Reproductive Toxicology 121 (2023) 108469

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Developmental neurotoxicity evaluation of three Chinese herbal medicines in zebrafish larvae by means of two behavioral assays: Touch-evoked response and light/dark transition

Noelia G. Romero [a,b], Gerard Gutierrez [a], Elisabet Teixidó [a,b], Lu Li [d,e,g], Jördis Klose [h], Ping Chung Leung [f], Salvador Cañigueral [c], Ellen Fritsche [h,j,k], Marta Barenys [a,b,i,*]

[a] GRET and Toxicology Unit, Department of Pharmacology, Toxicology and Therapeutic Chemistry, Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain
[b] Institute of Nutrition and Food Safety of the University of Barcelona (INSA-UB), Spain
[c] Unitat de Farmacologia, Farmacognòsia, i Terapèutica, Facultat de Farmàcia, i Ciències de l'Alimentació, Universitat de Barcelona, Av. Joan XXIII 27-31, 08028 Barcelona, Spain
[d] College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, China
[e] Department of Obstetrics & Gynaecology, Li Ka Shing Institute of Health Sciences, School of Biomedical Sciences, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong
[f] Institute of Chinese Medicine, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong
[g] National Key Laboratory of Chinese Medicine Modernization, Innovation Center of Yangtze River Delta, Zhejiang University, 314100, Jiaxing, China
[h] IUF - Leibniz Research Institute for Environmental Medicine, Düsseldorf, Germany
[i] German Centre for the Protection of Laboratory Animals (Bf3R), German Federal Institute for Risk Assessment (BfR), Berlin, Germany
[j] Medical Faculty, University of Düsseldorf, 40225 Düsseldorf, Germany
[k] DNTOX GmbH, 40223 Düsseldorf, Germany

ARTICLE INFO

Handling Editor: Dr. Bal-Price Anna

Keywords:
NAMs (New Approach Methodologies)
Herbal products
Traditional use
Pregnancy
Prenatal development
Alternative methods

ABSTRACT

Chinese herbal medicine (CHM) is used among pregnant women. However, the question of its safety during pregnancy remains unclear. The use of these products relies on history of use data but there are specific toxicities like developmental neurotoxicity that are clearly understudied. Here we use the zebfrafish embryo developmental toxicity assay (ZEDTA) in combination with two behavioral assays: touch-evoked response and Light/Dark (L/D) transition assay to evaluate the neuro/developmental toxicity of three herbal products commonly used in CHM [Chinese name (abbreviation; part of the plant and *Scientific name*]: tian ma (TM; tuber form *Gastrodia elata* Blume), lei gong teng (LGT; root and rhizome of *Tripterygium wilfordii* Hook.f) and cha ye (green tea, leaves from *Camellia sinensis* (L.) Kuntze). In case significant alterations were detected, single components with potential exposure during pregnancy were identified in the literature and further tested. TM had no neurodevelopmental toxic potential in zebrafish embryos, while LGT and its main compounds triptolide and celastrol induced significant alterations in behavior. Developmental exposure to EGCG, the main catechin of green tea, also produced significant alterations in zebrafish embryos behavior after developmental exposure. A combination of ZEDTA with L/D Transition assay is proposed as a useful combination of alternative methods for DNT assessment of CHM products together with other New Approach Methodologies (NAMs).

Abbreviations: AO, Adverse outcome; AOP, Adverse outcome pathway; CEEA, Ethic Committee for Animal Experimentation; CHM, Chinese herbal medicines; D_{intS}, Dysmorphogenesis interfering swimming; DMSO, Dimethyl sulfoxide; D_{not}, Dysmorphogenesis not interfering swimming; DNT, Developmental neurotoxicity; D_{total}, Total dysmorphogenesis; $EC_{50DTotal}$, Effective concentration 50 for total dysmorphogenesis; EFSA, European Food Safety Authority; EGCG, Epigallocatechin gallate; EMA, European Medicines Agency; GD, Gestational day; GDM, Gestational diabetes mellitus; HMPC, Herbal Medicinal Products Committee; hpf, hours post-fertilization; IR, Infrared radiation; IVB, in vitro battery; KE, key event; KNIME, Konstanz Information Miner; L/D, Light/Dark; LC_{50}, Lethal concentration 50; LGT, Lei gong teng; LOAEC, Lowest Observed Adverse Effect Level; NAM, New Approach Methodology; OECD, Organization for Economic Co-operation and Development; SEM, Standard error of the mean; TER, Touch-Evoked Response; TM, Tian ma; ZEDTA, Zebrafish embryo developmental toxicity assay.

* Corresponding author at: GRET and Toxicology Unit, Department of Pharmacology, Toxicology and Therapeutic Chemistry, Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain.
E-mail address: mbarenys@ub.edu (M. Barenys).

https://doi.org/10.1016/j.reprotox.2023.108469
Received 12 June 2023; Received in revised form 28 July 2023; Accepted 1 September 2023
Available online 4 September 2023

1. Introduction

Characterizing the general safety of herbal products is a critical public health need since the available information about them is very scarce [30]. Moreover, the characterization of their specific safety during prenatal neurodevelopment is even more understudied [4]. This is of particular concern because herbal products, and among them Chinese herbal medicines (CHM), are used as single products or in combinations to manage pregnancy-related discomforts or complications [26,33,41,9]. In general, these products are assumed "to be free of risks" because they are "natural" [12] moreover most pregnant women consider alternative and complementary medicine safer during pregnancy than conventional drugs [21]. However, depending on how they are placed in the market, these products are not held to the same safety standards and regulations than licensed medicinal products, and for some of them there is a too high degree of uncertainty to draw firm conclusions about their use during pregnancy [12]. Even less studied is the possibility of interactions in form of synergies among products consumed concomitantly, adding more uncertainty to the real exposure scenario.

To evaluate the potential adverse effects of substances in neurodevelopment, an OECD/EFSA (European Food Safety Authority) developmental neurotoxicity (DNT) in vitro battery (IVB) has been developed ([29]; EFSA PPR Panel et al., [10]). This battery has been designed following the paradigm shift in toxicology which moves from solely in vivo animal studies to combinations of New Approach Methodologies (NAMs) contributing to Adverse Outcome Pathway (AOP) descriptions. At the moment, it includes several human-cell based tests that cover a large variety of neurodevelopmental processes and allow the identification of alterations in specific key events. However, it lacks NAMs which are able to evaluate behavioral alterations. Since in nervous system related AOPs this is a critical Adverse Outcome, it is already under discussion if orthogonal assays (not part of the current DNT IVB) in alternative species (*C. elegans, Danio rerio*.) could support this in vitro evaluation [35]. Indeed, the OECD Expert Group on Developmental Neurotoxicity (Project 4.124: New Guidance Document on Developmental neurotoxicity (DNT) in vitro assays) is already working on identifying the critical points of behavioral evaluation in zebrafish and designing harmonized protocols for their performance [46] with the final aim to determine the additional value behavioral evaluation in zebrafish could have for the OECD/EFSA IVB [31].

In our study we focus on the evaluation of the neurodevelopmental toxicity potential of three CHM: tian ma (TM; steamed, fragmented and dried tuber of *Gastrodia elata* Blume, tall gastrodia tuber), lei gong teng (LGT; root and rhizome of *Tripterygium wilfordii* Hook.f, common threewingnut root) and cha ye (unfermented, stabilized and dried leaves of *Camellia sinensis* (L.) Kuntze, green tea). The use of these three CHM by pregnant women has been documented in different countries (TM: [41]; LGT: [40]; green tea: [9]). For TM and green tea there are no specific recommendations [20], but LGT use during pregnancy is restricted in the Chinese Pharmacopoeia although not in other restriction lists [8,20]. For all of them, some concerns about their safety during neurodevelopment have arisen from in vivo studies in rodents (green tea: [11]), from in vitro assays included in the OECD/EFSA IVB (TM and LGT: [22]) or from in vitro studies and clinical cases (LGT: [40,7]).

To perform this evaluation, we have used the zebrafish embryo developmental toxicity assay (ZEDTA) including the gold standard Light/Dark (L/D) Transition assay to assess behavioral alterations, which is one of the assays currently being harmonized and evaluated by the OECD Expert Group on DNT. Besides this test, we have included a second one which is not a reference test but is also broadly used: the Touch-Evoked Response (TER) test, which can be performed 24–48 h earlier than the L/D Transition assay and evaluates the response to mechanical instead of light stimuli. Since zebrafish embryos display different behaviors over time, with different onset and different complexity, the outcomes of both behavioral evaluations are integrated and compared to lethality and dysmorphogenic effects to draw conclusions on the DNT potential of the CHM tested. In case significant behavioral alterations are identified for a specific CHM, single compounds present in this CHM with potentially relevant single prenatal exposure are further tested with both methods. Finally, results of both behavioral tests are compared for all CHM/single compounds tested and discussed to support future decisions on the applicability of both assays in integrated methodological strategies.

