Toxicon 183 (2020) 51–60

Contents lists available at ScienceDirect

Toxicon

journal homepage: http://www.elsevier.com/locate/toxicon

Isolation of the *Tephrosia vogelii* extract and rotenoids and their toxicity in the RTgill-W1 trout cell line and in zebrafish embryos

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ARTICLE INFO

Keywords:
T. vogelii
Rotenoids
Cell-culture
Gill
Trout
Zebrafish

ABSTRACT

This study focused on identifying the rotenoids from the *Tephrosia vogelli* plant (fish-poison-bean), investigating the toxic potency of a crude *T. vogelii* extract and individual rotenoids (tephrosin, deguelin and rotenone) *in vitro* and *in vivo* and assessing the mode of action. A trout (*Onychorynhis mykiss*) gill epithelial cell line (RTgill-W1) was used to determine the cytotoxicity of rotenoids and effects on cell metabolism. Zebrafish (*Danio rerio*) aged from 3 h post fertilization (hpf) to 72 hpf were used for testing the developmental toxicity. The crude *T. vogelii* plant extract significantly decreased the cellular metabolic activity and was cytotoxic at lower concentrations (5 and 10 nM, respectively), while tephrosin, deguelin and rotenone showed these effects at concentrations $\geq$ 50 nM. The crude *T. Vogelli* extract had the highest toxic potency and induced adverse health effects in zebrafish including deformities and mortality at the lowest concentration (5 nM) compared to rotenone (10 nM) and deguelin and tephrosin (50 nM). These results indicate that the crude *T. Vogelii* extracts are highly potent and the bioactivity of these extracts warrant further investigation for their potential use to treat parasites in human and veterinary medicine and as a natural alternative to pesticides.

1. Introduction

Tephrosia vogelii (fish-poison-bean, locally named Utupa) is a small African tree of the family *Leguminosae*, which has bacteria associated with its roots that can fix atmospheric nitrogen and can be used to improve soil fertility (Nyirenda et al., 2011; Stevenson et al., 2012). The leaves of this plant contain known rotenoids, including rotenone, deguelin, and tephrosin, which are normal constituents in plants used in pesticidal preparations (Dzenda et al., 2007) or for protection against ecto- and endoparasites in humans and animals (Stevenson et al., 2012; Matovu and Olila, 2007). The pesticidal and insecticidal activities of *T. vogelii* have been suggested to be caused by rotenoids in the plant. It is however unclear whether there is an additive effect of the rotenoids, which may be contributing to the plant toxicity, as indicated in other plants (Nirma et al., 2012). In addition, the *T. vogelii* plant may also contain unknown bioactive compounds, which may contribute to the toxic effects, such as previously suggested (Deharo and Ginsburg, 2011).

The use of *T. vogelii* as a natural pesticide has been introduced into many different parts of the world. For example, in China it has been used to eliminate mosquito vectors of human and animal pathogens resistant to dichlorvos and chlorpyriphos (Li et al., 2015). The idea of using biologically active plant materials to control pests is also considered environmentally friendly, because they degrade more rapidly compared to synthetic pesticides and could be a useful alternative to commercial pesticides (Neuwinger, 2004; Sileshi et al., 2005; Sirrine and Shennan, 2012; Kamanula et al., 2011; Mafongoya and Kuntashula, 2005).

Rotenone is a natural plant toxin, which has been used by indigenous people for centuries in aquaculture, from harvesting fish, killing insects, as well as controlling fish populations and invasive species of fish as a fisheries management mechanism since the 1930s (Andel, 2000). Rotenone interferes with the cellular energy production by inhibiting NADH-ubiquinone reductase and thereby blocks mitochondrial electron transport (Singer and Ramsay, 1994). The resulting incomplete electron transfer within the mitochondrial respiratory chain leads to ATP depletion and in turn promotes the formation of ROS and thereby induces oxidative stress and apoptosis in cells. Moreover, rotenone can

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https://doi.org/10.1016/j.toxicon.2020.05.013
Received 27 December 2019; Received in revised form 12 April 2020; Accepted 19 May 2020
Available online 23 May 2020
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inhibit microtubule assembly through binding to tubulin leading to the inhibition of cell proliferation. In rotenone poisoned fish, reduced cellular uptake of blood oxygen results in increased blood partial oxygen pressure (pO_2), which results in blood acidosis, due to compensatory increase in cellular anaerobic metabolism and associated production of lactic acid (Fajt and Grizzle, 1998). Rotenone is reported to be extremely toxic in fish with the LC_{50} ranging from 1.94 μg/L for trout to 70 μg/L for the most resistant fish species (Fajt and Grizzle, 1993, 1998; Rach et al., 2009; Ling, 2003)

