine, dichlorodiphenyltrichloroethane (DDT), 2,4-dichlorophenoxyacetic acid (2,4-D), and dieldrin [43].

Despite the promising features of zebrafish embryos and larvae in developmental toxicity assays, there are important issues or limitations that need to be addressed before accepting the full potential of this model. For example, absorption, distribution, metabolism, and excretion (ADME) are important pharmacological factors that may affect the outcome of toxicity. Most zebrafish based developmental toxicity assays are based on waterborne exposure, whereby compounds uptake is by diffusion through the skin [44]. This may result in non-linear compound uptake; therefore internal concentration analysis is necessary to correlate toxic phenotypes with the actual concentration of the compounds within the larvae [15].

Our aim here is to compare the developmental toxicity of four types of a synthetic drug and plant extracts commonly used in the treatment of anxiety-related disorders. We assessed LC_{50}, mortality rate, and different phenotypic abnormalities after exposing the larvae to the drugs/ extracts. Abnormalities were scored on the basis of an assessment of various qualitative characters (**Table 1**). We chose qualitative characters because one of our objectives is to have a rapid method for assessing the toxicity of psychotropic drugs. The synthetic drugs tested were amitriptyline (SNRI), buspirone (serotonin receptor agonist), diazepam (a GABA agonist), and fluoxetine (SSRI). The four synthetic drugs used in the current study are commonly used as anxiolytics and/or antidepressants. Furthermore, during pregnancy, these types of drugs are commonly prescribed since they are a mainstay in the

management of panic disorders, anxiety disorders and depression [45-49]. The four plant species were *Hypericum perforatum*, *Passiflora incarnata*, *Valeriana officinalis*, and *Withania somnifera*. According to the World Health Organization (WHO) *Monographs on Selected Medicinal Plants* and the references therein, these plants and their products (extracts, decoctions, tinctures etc.) have long been used in traditional medicine to treat various mood disorders and psychological disturbances, including anxiety, anxiety-induced sleep disturbances, depression, nervous excitation, and stress [50-52].

Materials and methods

Ethics statement

Animal experimental procedures conducted in this study were all carried out in accordance with the Dutch Animals Act (http://wetten.overheid.nl/BWBR0003081/2014-12-18), the European guidelines for animal experiments (Directive 2010/63/EU; https://eur-lex.europa.eu/legal-content/NL/TXT/HTML/?uri=CELEX:32010L0063&qid=1531309204564&from=N) and institutional regulations.

Zebrafish husbandry

Male and female adult zebrafish (*Danio rerio*) of ABTL wild type strains were maintained in our facility according to standard protocols (zfin.org). Zebrafish eggs were obtained by random pairwise mating. Approximately 10 adult zebrafish (equal male to female ratio) were placed together in small breeding tanks the evening before eggs were required. The breeding tanks have mesh traps to prevent the eggs from being eaten by the adult fish. The eggs were harvested the following morning and transferred into 92 mm plastic Petri dishes (approximately 80 eggs per dish) containing 40 mL fresh embryo medium (EM). The procedure for the preparation of EM is based on a previously published protocol [40]. Unfertilized, unhealthy and dead embryos were identified and discarded using a plastic Pasteur pipette immediately after plating into Petri dishes.

At 1 dpf, the embryos were again screened and any dead or unhealthy embryos were removed. Live, healthy embryos were later dechorionated and transferred to 96

well plates together with 50 µl of EM, one embryo per well. Dechorionation was performed under a light microscope with a pair of watchmaker's forceps. We chose to dechorionate the embryos prior to the exposure to the drugs and herbal extracts since the chorion can act as a protective barrier [53-55]. Only several studies have used dechorionated embryos prior to exposure to the test compounds [42, 56], while most studies used non-dechorionated embryos in their toxicity studies [33, 41, 43, 57]. In studies that used non-dechorionated embryos, there could be an issue related to the exact dose of compounds that is uptaken by the embryos.

The outer wells of the 96 well plates were not used since a previous study in our laboratory showed high levels of evaporation in these wells [33]. Throughout all procedures, the embryos and the solutions were kept in an acclimatized room at 28 ± 0.5 °C, under a light-dark cycle of 14 hours light and 10 hours dark (lights on at 08:00).

Exposure to synthetic drugs and plant extracts

At 1 dpf, after dechorionation, zebrafish larvae were exposed to a set of test solutions comprising either synthetic drugs or plant extracts. The synthetic drugs were amitriptyline (Sigma-Aldrich, catalogue number PHR1384), buspirone (Sigma-Aldrich, catalogue number B7148), diazepam (Duchefa Farma, catalogue number 5372) and fluoxetine (Sigma-Aldrich, catalogue number F132). The plant extracts used in this study were *Hypericum perforatum*, *Passiflora incarnata*, *Valeriana officinalis* and, *Withania somnifera*.

Exposure of embryos to compounds or extracts

We used a series of concentrations for both synthetic drugs and plant extracts, whereby each concentration was double the next lowest value (i.e. a geometric range). The concentrations used are shown in Supplementary **Table S1**. In total we used four independent 96 well plates for each test compound or extract, with 24 embryos for each treatment group and 24 embryos for controls.

Amitriptyline, buspirone, and fluoxetine were dissolved directly in embryo medium. Diazepam and all plant extracts were dissolved in dimethylsulfoxide (DMSO). The highest concentration of diazepam (142.4 mg/L) was dissolved in DMSO at 1.0%. The highest concentration for *H. perforatum* (500 mg/L) was

dissolved in 0.16% DMSO, while 2% DMSO was used to dissolve the highest concentration used for the rest of extracts. All highest concentrations of DMSO described above also served as controls for the corresponding drug or extract. Since the DMSO concentrations used were different for diazepam and the plant extracts, we analysed the DMSO independently on zebrafish embryos and larvae. Exposure to the test solutions initiated at 1 dpf and the duration of exposure was 96 hours.

Morphological assessment of larvae

The morphological assessment was done at 5 dpf using a dissecting stereomicroscope. We scored for mortality rate and also different types of abnormalities. The abnormalities were either physiological (such as poor peripheral blood circulation), or morphological (including various kinds of abnormalities). These abnormalities and their criteria are given in **Table 1**.

Data analysis and interpretation

The abnormalities are presented as frequencies of occurrences in bar charts. The charts also show the mortality rate as a secondary line plot. LC_{50} was determined based on mortality scoring of four independent experiments from geometric series using Regression Probit analysis. This was achieved by using the dose-response curve (drc) package in RStudio© (version 1.1.456). N was 24 for controls, synthetic drugs, and herbal extracts treated embryos and larvae.

Results

We have examined the toxicity profiles of eight different psychotropic drugs commonly used in treating anxiety disorders (four synthetic and four herbal-based), in zebrafish larvae, after 48 and 96 h exposures. A normal 5-dpf larva is shown in **Figure 1** and larvae with selected abnormalities (BA, TF, SE, FD PO, YO, AP) after exposure to drugs are shown in **Figure 2.** The full classification and criteria for the various abnormalities are given in **Table 1**. We categorized all 16 abnormalities observed in the current study arbitrarily into four different groups of abnormalities: (a) circulatory-related defects (CD), developmental defects (DD), head defects (HD), and tissue defects (TD).

The description of our findings consider the incidence of abnormalities at the population level; it is beyond the scope of this study to look at the clustering of two or more abnormalities per larva since we are simply interested in comparing synthetic and herbal anxiolytics. A previous study done in our laboratory has reported clustering of abnormalities per larva [58].

Table 1. Qualitative criteria of abnormalities scored in 2 dpf embryos and 5 dpf larvae.

Category of abnormalities	Abnormalities	Qualitative criteria
Circulatory-related defects (CD)	Cardiomegaly (CM)	The heart appears abnormally bigger compared to the control groups
	Pericardial sac oedema (PO)	Pericardium is abnormally swollen with accumulation of pellucid fluid
	No circulation (NC)	No sustained blood flow in the peripheral circulation; only occasional heart contractions
	Impaired circulation (IC)	Minimal blood flow in the peripheral circulation, circulation in the tail absent
	Yolk sac oedema (YO)	Yolk sac is abnormally swollen due to the accumulation of pellucid fluid
Developmental defects (DD)	Bent body axis (BA)	Primary axis is abnormally flexed either dorsoventrally or laterally
	Growth retardation (GR)	Larvae appear smaller and less developed compared to the controls*
	Incomplete pigmentation (IP)	Larvae have dermal hypo-pigmentation compared to the control groups*
	Absence of pigments (AP)	Larvae have no dermal melanin
	Posterior truncation (PT)	The posterior part of larva (from cloaca to apex of caudal fin) appears truncated*
	Enlarged swim bladders (SB)	Swim bladder is abnormally distended compared to the control groups
Head defects (HD)	Facial defect (FD)	Larval jaws are malformed compared to the control groups
	Small eyes (SE)	The eyes appear abnormally small compared to the control groups
Tissue defects (TD)	Tail fin defect (TF)	The tail fin is either absent or truncated at the tip
	Necrosis head (NH)	Tissue necrosis of the head of the larvae**
	Necrosis body (NB)	Tissue necrosis of body of the larvae**

Key:
*These abnormalities were not quantified and only recorded qualitatively
**In these abnormalities, tissue appeared opaque and amorphous.