2. Material and methods

2.1. Plant extracts

Samples of dry root extracts of TM and LGT were prepared by the Institute of Chinese Medicine, Chinese University of Hong Kong under the supervision of Professor Ping Chung Leung and provided to our group by the Leibniz Research Institute for Environmental Medicine (IUF). Preparation procedures of TM and LGT aqueous dry extracts (with yield rates of 65.44 % and 6.55 %, respectively), as well as their quality specifications and the exclusion of pesticides and biological contaminants is described in Klose et al. [22]. For testing, TM and LGT dry extracts were diluted in 0.3X Danieaús solution, stock solutions of 1 mg/mL were prepared and stored at 4 °C for up to 3 days. Green tea was purchased from Amoros Nature Technology (Girona, Spain), with quality specifications detailed in Supplementary Material 1 excluding the presence of pesticides and biological contaminants. The aqueous extract was prepared according to the specifications detailed in the monograph of the HMPC of the EMA [17] for green tea traditional use. Specifically, 4.4 g of dried leaves were weighed and extracted by infusion with 210 mL of distilled water for 10 min. The hot solution was then filtered under vacuum using a Büchner funnel, the filtered solution was concentrated in a rotary evaporator (Büchi Rotavapor R-200) at a maximum temperature of 50 ºC and reduced pressure, and afterwards hermetically sealed and frozen. The frozen solutions were freeze-dried for 48 h and the dry extract was stored in airtight vials at room temperature. The yield rate was 10.7 %. A stock solution was prepared with 20 mg of extract and 20 mL of 0.3X Danieaús solution, corresponding to a concentration of 1 mg/mL. For each experiment a new stock solution was prepared and stored in the freezer. The ingredients present in the plant extracts used have already been published elsewhere (TM and LGT in Klöse et al., [22], and green tea extract in the monograph of the Herbal Medicinal Products Committee (HMPC) of the European Medicines Agency (EMA) [18]), and have been included in Supplementary Material 2 (Tables S2.1 and S2.2). An overview on the extracts and single compounds tested is presented in Fig. 1.

2.2. Single compounds

Celastrol (CAS: 34157-83-0; purity >98 %) and Triptolide (CAS: 38748-32-2; purity >98 %) were purchased from Cayman Chemical Company. These compounds were dissolved in dimethyl sulfoxide (DMSO) to a 0.7 μM solution. (−)-Epigallocatechin-3-gallate (EGCG, CAS: 989-51-5, purity > 98 %) was purchased from TransMIT PlantMetaChem (Giessen, Germany). EGCG was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich; ≥ 99.5 %) to a 5.6 μM solution. The final concentration of DMSO as solvent in all solutions was 0.1 % (v/v).

2.4. Zebrafish colony maintenance, egg harvest and selection

Adult zebrafish (*Danio rerio*; Tropical Center ICA S.A., Spain) were maintained in aquariums with a closed flow-through system in standardized water as specified in ISO 7346–3 (1996), at a temperature of 26 ± 1 °C, and a constant light:dark cycle of 14:10 h. Fish were daily fed with *Artemia salina* in the morning, and commercial flake food (Special Diets Services S-400, Batch Number 41900) in the afternoon. Fish were placed overnight in a mating tank with artificial plants and marbles to stimulate spawning. Next morning, after lights turned on, eggs were

collected by filtering water with a sieve, and afterwards washed a minimum of three times with standardized ISO 7346-3 water diluted 1:5. Fertilized, non-coagulated, and synchronously divided eggs were selected using a stereomicroscope (Motic SMZ168, Motic China group, LTD., China) and were transferred to a 6-well plate (10 embryos/well).

2.5. Exposure conditions

After egg selection (maximum at 2 hpf), water from each well was replaced by 0.3X Danieau's solution pH = 7.4, prepared from a concentrated solution (30X) (1740 mM NaCl, 21 Mm KCl, 12 mM $MgSO_4{\cdot}7H_2O$, 18 mM Ca $(NO_3)_2{\cdot}4H_2O$, 150 mM HEPES buffer, distilled water) or by solutions of plant extracts (TM; LGT; green tea) or compound solutions (Celastrol; Triptolide; EGCG) dissolved in 0.3X Danieau's solution. Exposure solutions of TM, LGT and green tea were obtained from the stock solution by 1:10 dilutions (0.001; 0.01; 0.1; 1; 10; 100 and 1000 μg/mL). Exposure solutions for celastrol (0.01; 0.02; 0.04; 0.08; 0.175; 0.35 and 0.7 μM), triptolide (0.002; 0.005; 0.01; 0.02; 0.04; 0.08; 0.175; 0. 35 and 0.7 μM) and EGCG (0.002; 0.005; 0.01; 0.02; 0.04; 0.08; 0.175; 0.35; 0.7; 1.4; 2.8 and 5.6 μM) were 1:2 dilutions from the respective stock solutions. Embryos were incubated at 26 ± 1 °C with photoperiod (14 h light and 10 h dark) and incubated according to the different exposure scenarios described in 2.5.1 or 2.5.2.

2.5.1. Developmental exposure (2–72 hpf) for Touch-evoked Response (TER) test

Selected eggs were exposed to 5 mL of the different concentrations of plant extracts or compound solutions, incubated at 26 ± 1 °C and every 24 h, after lethality (by coagulation and/or absence of heartbeat) and dysmorphogenesis recording, the medium was renewed with 5 mL of freshly prepared solutions (semi-static procedure; Fig. 2). Dysmorphogenesis observed in live larvae were classified into two groups: dysmorphogenesis that could interfere with the swimming response (D_{intS}, such as tail dysmorphogenesis, fin dysmorphogenesis or bent body axis) and dysmorphogenesis not interfering with swimming response (D_{not}, including pigmentation alterations and jaw dysmorphogenesis). The mean of total dysmorphogenesis (D_{Total}, in %), D_{intS} and D_{not} were calculated for at least 3 independent experiments. At 72 hpf, TER test was performed as described in [15]. Briefly, one embryo was transferred to a 2.9 cm diameter glass Petri dish with 2 mL of 0.3X Danieau's solution. The Petri dish was placed under a video camera and the embryo was allowed to acclimatize to the new environment for 1 min. After this

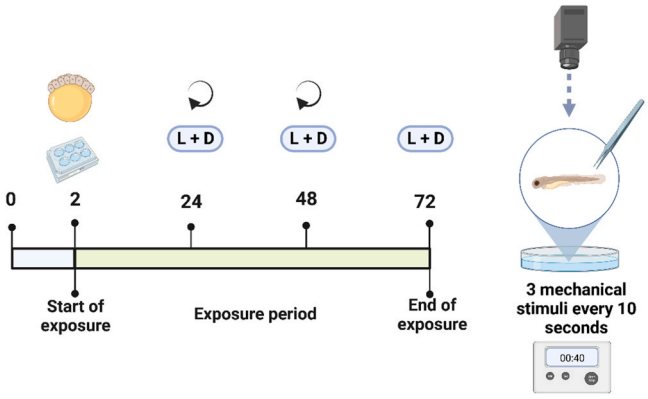


Fig. 2. Schematic representation of Developmental Exposure (2–72 hpf) for TER test. Fertilised eggs were exposed at 2 hpf and incubated in a 6-well plate. Accumulated Lethality (L) and total dysmorphogenesis (D) evaluation and the renewal of medium were performed every 24 h and TER test was performed at the end of exposure (72 hpf). The green line indicates exposure time. This graph was created with BioRender.com.

time, video recording was started (Casio EX-H30 camera, Japan) and at the 10th second the tail of the embryo was touched with the tip of a forceps (Dumont FST no. 5, Switzerland). The same procedure was repeated allowing 10 s after each touch, for a total of 3 touches per embryo. The total duration of the video was 40 s. A total of 6 larvae per concentration were evaluated, preferentially choosing those that had already hatched and exclusively selecting those that did not show D_{intS} among the 10 larvae/group. If there were not enough hatched larvae, they were manually dechorionated. This assay was only performed at concentrations where the accumulated mortality and/or D_{Total} per concentration at 72 hpf were ≤ 20 %. The whole assay was repeated at least 3 times on different days (3 independent experiments); therefore, a minimum of 18 larvae per concentration were evaluated.

2.5.2. Developmental exposure (2–96 hpf) for L/D Transition test and evaluation at 120 hpf

Selected eggs were exposed to 10 mL of the different concentrations of plant extracts or compound solutions, incubated at 26 ± 1 °C, and after 72 h, dysmorphogenesis (D_{intS}, D_{not} and D_{Total}) and lethality were observed, and medium was renewed with 5 mL of the same solutions

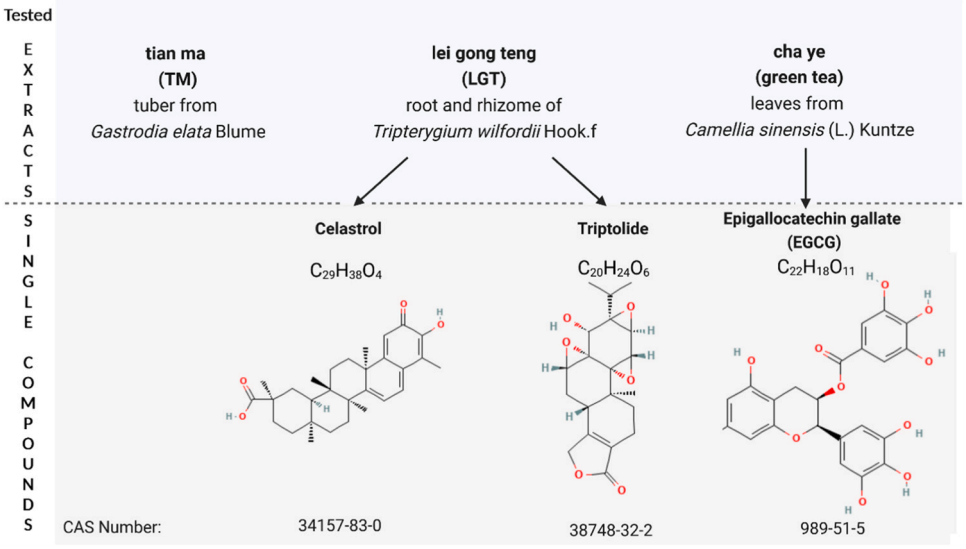


Fig. 1. Summary of the extracts and single compounds tested. This figure was created with BioRender.com, including Chemical Structure Depictions from PubChem (https://pubchem.ncbi.nlm.nih.gov/).