Rotenone is a registered pesticide in the U.S. under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), but the practice of its use is being discontinued (United States Environmenal Protection Agency, 2007). In contrast to US, the use of rotenone as pesticide is not approved by the European legislation (Reg. (EC) No 149/2008), because of various toxicological risks associated with its use, including high toxicity to fish and potential toxicity to honey bee colonies (Satta et al., 2008). Although rotenone is highly toxic to fish and insects (Dzenda et al., 2007), it has a relatively low toxicity to most mammals (Fukami et al., 1969), with lethal dose estimation for humans between 40 and 500 mg/kg (Ling, 2003). The mammalian toxicity of rotenone varies with species, sex (i.e. more toxic to females), mode of administration as well as type of formulation (Dzenda et al., 2007). Recently the effects of low chronic concentrations of rotenone were studied in adult zebrafish, whereby rotenone treatment reduced dopamine production and caused behavioural changes (Wang et al., 2017).

Deguelin has been reported to be the most abundant rotenoid in *T. Vogelli* extracts (Stevenson et al., 2012; Belmain et al., 2012). It is an enhancer of cell apoptosis and at 40 nM, deguelin was reported to be able to hinder cell propagation in malignant cell lines (Varughese et al., 2019), but also to hinder zebrafish embryonic development by targeting fibroblast growth factor receptor 4 (FGFR4), reducing cell proliferation at 100 nM and inducing apoptosis at 200 nM (Wu et al., 2016).

Together with deguelin, tephrosin was reported to be the most abundant in the leaves of the *T. Vogelii* plant (Matovu and Olila, 2007), however the exact mechanism of action of tephrosine has previously not been reported, albeit it is reported to be less toxic than deguelin (Belmain et al., 2012).

Although the toxicity of some rotenoids in fish has been extensively studied (Crosse and Fishery, 1984; Perry and Conway, 1977; Lindahl and Öberg, 1961; Hanisch et al., 2010; Xu et al., 2010; Rohan et al., 2015; Byrnes et al., 2018), there is insufficient data directly evaluating the biological activity of the crude *T. vogelii* extract compared to the individual rotenoids (rotenone, tephrosin and deguelin) (Kayange et al., 2019; Marango et al., 2017). The aim of this study was to identify and isolate major rotenoids in different specimens of *T.vogelii* and to determine the relative toxicity of the crude *T. vogelii* extract in itself, and compare the toxic potency with the individual rotenoids, rotenone, deguelin and tephrosin.

For the *in vitro* test, the trout (*Onychorynhis mykiss*) gill epithelial cell line (RTgill-W1) was used. This cell line has been extensively used in research (Dayeh et al., 2002; Lee et al., 2006, 2009; Brennan et al., 2012; Trubitt et al., 2015; Zeng et al., 2016) and a close correlation between RTgill-W1 cytotoxicity and acute fish toxicity has been demonstrated (Tanneberger et al., 2013). The mechanism, which underlies cytotoxicity is usually the disruption of the membrane integrity, therefore the use of *in vitro* models is recommended for better indication, and ranking of hazardous compounds (Bury et al., 2014).

Zebrafish (*Danio rerio*) was selected as a model organism for the *in vivo* test, as it is a well-known model for developmental toxicity assessment (Ton et al., 2006; Parng et al., 2007; Colwill and Creton, 2011). Zebrafish embryos from the blastula stage (3 hpf), when the cell fate specification onset takes place (Warga and Kimmel, 1990) were used and exposures continued up to 72 hpf, in order to test a range of doses and assess whether early embryonic exposures can impair development, which could lead to population decline.

2. Materials and methods

2.1. Sample collection

T. vogelii used in all bioassays was collected from Umbuji, Pagali and Bambi villages in Unguja Island, Zanzibar in Tanzania (Fig. 1), where it is cultivated by farmers for use as insecticide. The leaves were cut, and shade dried for three days just to reduce its freshness and avoid it to ferment before packed and transported to Norway, whereupon arrival they were stripped from branches and separately stored dry.