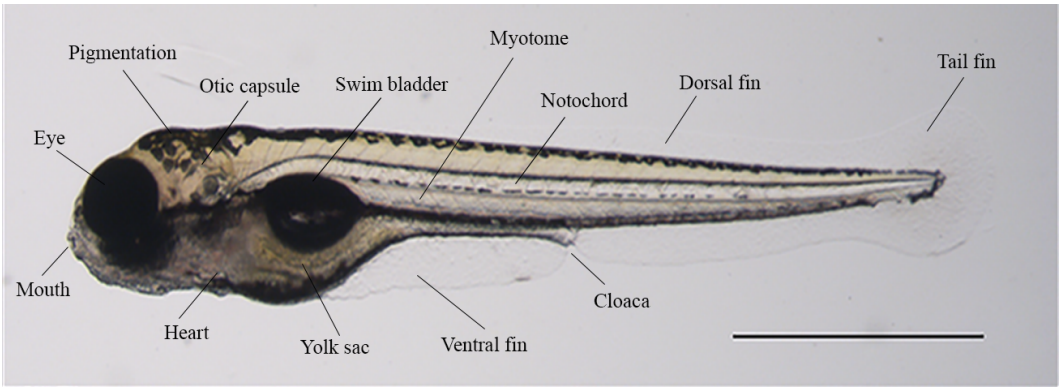


Figure 1. Photomicrograph of a normal (untreated) zebrafish larva at 5 dpf. Left lateral aspect. Rostral is to the left. Scale bar = 1 mm.

Figure 2. Selected phenotypic abnormalities observed in zebrafish larvae at 5 dpf. This figure shows some of the abnormalities scored after exposure to synthetic drugs or plant extracts. All images depict left lateral views. Rostral is to the left. Each larva is shown to the same scale, bar in A = 1 mm). A: Larva shows bent body axis (BA), small eyes (SE), facial defect (FD), and pericardial oedema (PO). B: Larva shows caudal fin abnormality (TF). C: This showing absence of pigment abnormality (AP) and BA. D: larva with yolk sac oedema (YO) and BA. Larvae in A, B and C come from a batch treated with amitriptyline (7.813 mg/L). Larva in D comes from batch treated with diazepam treated with 17.8 mg/L. The abnormalities shown here are representative of some of the individual abnormalities in Table 1.

All synthetic and herbal-based psychotropic drugs tested here show concentration-dependent mortality (**Figure 3 and Figure 4**). Interestingly, we noticed a higher incidence of abnormalities among larvae exposed to the four synthetic drugs than in those exposed to herbal extracts. For example, amitriptyline-treated larvae, in general, showed 12 different abnormalities in surviving individuals at both 2 dpf and 5 dpf stages (**Figure 3**). The 12 different abnormalities included pericardial oedema (PO), facial defect (FD), small eyes (SE), bent body axis (BA), yolk sac extension (YO),

enlarged swim bladder (SB), necrosis of body (NB), necrosis of head (NH), impaired circulation (IC), tail fin defect (TF), growth retardation (GR), and absence of pigments (AP). Moreover, most of the 12 abnormalities occurred at a higher frequency. Specifically, among 24 larvae, treatment with 7.813 mg/L amitriptyline resulted in PO, FD, and YO occurring at frequencies of 16, 9, and 7, respectively at 5 dpf.

Similar to the synthetic drugs, a group of multiple abnormalities occurred among surviving embryos and larvae at 312.5 mg/L after *Valerian officinalis* exposure (**Figure 4E** and **F**). The other three herbal extracts (*H. perforatum*, *P. incarnata* and *W. somnifera*), by contrast, showed little or no evidence of multiple abnormalities either at the highest concentrations or among surviving individuals (**Figure 4A-D**, **G** and **H**). To take one example, *H. perforatum* exposure was associated with only two types of abnormalities (PO and AP) at 2dpf (15.625 and 31.25 mg/L); and four types of abnormalities (PO, BA, GR, and AP) at 5 dpf (concentration range 3.906 to 62.5 mg/L). Moreover, the number of embryos and larvae that showed these abnormalities occurred were very low (**Figure 4A and B**).

Since diazepam and all four herbal extracts were dissolved in dimethylsulfoxide (DMSO), we decided to assess the potential toxicity of this solvent. We found that similar to the synthetic anxiolytics, DMSO also produced different types of abnormalities at higher frequencies than controls after both 48 and 96 h of exposure. DMSO caused six different abnormalities among surviving embryos after 48 h of exposure and seven different abnormalities among surviving larvae after 96 h of exposure (**Supplementary Figure S1**). The number of larvae with these abnormalities was high at both stages. Thus, in one example, all 24 larvae exposed to 56.64 g/L DMSO showed the TF abnormality at 2 dpf whereas the controls showed no abnormalities. Furthermore, in the same experiment, 12 larvae also showed BA, IC, no circulation (NC), and incomplete pigmentations (IP) abnormalities.

The LC_{50} values for the synthetic and herbal drugs at 2 dpf and 5 dpf are shown in **Table 2**. For all synthetic drugs, the LC_{50} values were dependent on the duration of exposure, such that longer exposure (96 h) resulted in lower LC_{50} values than shorter exposure (48 h). To give an example, the LC_{50} value for diazepam is 100.65 ± 246.83 mg/L after 48 h exposure; while after 96 h exposure, the LC_{50} value was 37.09 ± 5.94

mg/L. In contrast to synthetic drugs, two herbal extracts had similar LC_{50} values at 2 and 5 dpf. The LC_{50} value for *H. perforatum* was 45.49 ± 5.21 mg/L and 44.19 ± 5.0 mg/L at 2dpf and 5 dpf respectively. *V. officinalis* produced similar LC_{50} values after both 48 h and 96 h, which is 416.07 ± 34.03 mg/L.

Comparison of LC_{50} values between synthetic and herbal-based drugs revealed that the synthetic drugs had low LC_{50} values compared to *P. incarnata*, *V. officinalis*, and *W. somnifera*, consistent with their being more toxic than the herbals. The exception to this generalisation was *Hypericum perforatum*, which had relatively high toxicity (i.e. a low LC_{50}) more comparable with that of the four synthetics (**Table 2**).

Figure 5 depicts the incidence (as a percentage) of clustering of morphological abnormalities, arranged in four categories, observed after exposure to amitriptyline, buspirone and diazepam. We chose to show the results for these three synthetic compounds since they were associated with most of the 16 abnormalities scored in the current study. In addition, concentrations of drugs chosen to represent the clustering are shown in the legend of **Figure 5**. The outer ring represents the abnormalities by category, while the inner ring represents the 16 individual abnormalities.

Circulatory defects (CD) are the main category of abnormality observed in 2 dpf larvae after treatment with buspirone (77.14%) and diazepam (87.10%) (For a full list of the abbreviations used for abnormalities, see **Table 1**). This category of abnormality has the highest incidence at 5 dpf after exposure to each of the following three synthetic drugs: amitriptyline (45.01%), buspirone (55.71%), and diazepam (60%). Tissue defects (TD) scored the highest percentage incidence at 2 dpf in larvae treated with amitriptyline, with a percentage incidence of 65.39%. This category of abnormality was reduced dramatically in incidence at 5 dpf (19.99%).