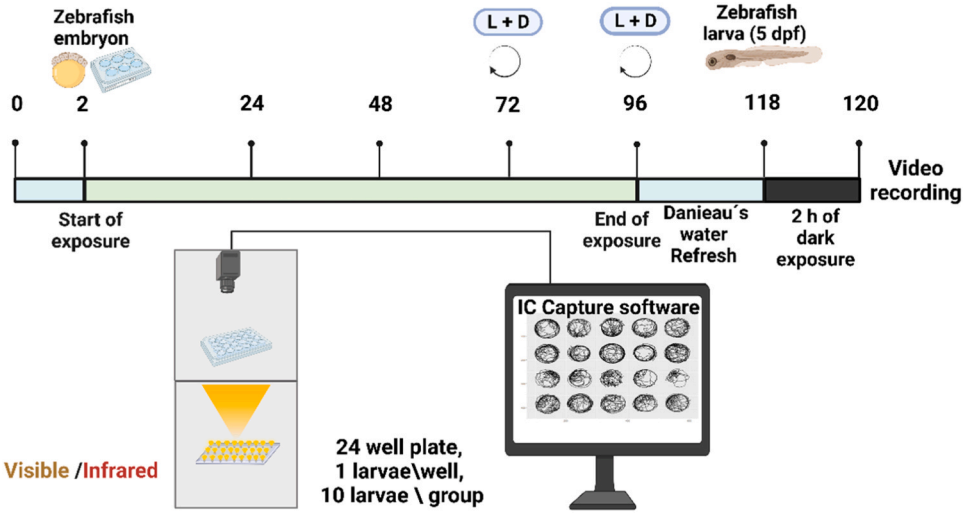


Fig. 3. Schematic representation of the developmental exposure (2–96 hpf) for the L/D Transition assay. Fertilised eggs were exposed at 2 hpf and incubated in a 6-well plate with 10 eggs per concentration. Accumulated lethality (L) and total dysmorphogenesis (D) evaluation were assessed at 72 and 96 hpf. At 72 hpf concentrations were renewed and at 96 hpf media were changed by 0.3X Danieaús solution. The L/D Transition assay was performed at 120 hpf. The green line indicates exposure time, and the light blue line indicates that embryos were incubated in 0.3X Danieaús solution without exposure. This graph was created with BioRender.com.

(Fig. 3). At 96 D_{intS}, D_{not}, D_{Total} and lethality were evaluated, and medium was exchanged for 5 mL of 0.3X Danieau's solution per well. At 118 hpf D_{intS}, D_{not}, D_{Total} and lethality were evaluated again, and larvae were transferred to 24-well plates and acclimated to the plate and the dark environment for 2 h. Plates were kept at 26 ± 1 °C under a camera (DMK 23UX236, The Imaging Source Europe GmbH, Germany) in a custom-made set-up previously described in [43] to record their locomotor activity at 30 frames per second. The light source was located at the bottom and provided both infrared (IR, 850 nm) and visible light (intensity: 7160 lumens). The process of switching from light to dark was fully automated by means of a control box. Briefly, larval trajectories based on X and Y coordinates, were used to calculate the total distance swum per minute/cycle and the total distance swum during the entire light period (3 cycles of 4 min: total 12 min) and during the entire dark period (2 cycles of 4 min: total 8 min). This assay was only performed at concentrations where the accumulated mortality and/or D_{Total} per concentration at 72 hpf were ≤ 20 %.

2.6. Data processing

Videos were analysed with ImageJ 1.47 v (32-bit) and Excel v.16.67 for the TER test. Data processing for TER was performed exactly as described in detail in [15]. For the L/D Transition assay, videos were cut into 4 min clips using VirtualDub software (v.1.10.4) and analysed to track individual larval movement. Larval trajectories were analysed using the KNIME® analytics platform. Briefly, video files are converted to images (frames) using ffmpeg software (Developers ffmpeg, 2016) as an external tool. Then the background is subtracted from all images by projecting the average intensity of all frames and a threshold is applied to identify the position of each larva in the well. From each binary image the centroid (x, y coordinates) is calculated. Euclidian distance between centroids at successive points in time (frames) and a moving average to smooth trajectories is used to calculate the travelled distance. The location of the larvae along the center of the well was also calculated by comparing the X and Y coordinates of the larval centroids to the X and Y coordinates of the well center. This information was used to calculate the thigmotaxis of larvae during the L/D Transition test measured as percentage of time spent in the outer zone and percentage of the total distance moved in the outer zone of the 24-well plate. The outer zone was defined as the region 4 mm (average body length of a 5 dpf larva) from the edge of the well. Thigmotactic behavior was only measured during the dark phase in robustly moving larvae since zone preference analysis requires exploration and inactivity could misrepresent thigmotaxis [36]. The workflow allows saving the data in Excel format, which contains row data, trajectory graphs of the larvae and the layout created (Supplementary Material 3).

2.7. Data analysis

Statistical analysis was performed with GraphPad Prism v.9. The mean of individual measurements was calculated for each exposure concentration group consisting in 10 embryos per group and afterwards, the mean of at least three independent experiments was obtained. For concentration–response curves, a sigmoidal (variable slope) curve fit was applied. The teratogenic index (TI) was calculated using the LC_{50} and $EC_{50DTotal}$ (Effective concentration 50 for total Dysmorphogenesis) values obtained in the concentration-response curves (TI= LC_{50}/EC_{50}). Based on [37], a TI ≥ 2 was established as the criteria to consider a compound as teratogenic. Statistical comparison was performed by one-way ANOVA with Dunnett's multiple comparison tests for concentration-effect analyses for total dysmorphogenesis and TER test. Two-way ANOVA followed by Dunnett's test using the same software was used to assess the effect of concentration and time over the entire test period in the L/D Transition assay and to assess the effect of concentration and time on the locomotion response within each time. A p-value < 0.05 was set as threshold for statistical significance. Error bars presented in all figures represent standard error of the mean (SEM).