2.2. Extraction, analysis, and isolation of rotenoids

Air dried leaves of *T. vogelii* from all three sampling sites were grinded together on a cutting mill (Retsch GM200). The grinded samples (20 g) were extracted with CHCl3 (150 mL) in a Beckman centrifuge tube (250 mL) overnight on a shaker (Edmund Bühler SM25, Tübingen, Germany) and centrifuged at 9000 g for 10 min (J2-MC; Beckman Instruments, Palo Alto, CA, USA). The supernatants were combined and evaporated to dryness in vacuo using a rotary evaporator (Büchi R-200, Flavil, Switzerland). The residue was re-dissolved in 10 mL ethyl acetate and concentrated further to 1 mL. The concentrated sample was fractionated by flash column chromatography using a glass column (10 × 130 mm), slurry-packed with 40 g silica gel (100–200 mesh) in AcOEt and conditioned with $CHCl_3$. The column was eluted using a 12 stepwise gradient of $CHCl_3$/MeOH 1:0–0:1) (200 mL per step). Aliquots (1 mL) of the fractions were evaporated and re-dissolved in MeCN: H_2O. Substance concentrations in the fractions were determined by MS, and accordingly, the fractions 4, 5, 6 and 7 were combined for further analysis. They were concentrated to 2 mL by evaporation in vacuo at 40 °C and in the following purified by preparative high-performance liquid chromatography (HPLC). Preparative HPLC was performed on a Phenomenex Luna C_{18} 100 Å, 250 × 10 mm semi-preparative column using the equipment described for the quantitative analysis. Eluents consisted of isocratic mixtures of acetonitrile and water (55:45), at 4 mL/min. Fractions were collected manually by monitoring absorbance at 295 nm. Fractions from 10 consecutive runs, which contained rotenone, deguelin and tephrosin, were combined and purified again by preparative HPLC and collected in 0.5 mL fractions using a 206-fraction collector (Gilson, Middelton WI, USA). Quantification of rotenoids using liquid chromatography was performed on a Phenomenex Evo C_{18} 100 Å 2.6 μm 2.1 × 100 mm column, using a UFLC-UV system (Shimatzu, Japan) equipped with two LC-20AD pumps, a SIL 20A automatic sample injector, and a SPD 20A DAD detector. Separation was achieved using isocratic elution with water- MeCN (45:55). Rotenone, deguelin and tephrosin were quantified using external four-point calibration curves in the concentration range 1–250 μg/L. The quantification of rotenoids in the CH_3Cl extract were based on the sum of rotenone, deguelin and tephrosin. The limit of detection (LOD) and the limit of quantification (LOQ) were defined as the minimum concentration generating a signal-to-noise ratios (S/N) equal to 3 and 10, respectively.

2.3. Liquid chromatography coupled to high-resolution mass spectrometry

Fractions collected with preparative HPLC were identified by high-resolution mass spectrometry (HRMS). The instrument used was a Thermo Fisher Scientific (Waltham, MA, US) Vanquish UHPLC interfaced to a Q-Exactive Fourier-transform high-resolution mass spectrometer, equipped with a heated electrospray interface. A Phenomenex Evo C18 100 Å 2.6 μm 2.1 × 100 mm column was employed for separation. The mobile phases were 0.1 % HCOOH (A) and 95 % acetonitrile with 0.1 % HCOOH (B). The column was eluted isocratically for 12 min using 55 % B, followed by linear gradient elution to 90 % B in 0.1 min. After flushing the column with 90 % B for 3 min, the mobile phase composition was returned to starting conditions and equilibrated for 5 min. The mass spectrometer was run in positive ion full-scan mode in the