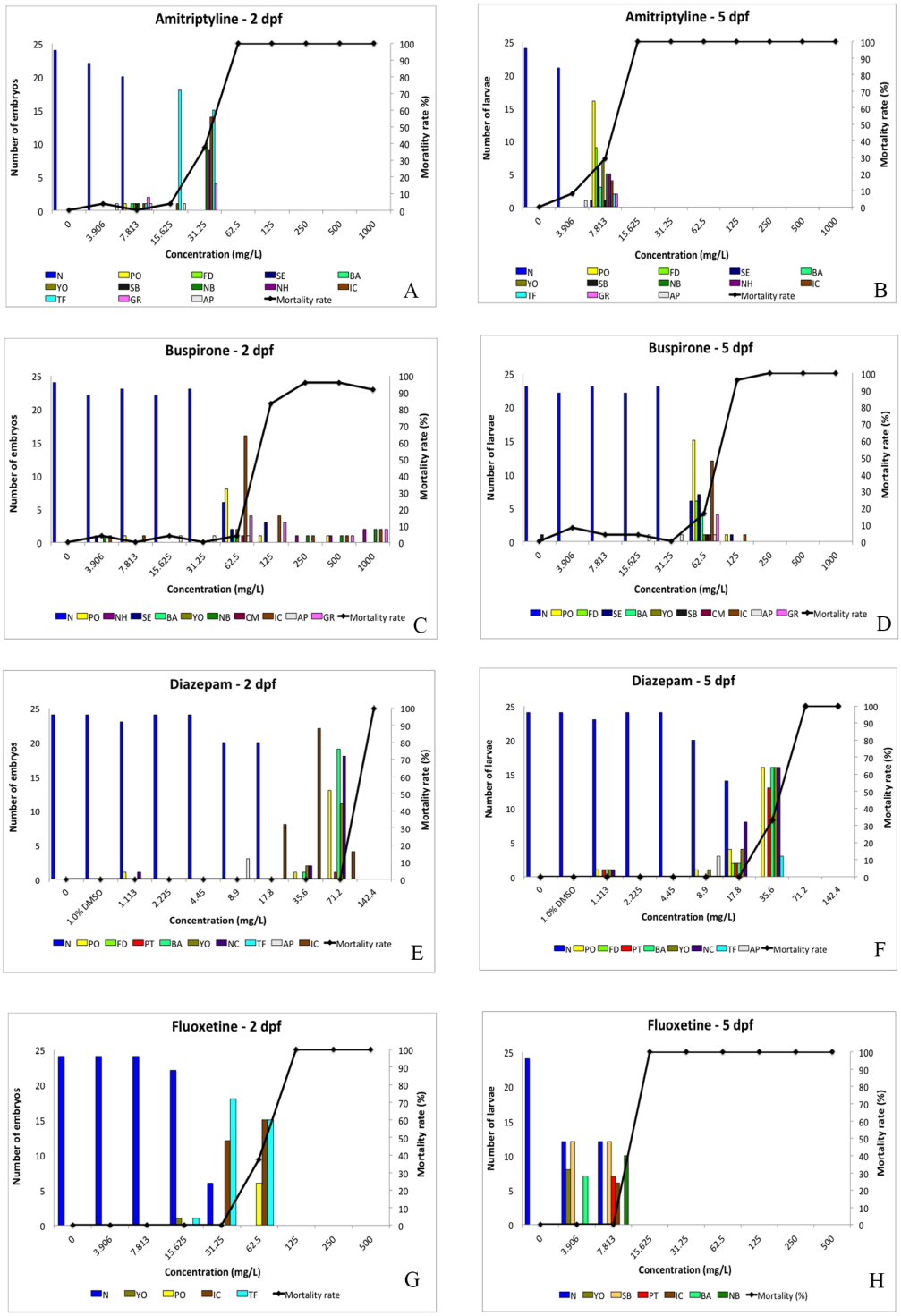


Figure 3. Incidence of abnormalities in zebrafish embryos and larvae after exposure to synthetic psychotropic drugs. A, C, E, and G: incidence of abnormalities at 2 dpf after exposure to amitriptyline, buspirone, diazepam and fluoxetine, respectively. B, D, F, and H: incidence of abnormalities observed at 5 dpf after exposure to amitriptyline, buspirone, diazepam and fluoxetine respectively. Secondary line chart: mortality rate. Diazepam was dissolved in 1% dimethylsulfoxide (DMSO). Refer to Table 1 for descriptions of the abbreviations used to describe the abnormalities.

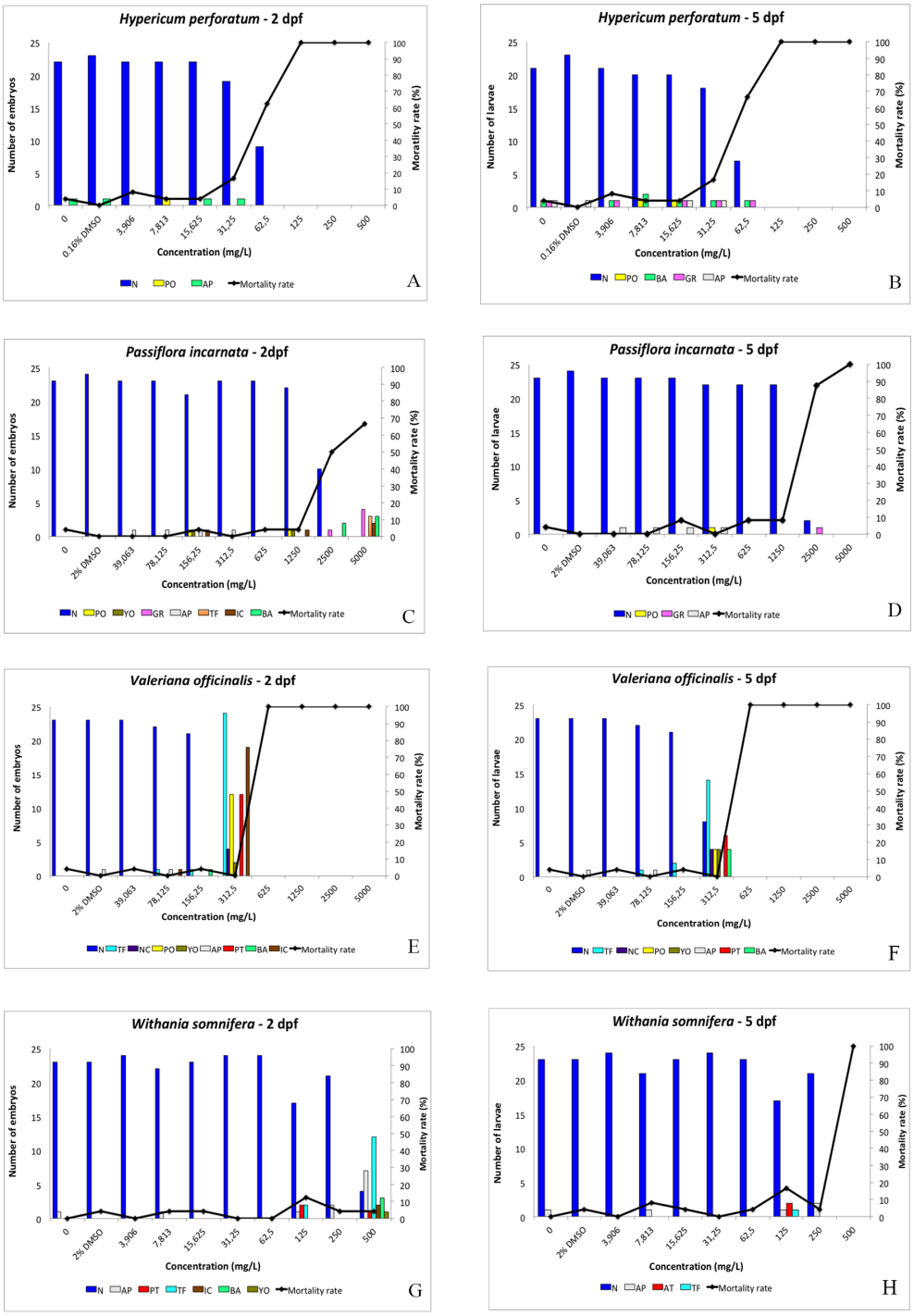


Figure 4. Incidence of scored abnormalities in zebrafish larvae after exposure to herbal extracts. A, C, E, and G: incidence of abnormalities observed at 2 dpf after exposure to *H. perforatum*, *P. incarnata*, *V. officinalis*, and *W. somnifera* respectively. B, D, F, and H: incidence of abnormalities observed at 5 dpf after exposure to *H. perforatum*, *P. incarnata*, *V. officinalis*, and *W. somnifera* respectively. Secondary line chart: mortality rate. Refer to Table 1 for descriptions of the abbreviations used to describe the abnormalities.

Table 2. LC_{50} values of synthetic and herbal based psychotropic drugs.