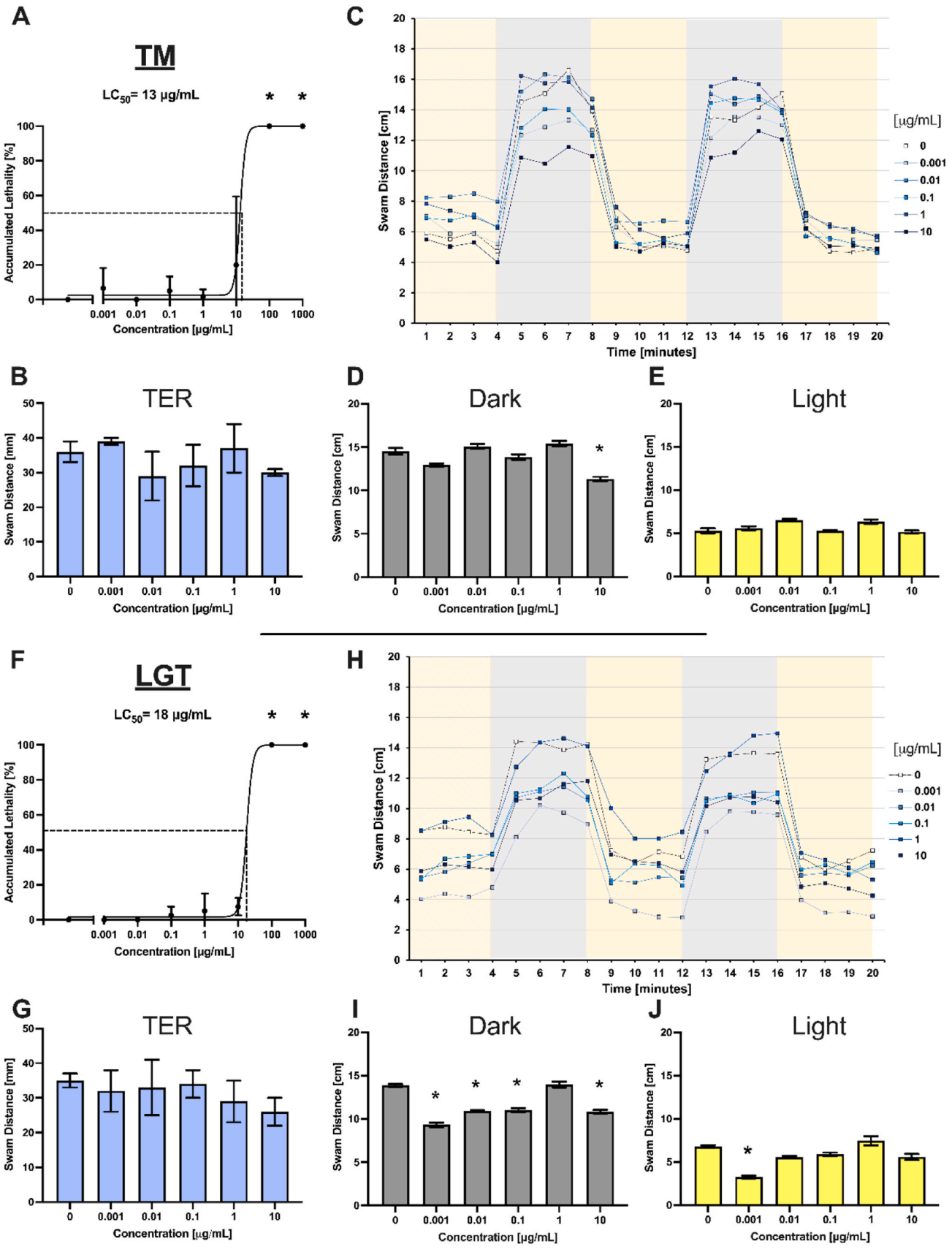
3. Results

3.1. Developmental exposure to LGT extract but not to TM extract causes neurodevelopmental adverse effects in zebrafish larvae

At 72 hpf, the LC_{50} of TM extract solutions in zebrafish embryos was 13 μg/mL (Fig. 4A), and total dysmorphogenesis evaluation showed no significant morphological alterations at the tested concentrations (Fig. S2.1A). At this timepoint, the TER test did not detect any significant difference among the concentrations evaluated in comparison to control (Fig. 4B).

At 120 hpf, the L/D Transition assay detected only a significant decrease at 10 μg/mL during the dark periods (Fig. 4C, D and E). This effect however, occurred at a concentration very close to the calculated LC_{50} value for this product (13 μg/mL). Putting these results in context, the effect observed at 10 μg/mL is considered to be related to general toxicity, and the common interpretation of the results of both behavioral tests, the TER and L/D Transition is that TM is not producing specific neurodevelopmental adverse effects in zebrafish larvae.

For LGT, however, although LC_{50} value and total dysmorphogenesis results were very similar to TM, LC_{50} = 18 μg/mL (Fig. 4F), and no significant morphological alterations at the tested concentrations



(caption on next page)

Fig. 4. Evaluation of TM and LGT. Accumulated lethality assessment (A, F) and TER assessment (B, G) after 72 h of exposure of zebrafish embryos exposed to TM (A, B) or LGT (F, G) representing the mean of at least 3 independent experiments (± standard error of the mean, SEM), including 6 larvae per concentration in each independent experiment (in total: at least 18 larvae). Locomotor activity of zebrafish larvae at 120 hpf after exposure to TM (C) or LGT (H) extract solutions including 5 cycles of 4 min, alternating light (3x) and dark (2x) cycles. Results are expressed as average distance swum per minute including 10 larvae per concentration in each independent experiment (in total: at least 30 larvae) (C, H), during dark cycles (D, I) or during light cycles (E, J). Yellow color indicates light cycles, grey color indicates dark cycles. In C) and H), the first yellow column is stripped to depict that the results obtained from the first cycle (adaptation period) are not included in the statistical evaluation. For the statistical analysis of A) and F) a sigmoidal curve fit (variable slope) was applied. In B) and G) a one-way ANOVA with Dunnett's multiple comparisons test was performed. In the L/D Transition assay, a two-way ANOVA followed by Dunnett's test was applied to assess the effect of concentration and time on the locomotion response. Results are indicated as significant (*) for $p < 0.05$ compared to the control group.

(Fig. S2.1. B), the behavioral evaluations showed a very different profile. In this case, the mean distance swum per larvae in the TER test showed a trend to decrease with increasing concentrations of LGT although these values were not statistically significant (Fig. 4G).

In the L/D Transition assay, after exposure to LGT extract from concentrations between 0.001 and 10 μg/mL, the swimming distance was significantly shorter than in the control group, and the effect was mainly observed during the dark cycles (Fig. 4H and I). Among these exposure groups, larvae exposed to 0.001 μg/mL LGT extract presented the shortest swimming distance per cycle, with a statistically significant difference in both light and dark cycles (Fig. 4J). These results point to the possibility that a compound present in LGT extract may cause developmental neurotoxicity at low concentrations, while at increasing concentrations other compounds present in the extract might compensate for this effect. The significant decrease observed at 10 μg/mL could again be related to general toxicity, as the LC_{50} value of this extract was calculated at 18 μg/mL.

3.2. Assessment of two compounds present in LGT extract: Triptolide reduces average swimming distance of larvae more potently than celastrol

In view of these results, we performed a literature research to identify compounds present in LGT extract which have been proposed to have beneficial effects when administered during pregnancy. Since a thorough investigation on all compounds present in LGT to identify the responsible/s one/s for the neurodevelopmental adverse effects observed would require a high amount of time and resources, the aim was to identify compounds present in LGT which rise interest for their future use during pregnancy to confirm or discard if they also induce the effect observed after exposure to LGT extract. This information would be relevant to contribute to future risk/benefit decisions for the further evaluation of these compounds, for example identifying potential synergistic effects among them.

From this literature research, two relevant compounds were identified: Triptolide and celastrol. In preclinical studies, triptolide attenuates kidney cyst formation and arrests cellular proliferation in a murine model of autosomal dominant polycystic kidney disease when administered from GD 10.5 until birth [25]. Celastrol attenuates intrahepatic cholestasis of pregnancy (which is a severe liver disorder during pregnancy) in rats when oral administered at the dose of 5 mg/kg bw daily for 5 consecutive days [14]. A developmental toxicity study of celastrol in zebrafish embryos was also found, but no neurodevelopmental endpoints were evaluated [45], therefore both compounds were included in our study.

After 72 h exposure of zebrafish embryos to triptolide solutions, an $LC_{50} = 0.10$ μM was obtained (Fig. 5A), and the evaluation of total dysmorphogenesis in live larvae showed no significant morphological alterations at any concentration tested (Fig. S2.1. C). After this exposure period a concentration-dependent decrease in the mean swimming distance was observed in the TER test with a LOAEC = 0.02 μM (Fig. 5B).

The results of the L/D Transition assay were in good agreement with this observation, with again a concentration-dependent adverse effect of reduced mean swimming distance and a LOAEC= 0.02 μM, but in this case the effect was only observed during the light cycles of the test (Fig. 5C, D and E). In this context, the high similarity of significant effects observed in both tests (Fig. 5B and E), and that these effects already occur at concentrations 4 and 3 times lower than the LC_{50}, and EC_{50} at 118 hpf, respectively (see Fig. S2.2, Fig. S2.3. and Table S2.3) at concentrations with mainly dysmorphogenesis not interfering with swimming response, strongly indicate that triptolide also induces neurodevelopmental alterations in zebrafish larvae.