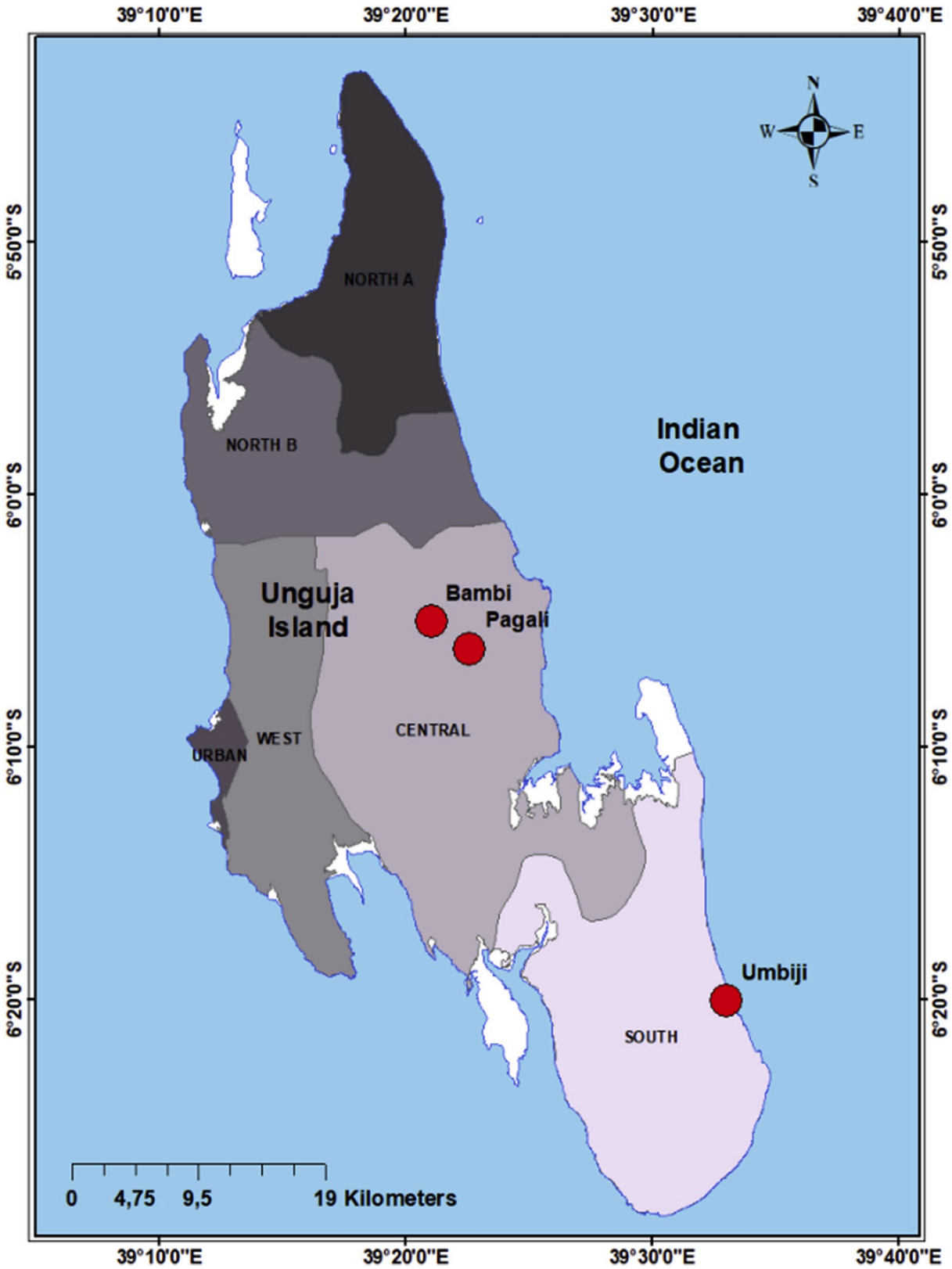


Fig. 1. A map of Unguja Island showing sampling sites (red circles). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mass range m/z 200–600. The mass resolution was set to 70,000 at m/z 200. The spray voltage was 2.8, the transfer capillary temperature was 280 °C, and the sheath and auxiliary gas flow rates were 35 and 10 units, respectively. The identity of target metabolites was verified using parallel reaction monitoring (targeted HRMS/MS) in the mass range m/z 200–2000. Precursor ions were selected in the quadrupole with an isolation width of 4 m/z and fragmented using higher-energy collision dissociation and normalized collision energy of 35 %. The mass

resolution for scanning of the product ions was set to 17,500. The Xcalibur™ 4.3 software (Thermo Fisher Scientific, Waltham, MA, US) was used for instrument control and calculation of mass errors and elemental compositions.

2.4. In vitro test

2.4.1. Cell culture and treatments

RTgill-W1 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and grown in Leibowitz's L-15 medium supplemented with 10 % FBS, 1 % glutamine and 1 % penicillin/streptomycin. The cells were cultured at 17 °C in a non-ventilated cell culture flask and sub-cultured 1:2 once a week using trypLE (Thermo Fisher, Carlsbad, CA, USA). For experiments, the cells were plated on black 98-well plates with clear bottom, with 40,000 cells/well the day before the experiments. The cell number gave full confluence at the day of experiment. The rotenoids were dissolved in DMSO and the final concentration of solvent in the cell culture was 0.1 % (Table S1). Final concentrations of 5, 10, 50, 100, 500 nM and controls containing the same amount of solvent were included in each experiment. The exposures lasted 48 h.