LC_{50} (mg/L)	Developmental stage of assessment	
	2 dpf (48 hpf)	**5 dpf (120 hpf)**
Amitriptyline	32.15 ± 2.81	8.48 ± 0.65
Buspirone	102.26 ± 12.14	72.22 ± 8.11
Diazepam	100.65 ± 246.83	37.09 ± 5.94
Fluoxetine	64.35 ± 9.14	11.06 ± 45.27
Hypericum perforatum	45.49 ± 5.21	44.19 ± 5.02
Passiflora incarnata	3232.31 ± 498.58	1625.2 ± 174.5
Valeriana officinalis	416.07 ± 34.03	416.07 ± 34.03
Withania somnifera	n.a	322.86 ± 57.67

Key:
hpf = hours post fertilization
n.a = Not applicable; LC_{50} could not be calculated as the concentrations used did not hit 100% lethality

Table 3. LC_{50} values of dimethylsulfoxide (DMSO).

Developmental stage of assessment	Dimethylsulfoxide (DMSO) LC_{50}	
	LC_{50} (g/L)	**LC_{50} (%)**
2 dpf (48 hpf)	76.48 ± 58.29	6.91 ± 0.53
5 dpf (120 hpf)	29.184 ± 37.65	2.64 ± 0.32

Key:
hpf = hours post fertilization

In amitriptyline treated larvae, CD incidence increased from 26.92% at 2 dpf to 45.01% at 5 dpf. In contrast, buspirone and diazepam showed decreased incidence between 2 dpf and 5 dpf for this category of abnormalities. For example, in buspirone-treated larvae, the incidence of CD decreased from 77.14% to 55.77%. Furthermore, the incidence of CD in diazepam-treated embryos/larvae dropped from 87.10% to 60%. All three of these synthetic drugs showed an increased incidence of developmental defects (DD) from 2 dpf to 5 dpf, with diazepam showing the highest difference in incidence between the two ages (33.02%). Both amitriptyline and buspirone showed a slight increase in the incidence of DD from 2 dpf to 5 dpf (2.31% and 2.08%, respectively).

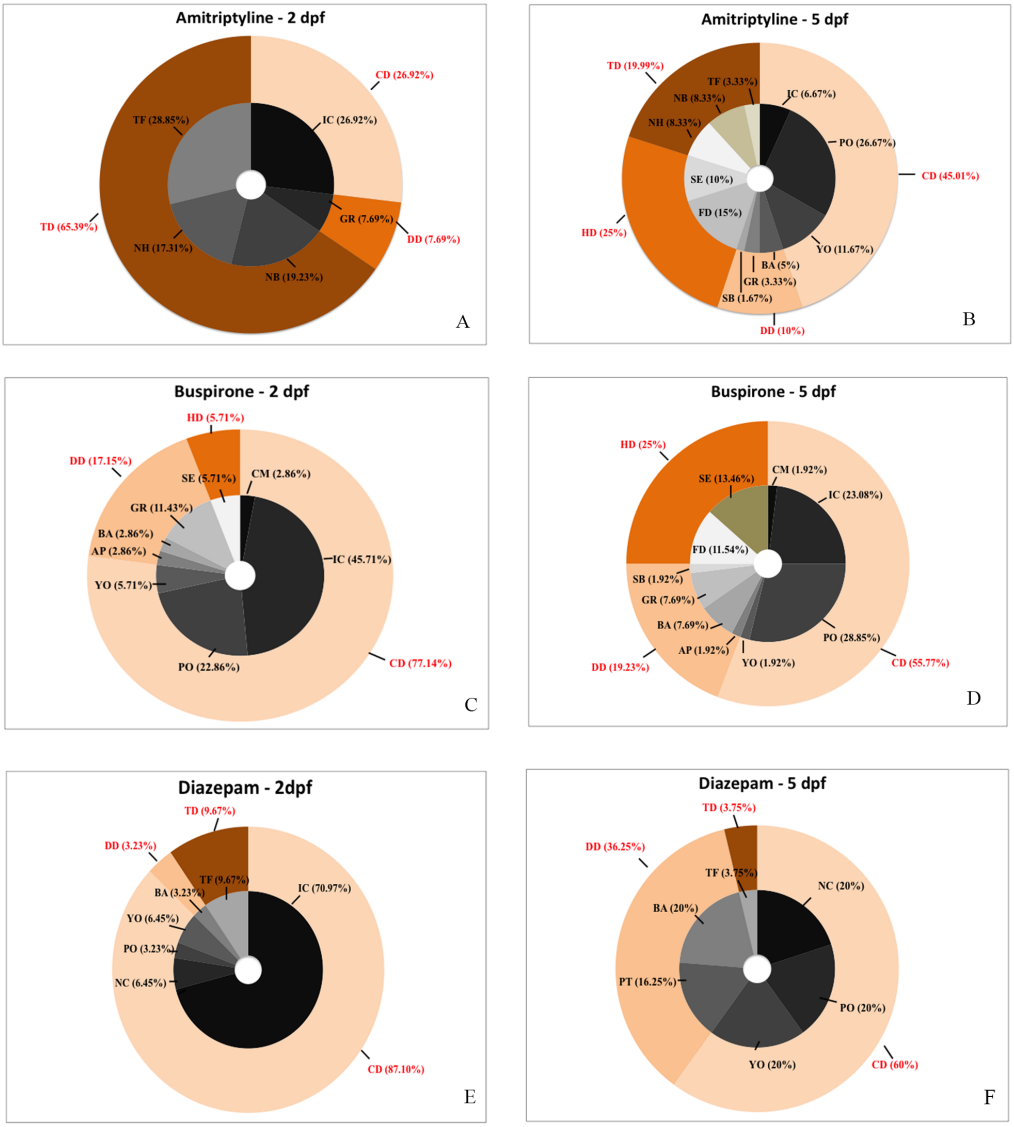


Figure 5. Doughnut chart representing the clustering of morphological abnormalities (according to four categories) after exposure to amitriptyline, buspirone, and diazepam. A, C, and E: abnormalities at 2 dpf. B, D, and F: abnormalities at 5 dpf. Outer ring, category of abnormalities; inner ring, individual abnormalities. Concentrations of drugs: amitriptyline (2 dpf = 31.25 mg/L; 5 dpf = 7.813 mg/L), buspirone (62.5 mg/L for both 2 and 5 dpf), and diazepam (35.6 mg/L for both 2 and 5 dpf). Refer to Table 1 for abbreviations. The data for fluoxetine have not been shown because the number of abnormalities was low.

Discussion

The potential developmental toxicity of psychotropic drugs, whether synthetic or herbal, is of considerable importance due to the fact that these drugs may be commonly used during pregnancy [12, 48, 59-61]. One striking finding from our study is that multiple abnormalities start to appear in the larvae only when the

concentration of synthetic or herbal drugs approaches lethal range. This could suggest that the abnormalities recorded here are the immediate phenotypic consequences of high toxicity in a dying embryo or larva. We did not observe these lethality-induced peaks of abnormalities in treatments with *Hypericum perforatum*, *Passiflora incarnata*, and *Withania somnifera* extracts. This finding of a relation between lethality and abnormalities was also found in a previous study from our laboratory. According to that study, among 43 water-soluble compounds, there was a strong correlation between teratogenicity and LC_{50} values [58].

Findings from our current study show that all four synthetic drugs are associated with some form of developmental toxicity in zebrafish embryos and larvae. Among the four drugs tested, amitriptyline showed the lowest LC_{50} and produced abnormalities at the lower concentration. Amitriptyline, a first generation tricyclic antidepressant (TCA) that has long been on the market, has already been linked with increased risk of congenital malformations with first-trimester exposure in humans [59, 62].

In addition, our findings on the four synthetic drugs are comparable with the numerous published reports of the developmental toxicity of these compounds in animal and clinical studies. For example, Beyer et al. found in hamsters that there was an increase in the foetal incidence of encephalocele[63], and bent tail[64], after the pregnant dam was exposed to amitriptyline on day 8 of pregnancy. Furthermore, a meta-analysis of case-controlled studies showed that benzodiazepine use during the first trimester of pregnancy was associated with orofacial clefts in new-borns born humans [65]. The findings on the developmental toxicity of fluoxetine are more of a mixed outcome than the other three. Numerous studies have found no convincing association between fluoxetine ingestion and perinatal abnormalities in humans, rabbits, and rats (for references see[66]). On the other hand, in other studies, this drug was reported to cause higher rates of prematurity and miscarriage in humans [67, 68].