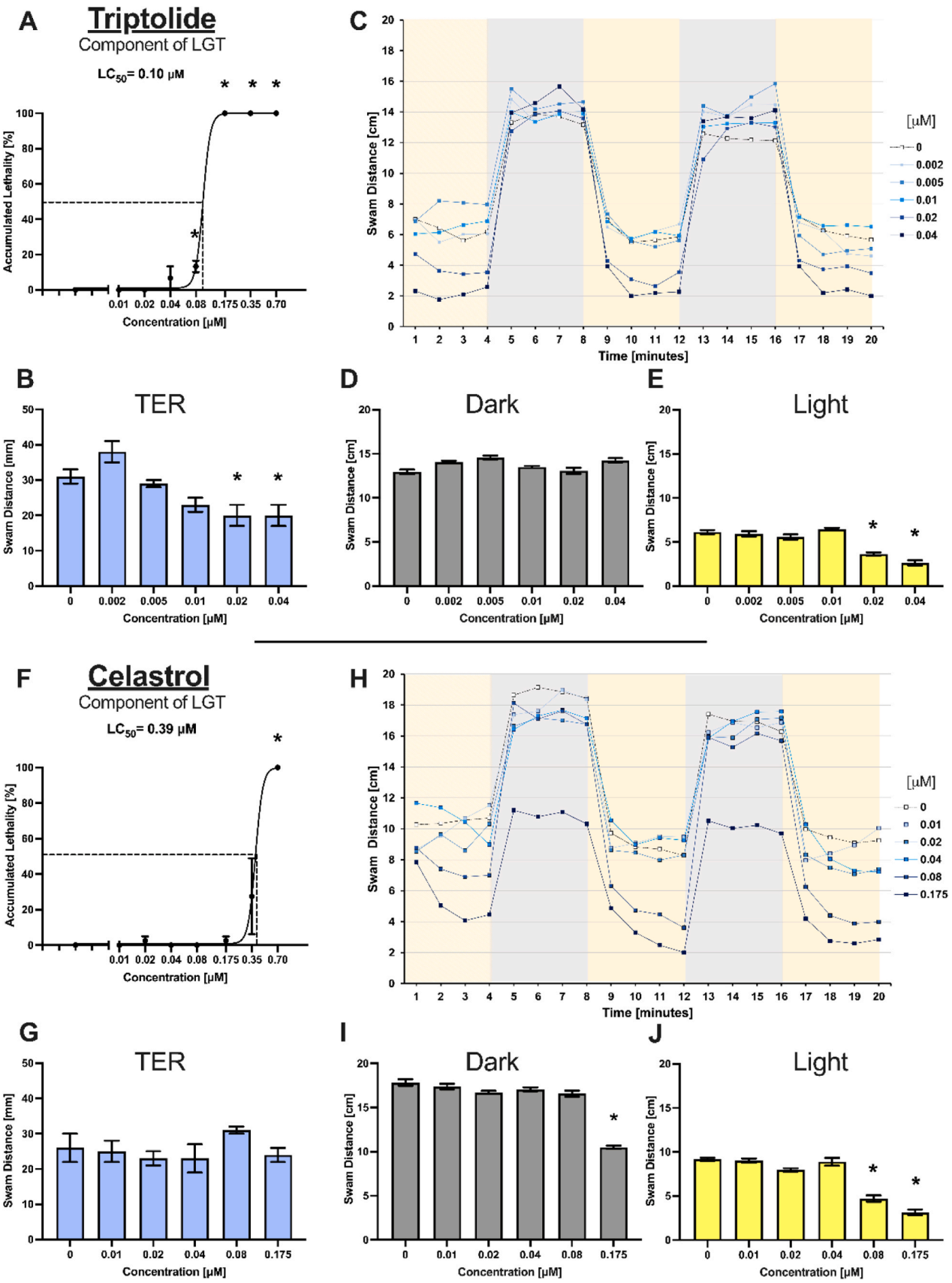
On the other hand, celastrol presented an $LC_{50} = 0.39$ μM (Fig. 5F) and induced significant effects on total dysmorphogenesis in live larvae at 0.35 μM after 72 h exposure (Fig. S2.1. D) with an $EC_{50DTotal}$ of 0.35 μM (Fig. S2.4). The lethality results are in good agreement with Wang et al. [45], since the authors demonstrated that the lethal effect of celastrol was concentration dependent with a $LC_{50} = 1.4$ μM after a 24 hpf exposure, which would be in good agreement with the $LC_{50} = 0.39$ μM value observed in our study after a longer exposure (72 hpf) of the embryos. The dysmorphogeneses observed were also very similar to the ones described by Wang et al. [45]: bent body axis and tail dysmorphogenesis (Table S2.4.). However, the calculation of the teratogenic index ($TI = LC_{50}/EC_{50} = 1.1$) at 72 hpf shows that the compound is not classified as teratogenic in zebrafish (because $TI < 2$; see Table S2.3.). In the TER test, no concentration-dependent effect was observed (Fig. 5G). A longer exposure however, unraveled concentration-dependent reductions in mean swimming distance in the L/D Transition assay with LOAEC= 0.08 μM during light cycles and 0.175 μM in dark cycles (Fig. 5H, I and J). The apparent disagreement between the results of both behavioral tests can be easily explained by the longer exposure to celastrol in the experimental design for L/D Transition assay, which could reasonably lead to stronger effects in L/D Transition assay than in TER test.

Our results indicate the presence of neurodevelopmental effects in larvae exposed to triptolide and celastrol. Both compounds induce a concentration-dependent hypoactivity but triptolide is approximately four times more potent than celastrol, and its adverse effects also occur after shorter times of exposure (already after 72 h). These two compounds, however, are probably not the only ones responsible for the effects observed after LGT extract exposure because, although in all cases an hypoactivity-related effect is observed, the pattern of significant alterations detected in the tests performed is different.

3.3. Green tea and EGCG induce hyperactivity in developing zebrafish embryos

The lethality evaluation resulted in a $LC_{50} = 359$ μg/mL after 72 hpf of exposure of zebrafish larvae to green tea solutions (Fig. 6A) and the evaluation of total dysmorphogenesis in live larvae showed no significant morphological alterations at any concentration tested (Fig. S2.1. E). At this stage, the TER test did not detect any significant difference between the tested green tea concentrations compared to the control (Fig. 6B).

After a longer exposure however, the L/D Transition assay revealed a significant increase in the distance swum at 1 μg/mL (Fig. 6C and D) during the dark cycles, and even at a lower concentration during the light cycles (0.01 μg/mL, Fig. 6E). The effects were not concentration-dependent possibly because at higher concentrations other compounds present in the mixture produced a compensating effect. A result supporting this interpretation is the significant decrease in the distance swum at 100 μg/mL, since at this concentration no lethality and no effects on total dysmorphogenesis were observed; therefore, this decrease



(caption on next page)

Fig. 5. Evaluation of triptolide and celastrol. Accumulated lethality assessment (A, F) and TER assessment (B, G) after 72 h of exposure of zebrafish embryos exposed to triptolide (A, B) or celastrol (F, G) representing the mean of at least 3 independent experiments (± standard error of the mean, SEM), including 6 larvae per concentration in each independent experiment (in total: at least 18 larvae). Locomotor activity of zebrafish larvae at 120 hpf after exposure to Tp (C) or Ce (H) extract solutions including 5 cycles of 4 min, alternating light (3x) and dark (2x) cycles. Results are expressed as average distance swum per minute including 10 larvae per concentration in each independent experiment (in total: at least 30 larvae) (C, H), during dark cycles (D, I) or during light cycles (E, J). Yellow color indicates light cycles, grey color indicates dark cycles. In C) and H), the first yellow column is stripped to depict that the results obtained from the first cycle (adaptation period) are not included in the statistical evaluation. For the statistical analysis of A) and F) a sigmoidal curve fit (variable slope) was applied. In B) and G) a one-way ANOVA with Dunnett's multiple comparisons test was performed. In the L/D Transition assay, a two-way ANOVA followed by Dunnett's test was applied to assess the effect of concentration and time on the locomotion response. Results are indicated as significant (*) for $p < 0.05$ compared to the control group.

in activity cannot be due to general toxicity or to the presence of malformations in the larvae.

To further investigate if the effects induced by green tea were due to one compound with potential relevant exposure during pregnancy, we selected EGCG because it has already been used in preclinical studies as potential prenatal treatment for pregnancy induced pre-eclampsia [38], in the prevention/treatment of brain development disorders due to Dowńs syndrome [24,39], fetal alcoholic syndrome [1,28,44] and EGCG has been shown to improve maternal and neonatal treatment of Gestational Diabetes Mellitus (GDM) [48]. Based on a literature research, the concentration range to be tested was decided so it would include the relevant in vivo concentrations (Fig. 6F). At this concentration range, LC_{50} was not reached, and no significant effects were found for total dysmorphogenesis (Fig. S2.1. F). At 72 hpf, again in the TER test there was no significant effect for the concentrations tested (Fig. 6G), but the L/D Transition assay evidenced alterations in behavior after a longer exposure. In this case, larvae presented a generalized hyperactivity, both in light and dark conditions (Fig. 6H), with LOAECs as low as 0.02 and 0.01 μg/mL in dark and light cycles respectively (Fig. 6I and J). In view of these results, it could be possible that the effects observed on motor activity in the L/D Transition assay for green tea could be related to the hyperactive effects induced by EGCG [49].

3.4. Thigmotactic behavior analysis did not bring additional information to the L/D Transition assay

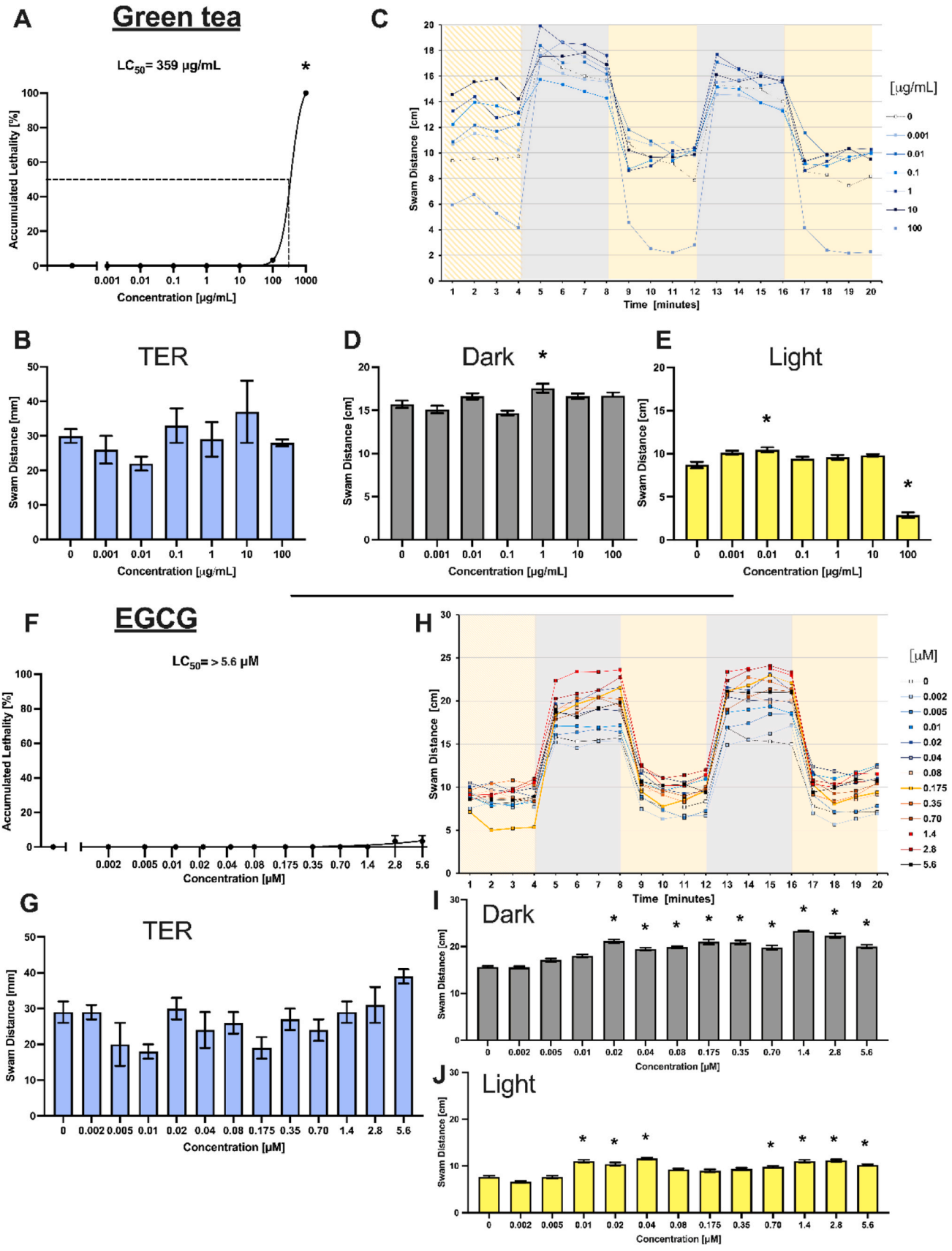
The analysis of the videos of the dark phases of the L/D Transition assay for thigmotactic behavior by means of 'distance in outer zone' (in %) and 'time in outer zone' (in %) did not identify any significant effect at any specific concentration of any of the products tested (Fig. S2.5 and S2.6).

4. Discussion

The evaluation of potential nervous system-related Adverse Outcomes after developmental exposure to CHM is necessary because a considerable proportion of women consume CHM during pregnancy [13,16,19,34]. However, evaluating neurodevelopmental adverse effects in classical guideline studies in rodents is time and resource consuming, which complicates screening high numbers of CHM and/or their single components in a short period of time. In this study we have used an alternative method to animal experimentation to evaluate behavioral alterations after developmental exposure to 6 different products: the ZEDTA combined with two behavioral tests, TER test and L/D Transition assay.

In our study we followed a two-step approach: first using a whole-mixture approach to test 3 CHM: TM, LGT and green tea extracts, followed, if necessary, by a single component-based approach testing 3 single compounds present in the corresponding CHM. Further studies could be done by testing mixtures of single components in different combinations and concentrations, but this was not the scope of this study. After a first overview comparing the results of both approaches, it is easy to identify one of the common consequences of testing complex mixtures in contrast to single compounds: the concentration-response pattern observed after exposure to CHM was, in general, not concentration-dependent, indicating that probably combination effects were occurring due to individual components having antagonistic interactions. In the single compound approach, concentration-dependent responses were identified for most endpoints, and the assessment of the outcome was clearer. While the first approach has the advantage of representing a more realistic exposure scenario when studying the consequences of CHM consumption during pregnancy, it does not provide specific information on which interactions are occurring. Besides that, since pregnant women could also be exposed to single compounds present in these CHM due to expected beneficial effects, if CHM results were significantly different than control results, those compounds were selected among the literature and tested separately in the second approach.

The first CHM tested was TM. In this case, no specific neurodevelopmental adverse effects were identified, and therefore, no single component present in TM was further evaluated. TM has been considered, in general, a non-toxic herb, it is regularly consumed by pregnant women in some countries [41], and no clinical cases relating it to neurodevelopmental adverse outcomes have been found. However, specific studies about neurodevelopmental toxicity of TM are very scarce in the literature. The only study evaluating these effects, identified an adverse effect of TM on developing oligodendrocytes at extract concentrations $\geq$ 500 μg/mL after 5 days of exposure without reducing nuclei number, viability or inducing cytotoxicity. This adverse effect was observed in human but not in rat neurospheres and was linked to the activation of a gene expression network related to oxidative stress [22]. At lower concentrations no adverse effect was found in any other neurodevelopmental endpoint studied. In our study, at 100 μg/mL, general toxicity is already significantly increased, and no specific neurodevelopmental effect is observed at lower concentrations. Other studies have reported a $LD_{50} = 39.8$ g/kg in mice after i.v. administration of TM [47], a dose that was not related to an experimentally measured C_{max}. Assuming a total distribution of the dose among the blood volume of a standard mouse (58.5 mL of blood per kg in mice according to National Centre for the Replacement, Refinement and Reduction of Animals in Research [32]) this doses might lead to a concentration of 680 μg/mL (and corrected for the 65.44 % yield rate of the extract would be 445 μg/mL). The study of extract concentrations of 500 μg/mL and above in isolated cells in vitro is useful to generate a worst-case scenario and an understanding for hazard, which can help to interpret results at lower concentrations, but does not represent a realistic exposure scenario in vivo, since reaching these concentrations in foetal brain would probably not occur in absence of general toxicity and is not possible after drinking a cup of TM preparation (with 3–10 g TM; [42]) or after ingesting capsules containing concentrated TM. Bioavailability data of TM in humans is not given [27], but pharmacokinetic studies of gastrodin, one of its main compounds, showed that oral doses of 200 mg lead to a C_{max} of 1.485 μg/mL [27]. This means - considering proportionality - when escalating doses, a 10,000 mg dose (which would be equivalent to the highest recommendation found in the internet) would only correspond to 74.25 μg/mL concentrations in blood (and corrected for the 65.44 % yield rate of the extract would correspond to 48.6 μg/mL). Although these calculations are not precise and need to be taken with caution, they are useful to show the relevant range of exposure in humans and to interpret the apparent discrepancy between neurospheres and zebrafish results, which is probably due to general toxicity effects in the whole organism evaluation. However, human



(caption on next page)

Fig. 6. Evaluation of green tea and EGCG. Accumulated lethality assessment (A, F) and TER assessment (B, G) after 72 h of exposure of zebrafish embryos exposed to green tea (A, B) or EGCG (F, G) representing the mean of at least 3 independent experiments (± standard error of the mean, SEM), including 6 larvae per concentration in each independent experiment (in total: at least 18 larvae). Locomotor activity of zebrafish larvae at 120 hpf after exposure to green tea (C) or EGCG (H) extract solutions including 5 cycles of 4 min, alternating light (3x) and dark (2x) cycles. Results are expressed as average distance swum per minute including 10 larvae per concentration in each independent experiment (in total: at least 30 larvae) (C, H), during dark cycles (D, I) or during light cycles (E, J). Yellow color indicates light cycles, grey color indicates dark cycles. In C) and H), the first yellow column is stripped to depict that the results obtained from the first cycle (adaptation period) are not included in the statistical evaluation. For the statistical analysis of A) and F) a sigmoidal curve fit (variable slope) was applied. In In B) and G) a one-way ANOVA with Dunnett's multiple comparisons test was performed. In the L/D Transition assay, a two-way ANOVA followed by Dunnett's test was applied to assess the effect of concentration and time on the locomotion response. Results are indicated as significant (*) for $p < 0.05$ compared to the control group.

exposure is a broad mixture of compounds from food, the environment and pharmaceuticals. Considering the large number of compounds that might converge on common neurodevelopmental key events [2,6], co-exposure to hazardous CHM might gain relevance in the mixture context.

The second CHM tested was LGT, which allowed to identify behavioral alterations derived from developmental exposure to LGT and to two of its main compounds celastrol and triptolide. For LGT extract, concentrations producing behavioral alterations were well in the low μg/mL range (0.001–0.1 μg/mL after 118 h, although the yield rate for this extract was very low =6.55 %, and the corrected concentration range for LGT would be 0.015–1.5 μg/mL), and were more in agreement with the concentrations described to produce alterations in human neurospheres (5 μg/mL after 72 h). A clinical report associated prenatal exposure to LGT with meningoencephalocele, a severe neurodevelopmental malformation [40], and a study in whole mice embryos revealed relevant neurodevelopmental alterations including open cranial neural tube, and malformations like twisted body axis [7]. No significant morphological malformations were detected in our zebrafish embryo study, but this could be a matter of the concentrations used and the big dilution steps (x10) among concentrations. Concerning embryolethality, in the same mouse embryo study, 100 and 200 μg/mL exposure of LGT extract produced a 100 % lethality in 9-day or 10-day old embryos, respectively [7]. Of note, concentrations producing neurodevelopmental related effects in zebrafish and in human neurospheres are at ranges of absence of embryolethality in zebrafish or in mouse embryos. All together, these results confirm the developmental neurotoxicity of LGT in zebrafish and support the claims for avoidance of use of this CHM during pregnancy.