Our findings with the herbal-based psychotropic drugs are interesting because very little has been reported in the literature about their toxicity [50-52]. We have shown here that zebrafish embryos and larvae treated with *H. perforatum* had lower LC_{50} values (higher lethality) than the other three extracts, which were comparable with the synthetics drugs. *V. officinalis* extract was associated with multiple

abnormalities at the concentrations close to its relatively high LC_{50}. Previous studies using animal models found no evidence that *Valeriana officinalis* extract, or its active constituents (valepotriates), were teratogenic after oral administration [69, 70]. However, there have been some concerns expressed about the use of this herbal drug during pregnancy due to variations in its composition between manufacturers [71, 72]. A very recent study highlighted pregnancy outcomes in psychiatric patients who had used *P. incarnata* [12]. A variety of adverse outcomes were seen in these pregnancies including neonatal death and various congenital anomalies, including premature rupture of membranes, pulmonary hypertension, and meconium aspiration syndrome.

Abnormality in pigmentation is interesting because various stressors and stimuli can disrupt pigmentation. A developing zebrafish can undergo aggregation or dispersion of pigments in response to different types of stimuli, including environmental, physical or chemical [41]. Hormonal mechanisms are thought to regulate these physiological changes [73]. Dispersion of melanocytes has been linked to activation of the stress mechanism in Arctic char (*Salvelinus alpinus*) species fish [74]. One of the two pigmentation abnormalities screened (AP, absence of pigment) was not actually found either in the case of the four synthetic or the four herbal extracts. We had included this abnormality on the screening list because it is commonly observed in various published studies. The other pigmentation abnormality that we did observe, however, was incomplete pigmentation (IP). This abnormality showed a marked increase in incidence in DMSO treated larvae. This is interesting because previous studies have shown that DMSO at subtoxic concentrations can increase heat-shock protein 70 (hsp 70, a marker for stress response [75]) levels in zebrafish larvae [76]. Thus, it is possible that DMSO induces pigmentation abnormalities through disruption of the stress pathway.

We used DMSO to dissolve the plant extracts and diazepam. The highest concentration used for herbal extracts was 2% and for diazepam was 1%. When we tested DMSO alone, we only found evidence of toxicity at $\geq$ 28.32 g/L ($\geq$ 2.56% v/v). Therefore, it is reasonable to assume that toxicity observed with the herbal extracts and diazepam in this study is not due to the presence of DMSO itself. Our findings relating to DMSO are comparable with previous studies, which showed zebrafish embryos and larvae to be tolerant to DMSO up to concentrations of 2% [76-78]. They are also in alignment with another study, which showed higher LC_{50} values (lower

lethality) of DMSO at earlier life stages in zebrafish [77]. Some abnormalities (BA, IP, IC, and PO) due to DMSO-exposure reported in our study were also found in previous studies [76, 78].

Our study indicates that the four synthetic psychotropic drugs examined here are capable of causing circulatory defects. Furthermore, such defects have the highest incidence among the four categories of abnormality for amitriptyline, buspirone, and diazepam treated embryos at 5 dpf. Interestingly, the clustering of abnormalities also showed that larvae with impaired circulation or no circulation have a higher incidence of pericardial and yolk sac oedema. In view of these findings, it would be interesting to look cardiotoxicity in more depth. Zebrafish larvae are good candidates for this type of analysis because their heart develops rapidly, with a beating heart formed by 22 hpf [28]. By 48 hpf, the cardiovascular system of zebrafish larvae is fully functional [79, 80]. It would be interesting to investigate if the synthetic drugs used in our study could affect the heart rate of developing zebrafish larvae, given the fact that some compounds has produced of arrhythmias and bradycardia in zebrafish larvae [43, 76, 81, 82]. Lee *et al.* recommended counting the heartbeat for a 30-second period beginning from 48 hpf when the heart is fully functional [83].

In addition, there are some concerns that we would like to highlight regarding the comparison of lethality between zebrafish and other species. Previous studies [33, 84] have examined the correlation between larval LC_{50} and rodent LD_{50} values and found that the toxicity of compounds in zebrafish embryos and larvae correlated well with values reported from rodent studies. Hence, zebrafish embryos and larvae could be used as a predictive model for the developmental toxicity of compounds. However, one of the two studies [33] (see above) has suggested the presence of various methodological factors that may affect the outcome of such studies. The factors include differences in exposure time, developmental stage, and route of administration. Therefore, correlating larval LC_{50} with rodent LD_{50} is not conclusive. Furthermore, in rodents, the amount of drug used is determined by the weight of the animal (LD_{50} expressed as mg/kg), while this is not the case in zebrafish larvae (where LC_{50} is expressed as mg/L or mmol/L of swimming water). Hence, there remains an issue regarding extrapolation of data acquired from the zebrafish model to humans [85].

Another major limitation of zebrafish embryo and larval-based toxicity assays is that there is no consensus on optimal protocol [15]. Elements of the protocol that can vary between workers include the scoring system for abnormalities, the duration of exposure and age of embryos/larvae at which the abnormalities were scored [15]. However, there are common interests among scientists to harmonize zebrafish-based developmental toxicity assays so that concordance with mammalian data and inter-laboratory reproducibility are ensured [86].

Future directions

Our results show that assays using zebrafish embryos and larvae are capable uncovering developmental of synthetic and herbal psychotropic drugs. Nevertheless, it is necessary to include some further analyses that can yield in-depth understanding of how the psychotropic drugs can induce developmental toxicity. The current study did not characterise the abnormalities observed in detail or examine their mechanism of action at the cellular or molecular level. In addition to reporting LC_{50} values, it is also would be interesting to evaluate the teratogenicity of the pure compounds at every developmental stage. This information could be very useful in determining whether specific toxicity is due to general developmental toxicity or was specific to the biological system.

A previous study reported teratogenicity index (TI) as the ratio of LC_{50}/EC_{50} values and this ratio was used to rank teratogenic compounds, with most teratogenic compounds showing higher TI values [57]. Since we could not determine EC_{50} values in the present study, teratogenicity index could not be determined. Several studies have reported the use of larval zebrafish in assessing teratogenicity of small molecules. One study demonstrated that 36/41 mammalian teratogens were teratogenic in zebrafish embryos [87].

In addition, a study done previously in our laboratory showed that among 43 water-soluble compounds tested, there was a variable correlation between teratogenicity LC_{50} values [58]. Some compounds were relatively teratogenic but had low lethality and other compounds only showed abnormalities near the lethal dose. We previously reported that amitriptyline was teratogenic at doses well below the lethal dose. It would be interesting to test the synthetic drugs and herbal extracts on

the embryos and larvae using linear concentration ranges. This could be useful in determining EC_{50} and TI values.

Zebrafish larvae develop rapidly, especially at early stages (< 5 dpf) [88]. To make future studies more robust, it might be interesting to collect the readout (that is, of screening or scoring of abnormalities) at a different time point of development. Moreover, it might be also desirable in the future to use a more finely tuned series of exposures regime within this crucial 5-dpf range in order to more closely resolve the teratogenic and lethal exposure ranges.

Conclusion

In conclusion, we have demonstrated that zebrafish embryos (2 dpf) and larvae (5 dpf) are good models for assessing the developmental toxicity of synthetic and herbal-based psychotropic drugs. The assay performed in the current study has potential as a high-throughput screening assay. It could, in principle, be implemented during the early drug development stage for the assessment of safety/toxicology of candidate psychotropic drugs. This could reduce or complement the usage of mammalian models. In addition, it is also essential to know the ADME properties of these compounds and extracts; these would provide scientific information on the stages of development most sensitive to the toxic effects of drugs. Due to our incomplete knowledge of the developmental toxicity of plant extracts such as *Hypericum* and *Valerian* products, which are widely available over the counter, we recommend more studies into the pharmacology of these plants.

References

[1] A.J. Baxter, T. Vos, K.M. Scott, A.J. Ferrari, H.A. Whiteford. The global burden of anxiety disorders in 2010. Psychol. Med. 2014;44(11) 2363-74.

[2] A.P. Association. Anxiety disorders. Diagnostic and statistical manual of mental disorders, American Psychiatric Publishing, Arlington, VA, 2013, pp. 189-233.

[3] C. Lochner, M. Mogotsi, P.L. du Toit, D. Kaminer, D.J. Niehaus, D.J. Stein. Quality of life in anxiety disorders: a comparison of obsessive-compulsive disorder, social anxiety disorder, and panic disorder. Psychopathology 2003;36(5) 255-62.

[4] M.P. Austin, L. Tully, G. Parker. Examining the relationship between antenatal anxiety and postnatal depression. J. Affect. Disord. 2007;101(1-3) 169-74.