Further evaluation around LGT exposure brought us to study the effects of celastrol and triptolide, two of its main compounds which are, moreover, postulated in the literature to have potential beneficial effects during pregnancy. On the one hand, our study revealed teratogenic effects of celastrol at 72 hpf at concentrations where lethality was also present (TI<2; Table S2.3). The dysmorphogeneses were related to bent body axis and malformed tail (Table S2.4), which points to the possibility of celastrol being the compound responsible of the teratogenic effects observed in mouse embryos after LGT extract exposure also at lethal concentrations [7]. On the other hand, lower concentrations of triptolide induced neurodevelopmental related behavioral alterations and triptolide was found to be four times more potent than celastrol in this aspect, while the malformations induced by triptolide were unspecific and mainly not interfering with swimming response. These results should be considered as highly relevant and strongly warn against the use of both compounds during pregnancy.

The third CHM under study was green tea. Minor behavioral effects were observed, and they were not following a concentration-response relationship. Although these results were difficult to interpret and pointed to no biological relevance of isolated minor effects, further investigations brought us to discover a clear neurodevelopmental adverse outcome after exposure to EGCG. Effective concentrations of EGCG were again in the low μM range and are well covered by the range of relevant concentrations in vivo after intake of EGCG food supplements (relevant concentrations discussed in Barenys et al. [4]. To the best of our knowledge, although there are several studies relating EGCG developmental exposure to disturbed cellular KEs [3,23], this is the first time that it is clearly related to a DNT AO. The link between the different observed KE in previous publications (AOP described in AOP-Wiki n. 486; https://aopwiki.org/; and updated in [23]) and the AO still needs clarification. In this regard, it is surprising that LGT and EGCG presented the same adverse effect in vitro in neurospheres [22] same KE: disturbed neural progenitor cell migration), while a different AO was observed in our study. Why in one case the exposure leads to hypoactivity and in the other to hyperactivity in zebrafish, while the same effects are observed at a cellular level, needs to be clarified, however our results support previous evaluations pointing to DNT effects of EGCG high-dose exposure [3,23].

From a methodological point of view, an added value of our study is that behavioral alterations were evaluated by using two different methods. In most of the cases (67 %; 4 out of 6) the results of both tests were not in agreement. Here, in all cases (100 %, 4 out of 4) the difference resulted from a less sensitive TER test in comparison to the L/D Transition assay. Yet it has to be pointed out that the number of test compounds in this study is low. The lower sensitivity of the TER test in comparison to the L/D Transition test might have a very simple explanation, because the exposure period for the TER test was shorter than for the L/D Transition assay. However, longer exposure periods for the TER test would lead to already described assay performance difficulties due to increased variability in results when the swim bladder inflates [15]. Contrarily, the low variability among results is a strong point of the L/D Transition assay compared to the TER test, although the lower number of larvae included per concentration in this test compared to the L/D Transition assay (6 vs. 10) can also have been a limitation for a precise comparison. Since the L/D Transition assay is being evaluated by the OECD Expert Group on Developmental Neurotoxicity (Project 4.124: New Guidance Document on Developmental neurotoxicity (DNT) in vitro assays), we contribute to the field by sharing an experimental design (Fig. 3) that leads to low variability in behavioral results, which could be of interest for future decisions on standardization/harmonization of protocols among laboratories. We also contribute by providing information that performing a TER assay in our conditions, or including thigmotactic behavior analysis to the L/D Transition assay does not bring any added value to performing the L/D Transition assay alone. Moreover, we share an open-access workflow in KNIME (with instructions on how to use it in Supplementary Material 3, https://hub.knime.com/-/spaces/-/latest/~yYh3203yR7_047XN/) for the evaluation of L/D Transition assay results which allows the performance of the test without the need to invest in high-cost equipment/software.

The inclusion of zebrafish L/D Transition assay into the IVB would contribute with the possibility to assess behavioral final Adverse Outcomes, but it would be interesting to test if the parallel inclusion of embryonic zebrafish neural tissue cultures to the battery could further help to interpret the relevance of behavioral results for the human species following a parallelogram approach, similar to the previously published rat-human comparison [5].

5. Conclusions

The main specific conclusions of our work are that 1) TM has no neurodevelopmental toxic potential in zebrafish embryos, 2) exposure to LGT and its two main compounds triptolide and celastrol during

pregnancy is highly inadvisable due to their developmental neurotoxic potential, 3) EGCG developmental exposure produces behavioral alterations in zebrafish embryos, and 4) L/D Transition assay was more sensitive than TER assay for the detection of neurodevelopmental toxicity of CHM and their single compounds. These conclusions lead us to state that, although the number of CHM products and single compounds tested is not enough to conclude it robustly, our results indicate that the ZEDTA assay in combination to L/D Transition assay could be a useful combination of alternative methods for DNT assessment of CHM products and their main compounds in the context of cell-based DNT IVB NAMs.

Funding

This project was funded by Fundació Bosch i Gimpera, Catalonia, Spain (project number: 300155). NGR received a scholarship from the "Carlos Antonio López" Scholarship Programme- BECAL of the Government of Paraguay, Paraguay. ET is a Serra Húnter Fellow, Serra Húnter Programme, Catalonia, Spain.

Ethical statement

Experiments were conducted in accordance with the Ethical Committee for Animal Experimentation of the University of Barcelona (CEEA). The maintenance of the adult zebrafish colony was accepted with license number 334/18 by the Department of Environment and Housing of the Generalitat de Catalunya.

Credit authorship contribution statement

Noelia Giselle Romero: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Validation, Writing – original draft. **Gerard Gutierrez:** Investigation. **Elisabet Teixidó:** Data curation, Formal analysis, Software, Validation, Supervision, Review. **Lu Li:** Resources, Review. **Jördis Klose:** Resources, Review. **Ping Chung Leung:** Resources, Review. **Salvador Cañigueral:** Resources, Methodology, Review. **Ellen Fritsche:** Conceptualization, Resources, Review. **Marta Barenys:** Conceptualization, Data curation, Formal analysis, Investigation, Funding acquisition, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank Laura Castro and the staff of the zebrafish facility (CCiTUB) for their excellent technical support, Dr. Gómez-Catalán for fruitful discussion and INSA-UB Maria de Maeztu Unit of Excellence (Grant CEX2021–001234-M) funded by MICIN/AEI/FEDER, UE for institutional support.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.reprotox.2023.108469.