[5] J. Martini, S. Knappe, K. Beesdo-Baum, R. Lieb, H.U. Wittchen. Anxiety disorders before birth and self-perceived distress during pregnancy: associations with maternal depression and obstetric, neonatal and early childhood outcomes. Early Hum. Dev. 2010;86(5) 305-10.

[6] J.C. van Bussel, B. Spitz, K. Demyttenaere. Anxiety in pregnant and postpartum women. An exploratory study of the role of maternal orientations. J. Affect. Disord. 2009;114(1-3) 232-42.

[7] V. Glover. Maternal depression, anxiety and stress during pregnancy and child outcome; what needs to be done. Best Pract. Res. Clin. Obstet. Gynaecol. 2014;28(1) 25-35.

[8] J. Martini, J. Petzoldt, F. Einsle, K. Beesdo-Baum, M. Hofler, H.U. Wittchen. Risk factors and course patterns of anxiety and depressive disorders during pregnancy and after delivery: a prospective-longitudinal study. J. Affect. Disord. 2015;175385-95.

[9] A. Waqas, N. Raza, H.W. Lodhi, Z. Muhammad, M. Jamal, A. Rehman. Psychosocial Factors of Antenatal Anxiety and Depression in Pakistan: Is Social Support a Mediator? PLoS One 2015;10(1) e0116510.

[10] D. Ram, S. Gandotra. Antidepressants, anxiolytics, and hypnotics in pregnancy and lactation. Indian J. Psychiatry 2015;57(Suppl 2) S354-S371.

[11] L.J. John, N. Shantakumari. Herbal Medicines Use During Pregnancy: A Review from the Middle East. Oman Med. J. 2015;30(4) 229-236.

[12] Z. Ozturk, C. Colak Kalayci. Pregnancy outcomes in psychiatric patients treated with passiflora incarnata. Complement. Ther. Med. 2018;3630-32.

[13] S. Marchetti, J.H.M. Schellens. The impact of FDA and EMEA guidelines on drug development in relation to Phase 0 trials. Br. J. Cancer 2007;97(5) 577-581.

[14] A.H. Piersma. Validation of alternative methods for developmental toxicity testing. Toxicol. Lett. 2004;149(1-3) 147-53.

[15] E. Teixido, E. Piqué, N. Boix, J. Llobet, J. Gomez. Zebrafish as a model for developmental toxicity assessment. 2015, pp. 65-83.

[16] N. Shanks, R. Greek, J. Greek. Are animal models predictive for humans? Philos. Ethics Humanit. Med. 2009;42.

[17] J. Bailey, A. Knight, J. Balcombe. The future of teratology research is in vitro. Biog. Amines 2005;1997-145.

[18] D. Kokel, R.T. Peterson. Using the zebrafish photomotor response for psychotropic drug screening. Methods Cell Biol. 2011;105517-24.

[19] J. Rihel, D.A. Prober, A. Arvanites, K. Lam, S. Zimmerman, S. Jang*, et al.* Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. Science 2010;327(5963) 348-51.

[20] T. Ito, H. Ando, T. Suzuki, T. Ogura, K. Hotta, Y. Imamura*, et al.* Identification of a primary target of thalidomide teratogenicity. Science 2010;327(5971) 1345-50.

[21] C. Harper, C. Lawrence. The Laboratory Zebrafish, CRC Press2016.

[22] G.J. Lieschke, P.D. Currie. Animal models of human disease: zebrafish swim into view. Nat. Rev. Genet. 2007;8(5) 353-67.

[23] M.J. Airhart, D.H. Lee, T.D. Wilson, B.E. Miller, M.N. Miller, R.G. Skalko. Movement disorders and neurochemical changes in zebrafish larvae after bath exposure to fluoxetine (PROZAC). Neurotoxicol. Teratol. 2007;29(6) 652-64.

[24] M.J. Carvan, 3rd, E. Loucks, D.N. Weber, F.E. Williams. Ethanol effects on the developing zebrafish: neurobehavior and skeletal morphogenesis. Neurotoxicol. Teratol. 2004;26(6) 757-68.

[25] T.D. Irons, R.C. MacPhail, D.L. Hunter, S. Padilla. Acute neuroactive drug exposures alter locomotor activity in larval zebrafish. Neurotoxicol. Teratol. 2010;32(1) 84-90.

[26] D. Kokel, J. Bryan, C. Laggner, R. White, C.Y. Cheung, R. Mateus*, et al.* Rapid behavior-based identification of neuroactive small molecules in the zebrafish. Nat. Chem. Biol. 2010;6(3) 231-237.

[27] J.I. Matsui, A.L. Egana, T.R. Sponholtz, A.R. Adolph, J.E. Dowling. Effects of ethanol on photoreceptors and visual function in developing zebrafish. Invest. Ophthalmol. Vis. Sci. 2006;47(10) 4589-4597.

[28] C. Parng. In vivo zebrafish assays for toxicity testing. Curr Opin Drug Discov Devel 2005;8(1) 100-6.

[29] C.B. Kimmel, W.W. Ballard, S.R. Kimmel, B. Ullmann, T.F. Schilling. Stages of embryonic development of the zebrafish. Dev. Dyn. 1995;203(3) 253-310.

[30] D. Voelker, C. Vess, M. Tillmann, R. Nagel, G.W. Otto, R. Geisler*, et al.* Differential gene expression as a toxicant-sensitive endpoint in zebrafish embryos and larvae. Aquat. Toxicol. 2007;81(4) 355-64.

[31] K. Howe, M.D. Clark, C.F. Torroja, J. Torrance, C. Berthelot, M. Muffato*, et al.* The zebrafish reference genome sequence and its relationship to the human genome. Nature 2013;496(7446) 498-503.

[32] C. Singleman, N.G. Holtzman. Growth and maturation in the zebrafish, Danio rerio: a staging tool for teaching and research. Zebrafish 2014;11(4) 396-406.

[33] S. Ali, H.G.J.v. Mil, M.K. Richardson. Large-Scale Assessment of the Zebrafish Embryo as a Possible Predictive Model in Toxicity Testing. PLoS One 2011;6(6) e21076.

[34] J.M. Spitsbergen, M.L. Kent. The state of the art of the zebrafish model for toxicology and toxicologic pathology research--advantages and current limitations. Toxicol. Pathol. 2003;31 Suppl62-87.

[35] F. Labrot, J.F. Narbonne, P. Ville, M. Saint Denis, D. Ribera. Acute toxicity, toxicokinetics, and tissue target of lead and uranium in the clam Corbicula fluminea and the worm Eisenia fetida: comparison with the fish Brachydanio rerio. Arch. Environ. Contam. Toxicol. 1999;36(2) 167-78.

[36] K. Kumar, B.A. Ansari. Malathion toxicity: Effect on the liver of the fish Brachydanio rerio (cyprinidae). Ecotoxicol. Environ. Saf. 1986;12(3) 199-205.

[37] P.F. Lanzky, B. Halting-Sørensen. The toxic effect of the antibiotic metronidazole on aquatic organisms. Chemosphere 1997;35(11) 2553-2561.

[38] G. Görge, R. Nagel. Toxicity of lindane, atrazine, and deltamethrin to early life stages of zebrafish (Brachydanio rerio). Ecotoxicol. Environ. Saf. 1990;20(3) 246-255.

[39] S. George, T. Xia, R. Rallo, Y. Zhao, Z. Ji, S. Lin*, et al.* Use of a high-throughput screening approach coupled with in vivo zebrafish embryo screening to develop hazard ranking for engineered nanomaterials. ACS nano 2011;5(3) 1805-17.

[40] S. Ali, D.L. Champagne, M.K. Richardson. Behavioral profiling of zebrafish embryos exposed to a panel of 60 water-soluble compounds. Behav. Brain Res. 2012;228(2) 272-83.

[41] S. Ali, D.L. Champagne, A. Alia, M.K. Richardson. Large-Scale Analysis of Acute Ethanol Exposure in Zebrafish Development: A Critical Time Window and Resilience. PLoS One 2011;6(5) e20037.

[42] S.M. Bugel, J.A. Bonventre, R.L. Tanguay. Comparative Developmental Toxicity of Flavonoids Using an Integrative Zebrafish System. Toxicol. Sci. 2016;154(1) 55-68.

[43] C. Ton, Y. Lin, C. Willett. Zebrafish as a model for developmental neurotoxicity testing. Birth Defects Res. A Clin. Mol. Teratol. 2006;76(7) 553-67.

[44] H. Diekmann, A. Hill. ADMETox in zebrafish. Drug Discov. Today Dis. Models 2013;10(1) e31-e35.

[45] S. Alwan, J. Reefhuis, S.A. Rasmussen, J.M. Friedman. Patterns of antidepressant medication use among pregnant women in a United States population. J. Clin. Pharmacol. 2011;51(2) 264-70.

[46] M.K. Bakker, P. Kolling, P.B. van den Berg, H.E. de Walle, L.T. de Jong van den Berg. Increase in use of selective serotonin reuptake inhibitors in pregnancy during the last decade, a population-based cohort study from the Netherlands. Br. J. Clin. Pharmacol. 2008;65(4) 600-6.

[47] R.A. Charlton, S. Jordan, A. Pierini, E. Garne, A.J. Neville, A.V. Hansen*, et al.* Selective serotonin reuptake inhibitor prescribing before, during and after pregnancy: a population-based study in six European regions. BJOG 2015;122(7) 1010-20.

[48] M. Leppée, Čulig, M. Erić, S. Sijanovic. The effects of benzodiazepines in pregnancy. Acta Neurol. Belg. 2010;110(2) 163-7.

[49] S.E. Andrade, M.A. Raebel, J. Brown, K. Lane, J. Livingston, D. Boudreau*, et al.* Use of antidepressant medications during pregnancy: a multisite study. Am. J. Obstet. Gynecol. 2008;198(2) 194.e1-5.

[50] W.H. Organization. WHO Monographs on Selected Medicinal Plants - Volume 1, World Health Organization1999.

[51] W.H. Organization. WHO Monographs on Selected Medicinal Plants - Volume 2, World Health Organization1999.

[52] W.H. Organization. WHO monographs on selected medicinal plants - Volume 3, 2006.

[53] G. Gellert, J. Heinrichsdorff. Effect of age on the susceptibility of zebrafish eggs to industrial wastewater. Water Res. 2001;35(15) 3754-7.

[54] M. Hagedorn, F.W. Kleinhans, D. Artemov, U. Pilatus. Characterization of a major permeability barrier in the zebrafish embryo. Biol. Reprod. 1998;59(5) 1240-50.

[55] K. Henn, T. Braunbeck. Dechorionation as a tool to improve the fish embryo toxicity test (FET) with the zebrafish (Danio rerio). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 2011;153(1) 91-8.

[56] S. Lantz-McPeak, X. Guo, E. Cuevas, M. Dumas, G.D. Newport, S.F. Ali*, et al.* Developmental toxicity assay using high content screening of zebrafish embryos. J. Appl. Toxicol. 2015;35(3) 261-72.

[57] I.W. Selderslaghs, A.R. Van Rompay, W. De Coen, H.E. Witters. Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. Reprod. Toxicol. 2009;28(3) 308-20.

[58] J. Aalders, S. Ali, T.J. de Jong, M.K. Richardson. Assessing Teratogenicity from the Clustering of Abnormal Phenotypes in Individual Zebrafish Larvae. Zebrafish 2016;13(6) 511-522.

[59] A. Bérard, J.-P. Zhao, O. Sheehy. Antidepressant use during pregnancy and the risk of major congenital malformations in a cohort of depressed pregnant women: an updated analysis of the Quebec Pregnancy Cohort. BMJ Open 2017;7(1) e013372.

[60] M.H. Mabina, S.B. Pitsoe, J. Moodley. The effect of traditional herbal medicines on pregnancy outcome. The King Edward VIII Hospital experience. S. Afr. Med. J. 1997;87(8) 1008-10.

[61] S.W. Wen, M. Walker. Risk of fetal exposure to tricyclic antidepressants. J. Obstet. Gynaecol. Can. 2004;26(10) 887-92.

[62] M.B. Bracken, T.R. Holford. Exposure to prescribed drugs in pregnancy and association with congenital malformations. Obstet. Gynecol. 1981;58(3) 336-44.

[63] J.A. Al-Tubaikh, M.F. Reiser. Congenital Diseases and Syndromes: An Illustrated Radiological Guide, Springer Berlin Heidelberg2009.

[64] B.K. Beyer, M.S. Guram, W.F. Geber. Incidence and potentiation of external and internal fetal anomalies resulting from chlordiazepoxide and amitriptyline alone and in combination. Teratology 1984;30(1) 39-45.

[65] L.R. Dolovich, A. Addis, J.M. Vaillancourt, J.D. Power, G. Koren, T.R. Einarson. Benzodiazepine use in pregnancy and major malformations or oral cleft: meta-analysis of cohort and case-control studies. BMJ 1998;317(7162) 839-43.

[66] T.H. Shepard. Catalog of Teratogenic Agents, The John Hopkins University Press, Baltimore, Maryland, 2010.

[67] C.D. Chambers, K.A. Johnson, L.M. Dick, R.J. Felix, K.L. Jones. Birth outcomes in pregnant women taking fluoxetine. N. Engl. J. Med. 1996;335(14) 1010-5.

[68] R. Rahimi, S. Nikfar, M. Abdollahi. Pregnancy outcomes following exposure to serotonin reuptake inhibitors: a meta-analysis of clinical trials. Reprod. Toxicol. 2006;22(4) 571-5.

[69] P. Morazzoni, E. Bombardelli. Valeriana officinalis: Traditional use and recent evaluation of activity, 1995.

[70] S. Tufik, K. Fujita, L. Seabra Mde, L.L. Lobo. Effects of a prolonged administration of valepotriates in rats on the mothers and their offspring. J. Ethnopharmacol. 1994;41(1-2) 39-44.

[71] T.B. Klepser, M.E. Klepser. Unsafe and potentially safe herbal therapies. Am. J. Health Syst. Pharm. 1999;56(2) 125-38; quiz 139-41.

[72] A.H.C. Wong, M. Smith, H.S. Boon. Herbal Remedies in Psychiatric Practice. Arch. Gen. Psychiatry 1998;55(11) 1033-1044.

[73] R. Fujii. The regulation of motile activity in fish chromatophores. Pigment Cell Res. 2000;13(5) 300-19.

[74] E. Hoglund, P.H. Balm, S. Winberg. Skin darkening, a potential social signal in subordinate arctic charr (Salvelinus alpinus): the regulatory role of brain monoamines and pro-opiomelanocortin-derived peptides. J. Exp. Biol. 2000;203(Pt 11) 1711-21.

[75] M.E. Feder, G.E. Hofmann. HEAT-SHOCK PROTEINS, MOLECULAR CHAPERONES, AND THE STRESS RESPONSE: Evolutionary and Ecological Physiology. Annu. Rev. Physiol. 1999;61(1) 243-282.

[76] A. Hallare, K. Nagel, H.R. Kohler, R. Triebskorn. Comparative embryotoxicity and proteotoxicity of three carrier solvents to zebrafish (*Danio rerio*) embryos. Ecotoxicol. Environ. Saf. 2006;63(3) 378-88.

[77] Y. Huang, R. Cartlidge, M. Walpitagama, J. Kaslin, O. Campana, D. Wlodkowic. Unsuitable use of DMSO for assessing behavioral endpoints in aquatic model species. Sci. Total Environ. 2018;615107-114.

[78] J. Maes, L. Verlooy, O.E. Buenafe, P.A.M. de Witte, C.V. Esguerra, A.D. Crawford. Evaluation of 14 Organic Solvents and Carriers for Screening Applications in Zebrafish Embryos and Larvae. PLoS One 2012;7(10) e43850.

[79] D. Sedmera, M. Reckova, A. deAlmeida, M. Sedmerova, M. Biermann, J. Volejnik*, et al.* Functional and morphological evidence for a ventricular conduction system in zebrafish and Xenopus hearts. Am. J. Physiol. Heart Circ. Physiol. 2003;284(4) H1152-60.

[80] C. Thisse, L.I. Zon. Organogenesis--heart and blood formation from the zebrafish point of view. Science 2002;295(5554) 457-62.

[81] D.J. Milan, T.A. Peterson, J.N. Ruskin, R.T. Peterson, C.A. MacRae. Drugs That Induce Repolarization Abnormalities Cause Bradycardia in Zebrafish. Circulation 2003;107(10) 1355-1358.

[82] S.W. Mittelstadt, C.L. Hemenway, M.P. Craig, J.R. Hove. Evaluation of zebrafish embryos as a model for assessing inhibition of hERG. J. Pharmacol. Toxicol. Methods 2008;57(2) 100-105.