References

[1] L. Almeida-Toledano, et al., 'Epigallocatechin gallate ameliorates the effects of prenatal alcohol exposure in a fetal alcohol spectrum disorder-like mouse model', Int. J. Mol. Sci. 22 (2) (2021) 1–24, https://doi.org/10.3390/ijms22020715.
[2] A. Bal-Price, et al., 'Putative adverse outcome pathways relevant to neurotoxicity', Crit. Rev. Toxicol. Inf. Healthc. (2015) 83–91, https://doi.org/10.3109/10408444.2014.981331.
[3] M. Barenys, et al., 'Epigallocatechin gallate (EGCG) inhibits adhesion and migration of neural progenitor cells in vitro', Arch. Toxicol. 91 (2) (2017) 827–837, https://doi.org/10.1007/s00204-016-1709-8.
[4] M. Barenys, S. Masjosthusmann, E. Fritsche, 'Is Intake of Flavonoid-Based Food Supplements During Pregnancy Safe for the Developing Child? A Literature Review', Curr. Drug Targets (2017) 196–231, https://doi.org/10.2174/1389450116666150804110049.
[5] J. Baumann, et al., 'Comparative human and rat neurospheres reveal species differences in chemical effects on neurodevelopmental key events', Arch. Toxicol. 90 (6) (2016) 1415–1427, https://doi.org/10.1007/s00204-015-1568-8.
[6] J. Blum, et al., 'Establishment of a human cell-based in vitro battery to assess developmental neurotoxicity hazard of chemicals', Chemosphere (2023) 311, https://doi.org/10.1016/j.chemosphere.2022.137035.
[7] W.Y. Chan, T.B. Ng, 'Adverse effect of Tripterygium wilfordii extract on mouse embryonic development', Contraception 51 (1) (1995) 65–71, https://doi.org/10.1016/0010-7824(94)00001-D.
[8] Chinese Pharmacopoeia Commission (2020) 'Chinese Pharmacopoeia Commission Pharmacopoeia of the People's Republic of China'.
[9] L. Dimene, et al., 'A cross-sectional study to determine the use of alternative medicines during pregnancy in the district hospitals in manicaland, zimbabwe', Afr. Health Sci. 20 (1) (2020) 64–72, https://doi.org/10.4314/ahs.v20i1.11.
[10] EFSA PPR Panel (EFSA Panel on Plant Protection Products and their Residues), et al., 'Development of Integrated Approaches to Testing and Assessment (IATA) case studies on developmental neurotoxicity (DNT) risk assessment', EFSA J. 19 (6) (2021) https://doi.org/10.2903/j.efsa.2021.6599.
[11] H.T. El-Borm, A.S. Abd El-Gaber, 'Effect of prenatal exposure of green tea extract on the developing central nervous system of rat fetuses; histological, immune-histochemical and ultrastructural studies', Saudi J. Biol. Sci. 28 (8) (2021) 4704–4716, https://doi.org/10.1016/J.SJBS.2021.04.084.
[12] E. Ernst, 'Herbal medicinal products during pregnancy: Are they safe, BJOG: Int. J. Obstet. Gynaecol. (2002) 227–235, https://doi.org/10.1111/j.1471-0528.2002.t01-1-01009.x.
[13] D.D. Glover, et al., 'Prescription, over-the-counter, and herbal medicine use in a rural, obstetric population', Am. J. Obstet. Gynecol. 188 (4) (2003) 1039–1045, https://doi.org/10.1067/MOB.2003.223.
[14] J. Guo, et al., 'Celastrol attenuates intrahepatic cholestasis of pregnancy by inhibiting matrix metalloproteinases-2 and 9', Ann. Hepatol. 18 (1) (2019) 40–47, https://doi.org/10.5604/01.3001.0012.7860.
[15] L. Guzman, et al., 'Evaluation of the effects of acetylcholinesterase inhibitors in the zebrafish touch-evoked response: quantitative vs. qualitative assessment', Environ. Sci. Eur. 32 (1) (2020) 145, https://doi.org/10.1186/s12302-020-00421-7.
[16] E. Hemminki, et al., 'A Survey on the use of Alternative Drugs During Pregnancy', Scand. J. Soc. Med. 19 (3) (1991) 199–204, https://doi.org/10.1177/140349489101900310.
[17] HMPC (2012) Community herbal monograph on *Camellia sinensis* (L.) Kuntze, non fermentatum folium. ⟨www.ema.europa.eu⟩.
[18] HMPC (2013) Assessment report on Camellia sinensis (L.) Kuntze, non fermentatum folium. ⟨www.ema.europa.eu⟩.
[19] Hollyer, T. et al. (2002) The use of CAM by women suffering from nausea and vomiting during pregnancy. ⟨http://www.biomedcentral.com/1472–6882/2/5⟩.
[20] P. Jun, et al., 'Traditional Chinese Medicine and Traditional Indonesian Medicine: A Comparative Review of Herbal Medicines Restricted in Pregnancy', Chin. J. Integr. Med. Springe Sci. Bus. Media Dtschl. GmbH (2021) 794–800, https://doi.org/10.1007/s11655-021-3487-7.
[21] Kennedy, D.A. et al. (2013) Herbal medicine use in pregnancy: results of a multinational study. ⟨http://www.biomedcentral.com/1472–6882/13/355⟩.
[22] J.ördis Klose, et al., 'Application of the adverse outcome pathway concept for investigating developmental neurotoxicity potential of Chinese herbal medicines by using human neural progenitor cells in vitro, AOP Framew. Causal Connects Toxicol. Mech. information', Cell Biol. Toxicol. 1 (2022) 3, https://doi.org/10.1007/s10565-022-09730-4.
[23] B.A. Kühne, et al., 'Application of the adverse outcome pathway to identify molecular changes in prenatal brain programming induced by IUGR: Discoveries after EGCG exposure', Food Chem. Toxicol. 170 (2022), 113506 https://doi.org/10.1016/J.FCT.2022.113506.
[24] R. De la Torre, et al., 'Epigallocatechin-3-gallate, a DYRK1A inhibitor, rescues cognitive deficits in Down syndrome mouse models and in humans', Mol. Nutr. Food Res. 58 (2) (2014) 278–288, https://doi.org/10.1002/mnfr.201300325.
[25] Leuenroth, S.J. et al. (2007) Triptolide is a traditional Chinese medicine-derived inhibitor of polycystic kidney disease. ⟨www.pnas.org/cgi/content/full/⟩.
[26] L. Li, et al., 'Systematic review of Chinese Medicine for miscarriage during early pregnancy', Evid. -Based Complement. Altern. Med. (2014) https://doi.org/10.1155/2014/753856.
[27] Y. Liu, et al., 'A review on central nervous system effects of gastrodin', Front. Pharmacol. Front. Media S. A (2018) https://doi.org/10.3389/fphar.2018.00024.
[28] L. Long, et al., 'The preventive effect of oral EGCG in a fetal alcohol spectrum disorder mouse model', Alcohol.: Clin. Exp. Res. 34 (11) (2010) 1929–1936, https://doi.org/10.1111/j.1530-0277.2010.01282.x.

[29] S. Masjosthusmann, et al., 'Establishment of an a priori protocol for the implementation and interpretation of an in-vitro testing battery for the assessment of developmental neurotoxicity', EFSA Support. Publ. 17 (10) (2020) https://doi.org/10.2903/sp.efsa.2020.en-1938.
[30] C.A. Mitchell, et al., 'The Botanical Safety Consortium: A public-private partnership to enhance the botanical safety toolkit'. Regulatory Toxicology and Pharmacology, Academic Press Inc,, 2022 https://doi.org/10.1016/j.yrtph.2021.105090.
[31] Muriana, A. et al. (2021) An Inter-laboratory Case Study to Harmonize Zebrafish Light-Dark Transition Test to Predict Developmental Neurotoxicity (WC11).
[32] National Centre for the Replacement, R. and R. of A. in R, Approaches for sampling blood in the mouse, covering non-surgical, surgical and terminal techniques. Blood sampling: Mouse, 2013. ⟨https://nc3rs.org.uk/3rs-resources/blood-sampling/blood-sampling-mouse⟩. Accessed: 29 March 2023.
[33] E. Nazik, G. Eryilmaz, 'Incidence of pregnancy-related discomforts and management approaches to relieve them among pregnant women', J. Clin. Nurs. 23 (11–12) (2014) 1736–1750, https://doi.org/10.1111/jocn.12323.
[34] H. Nordeng, G.C. Havnen, 'Use of herbal drugs in pregnancy: A survey among 400 Norwegian women', Pharmacoepidemiol. Drug Saf. 13 (6) (2004) 371–380, https://doi.org/10.1002/pds.945.
[35] OECD (2022) 'Guidance evaluation of data developmental neurotoxicity in vitrotesting'.
[36] S.J. Schnörr, P.J. Steenbergen, M.K. Richardson, D.L. Champagne, Measuring thigmotaxis in larval zebrafish, Behav. Brain Res. 228 (2) (2012) 367–374, https://doi.org/10.1016/j.bbr.2011.12.016.
[37] I.W.T. Selderslaghs, R. Bust, H.E. Witters, 'Feasibility study of the zebrafish assay as an alternative method to screen for developmental toxicity and embryotoxicity using a training set of 27 compounds', Reprod. Toxicol. 33 (2) (2012) 142–154, https://doi.org/10.1016/j.reprotox.2011.08.003.
[38] D.D. Shi, et al., 'Epigallocatechin gallate enhances treatment efficacy of oral nifedipine against pregnancy-induced severe pre-eclampsia: A double-blind, randomized and placebo-controlled clinical study', J. Clin. Pharm. Ther. 43 (1) (2018) 21–25, https://doi.org/10.1111/jcpt.12597.
[39] B. Souchet, et al., 'Prenatal treatment with EGCG enriched green tea extract rescues GAD67 related developmental and cognitive defects in Down syndrome mouse models', Sci. Rep. 9 (1) (2019) https://doi.org/10.1038/s41598-019-40328-9.
[40] A. Takei, et al., 'Meningoencephalocele Associated with Tripterygium wilfordii Treatment', Pediatr. Neurosurg. 27 (1) (1997) 45–48, https://doi.org/10.1159/000121224.
[41] L. Tang, et al., 'Consumption of Chinese herbal medicines during pregnancy and postpartum: A prospective cohort study in China', Midwifery 34 (2016) 205–210, https://doi.org/10.1016/j.midw.2015.11.010.
[42] TCM Treatment (2011) TCM China: Introduction To Gastrodia Tuber (tian ma). ⟨https://www.tcmtreatment.com/herbs/0-tianma.htm⟩ (Accessed: 29 March 2023).
[43] E. Teixidó, et al., 'Assessment of developmental delay in the zebrafish embryo teratogenicity assay', Toxicol. Vitr. 27 (1) (2013) 469–478, https://doi.org/10.1016/J.TIV.2012.07.010.
[44] V. Tiwari, A. Kuhad, K. Chopra, 'Epigallocatechin-3-gallate ameliorates alcohol-induced cognitive dysfunctions and apoptotic neurodegeneration in the developing rat brain', Int. J. Neuropsychopharmacol. 13 (8) (2010) 1053–1066, https://doi.org/10.1017/S146114571000060X.
[45] S. Wang, et al., 'Toxic effects of celastrol on embryonic development of zebrafish (Danio rerio, Drug Chem. Toxicol. 34 (1) (2011) 61–65, https://doi.org/10.3109/01480545.2010.494664.
[46] Work Plan TGP (2021) Work plan for the Test Guidelines Programme (TGP).
[47] H.D. Zhan, et al., 'The rhizome of Gastrodia elata Blume – An ethnopharmacological review', J. Ethnopharmacol. 189 (2016) 361–385, https://doi.org/10.1016/J.JEP.2016.06.057.
[48] H. Zhang, et al., 'Dietary epigallocatechin 3-gallate supplement improves maternal and neonatal treatment outcome of gestational diabetes mellitus: a double-blind randomised controlled trial', J. Hum. Nutr. Diet. 30 (6) (2017) 753–758, https://doi.org/10.1111/jhn.12470.
[49] Q. Zhang, et al., 'Evaluation of the effect of green tea and its constituents on embryo development in a zebrafish model', J. Appl. Toxicol. [Prepr.] (2022) https://doi.org/10.1002/jat.4380.