[83] K.Y. Lee, G.H. Jang, C.H. Byun, M. Jeun, P.C. Searson, K.H. Lee. Zebrafish models for functional and toxicological screening of nanoscale drug delivery systems: promoting preclinical applications. Biosci. Rep. 2017;37(3).

[84] C. Parng, W.L. Seng, C. Semino, P. McGrath. Zebrafish: a preclinical model for drug screening. Assay Drug Dev. Technol. 2002;1(1 Pt 1) 41-8.

[85] A.H. Piersma, G. Janer, G. Wolterink, J.G. Bessems, B.C. Hakkert, W. Slob. Quantitative extrapolation of in vitro whole embryo culture embryotoxicity data to developmental toxicity in vivo using the benchmark dose approach. Toxicol. Sci. 2008;101(1) 91-100.

[86] A.L. Gustafson, D.B. Stedman, J. Ball, J.M. Hillegass, A. Flood, C.X. Zhang*, et al.* Inter-laboratory assessment of a harmonized zebrafish developmental toxicology assay - progress report on phase I. Reprod. Toxicol. 2012;33(2) 155-64.

[87] R. Nagel. DarT: The embryo test with the Zebrafish Danio rerio--a general model in ecotoxicology and toxicology. Altex 2002;19 Suppl 138-48.

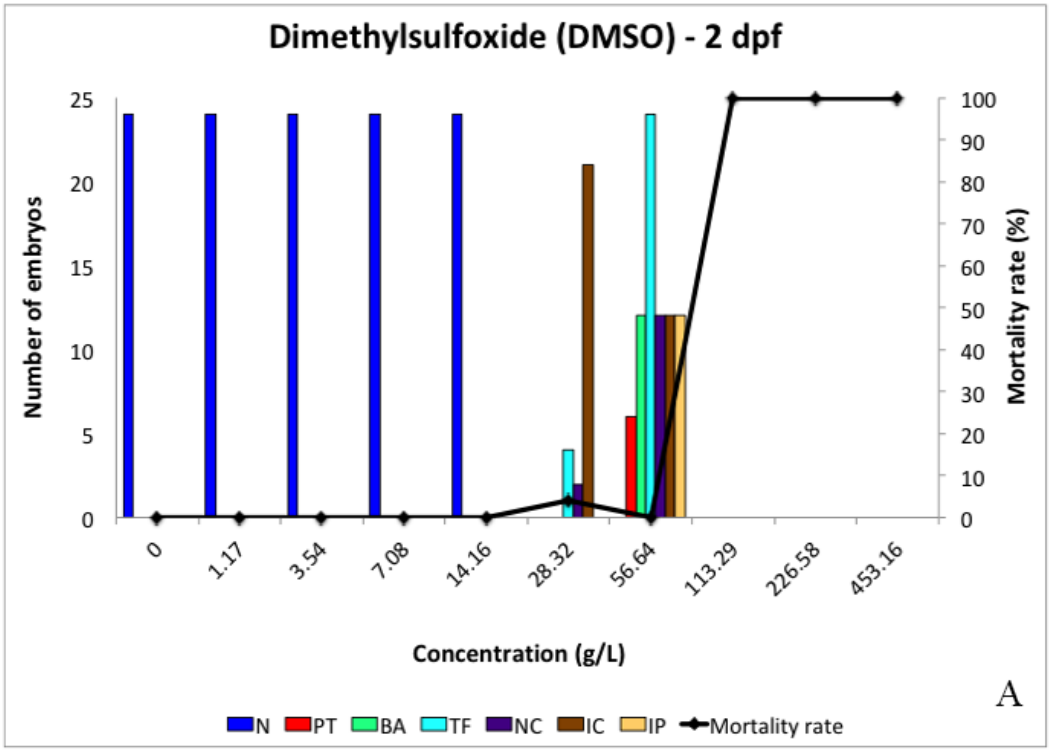
[88] R. Dahm, R. Geisler. Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species. Mar. Biotechnol. (N. Y.) 2006;8(4) 329-45.

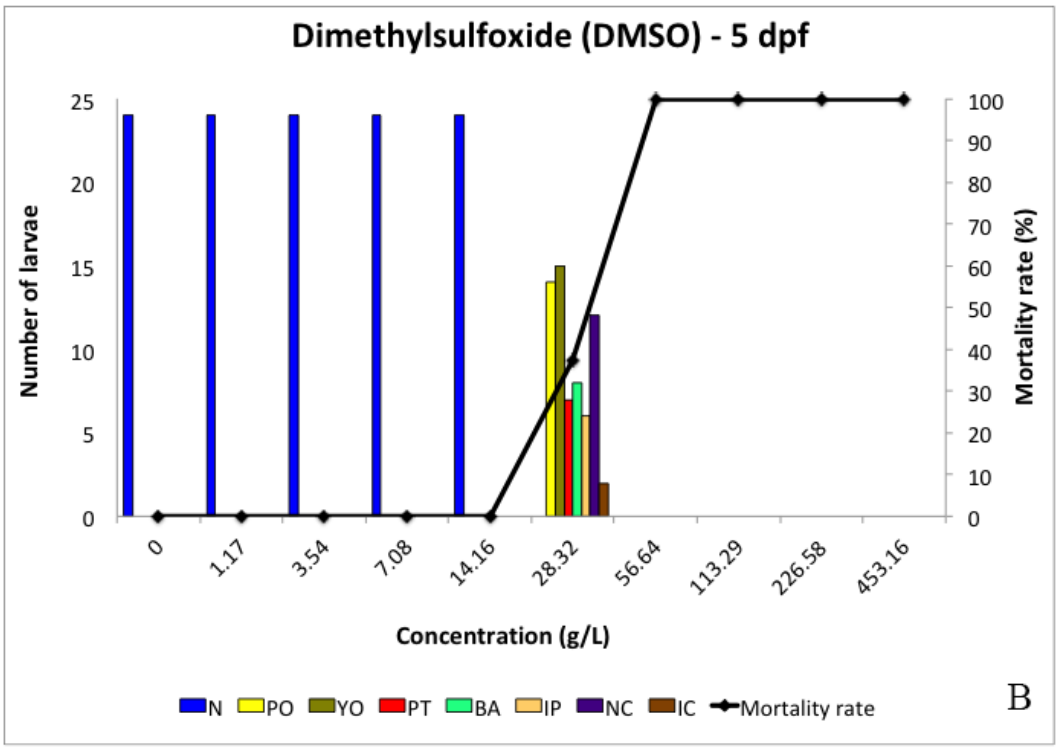
Supplementary materials:

Supplementary Table S1. Concentrations used in geometric series in the current study for compounds and plant extracts. N = 24 for both controls and untreated larvae.

Compounds/ Extract	**Concentrations in geometric series (mg/L)**									
	C0	***C1***	***C2***	***C3***	***C4***	***C5***	***C6***	***C7***	***C8***	***C9***
Amitriptyline	0	3.906	7.813	15.625	31.25	62.5	125	250	500	1000
Buspirone	0	3.906	7.813	15.625	31.25	62.5	125	250	500	1000
Diazepam	0	1.0% DMSO	1.113	2.225	4.45	8.9	17.8	35.6	71.2	142.4
Fluoxetine	0	3.906	7.813	15.625	31.25	62.5	125	250	500	n.a
H. perforatum	0	0.16% DMSO	3.906	7.813	15.625	31.25	62.5	125	250	500
P. incarnata	0	2.0% DMSO	39.063	78.125	156.25	312.5	625	1250	2500	5000
V. officinalis	0	2.0% DMSO	39.063	78.125	156.25	312.5	625	1250	2500	5000
W. somnifera	0	2.0% DMSO	3.906	7.813	15.625	31.25	62.5	125	250	500
Dimethylsulfoxide* (in g/L)	0	1.17 (0.16)	3.54 (0.32)	7.08 (0.64)	14.16 (1.28)	28.32 (2.56)	56.64 (5.12)	113.29 (10.24)	226.58 (20.48)	453.16 (40.96)

Key:
*Concentration of DMSO is also show in percentage inside parentheses; DMSO concentration represented in g/L unit
n.a = not applicable





Supplementary Figure S1. Incidence of abnormalities in zebrafish embryos and larvae after exposure to dimethylsulfoxide (DMSO). A and B: incidence of abnormalities observed after exposure to DMSO at 2 dpf and 5 dpf respectively. Secondary line chart: mortality rate. Refer to the Table 1 for descriptions of the abbreviations used to describe the abnormalities. Figure only shows concentrations in g/L unit, for the corresponding concentration in percentage, refer to the Supplementary Table S1.