2.4.2. Cell metabolism and cytotoxicity

Metabolic activity of the cells was measured using the Alamar Blue assay according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, US). The dark blue oxidized form of Alamar Blue is reduced to a highly fluorescent form and the measured fluorescence intensity is thus proportional to the number of viable cells. The fluorescence (Ex/Em 555/585 nm) was quantified using Spectramx i3x plate reader (Molecular Devices, San Jose, Ca, USA).

The CellTox green is a non-toxic dye that stains DNA of cells with impaired membrane integrity and was used to assess cytotoxicity. The binding interactions with DNA produce a fluorescence signal that is proportional with cytotoxicity (necrotic and late apoptotic cells). CellToxTM Green Dye (1:2000) (Promega, Madison, WI, USA) was added to the cells as described by the manufacturer and fluorescence (Ex/Em 485/520 nm) quantified by Spectramx i3x plate reader, using the well scan function where 80 different points were read in each well.

The concentration at which 50 % of the cells showed a reduction in metabolic activity (IC_{50}) or induced 50 % cytotoxicity (EC_{50} values) were retrieved from the dose-response curves by non-linear regression analysis with dynamic fitting (Sigma plot, version 14.0 Systat Software Inc. San Jose, CA, USA) (Table 1).

2.5. Zebrafish embryo development test

AB wild-type strain zebrafish were maintained at 28 °C under a 14:10 light/dark photoperiod. Adult care and breeding were in accordance with the local protocols previously described in Hurem et al. (2018). To generate embryos, adult fish were placed in spawning tanks in the afternoon and spawned following the cessation of light (08:00) the next day. The embryos were collected at 09:00 and maintained in embryo media (60 μg/mL Instant Ocean® sea salts) until exposure. All embryo exposures were performed in 24-well plates (Nunc™, Thermo-Fischer Scientific) with 5 embryos aged 3 hpf at start of exposure in a 2 mL volume resulting in a total of 15–20 embryos per each rotenoid concentration in 4 technical replicate wells (0, 5, 10, 50, 100 and 500 nM) (Table S1). In order to determine the toxic effects of rotenoids on the development of the embryos and larva, a modified zebrafish embryo toxicity test was applied (OECD, 2013). Adverse health effects in exposed zebrafish embryos and larvae compared to control, i.e. death, deformities at different concentrations were assessed at 24, 48, and 72 hpf (corresponding to 21, 45 and 69 h of exposure). The larvae were euthanized at 96 hpf as the results at this time-point did not differ from the 72 hpf. The EC_{50} values were estimated using non-linear regression ($y = 100*x/(EC_{50}+x)$) (GraphPad Prism 8.2.1 Software Inc., La Jolla, CA, USA). The hatching time was assessed in live embryos from > 48 hpf and exposed to concentrations < 50 nM.

Table 1
Estimated IC_{50} (metabolic activity) and EC_{50} (cytotoxicity) values of rotenoid effects on the RTgill-W1 cell line. The values are retrieved from the data presented in Figs. 2 and 3 respectively.

	Metabolic activity IC_{50} (nM)		Cytotoxicity EC_{50} (nM)	
Time point	**24 h**	**48 h**	**24 h**	**48 h**
T. vogelii	19	4.8	198	6.3
Tephrosin	59	19	–	26
Deguelin	322	81	–	109
Rotenone	204	32	–	33

2.6. Statistical analysis

After arranging the data in MS Excel, the data was tested for normality and passed the normality criteria (Shapiro-Wilk test $p > 0.07$, Brown-Forsythe test $p > 0.1$ for the *in vitro* results, while the *in vivo* test results showed a normal quantile (QQ) plot, Shapiro-Wilk $p > 0.07$, Kolmogorov-Smirnov $p > 0.06$ and an overall probability of normal distribution of > 80 %). This data was further analysed using one-way-ANOVA, followed by the Holm Sidak's multiple comparisons test. The Sigma Plot (Version 14.0 Systat Software Inc. San Jose, CA, USA) and GraphPad Prism® Software (Version 8.2.1, Inc., La Jolla, CA, USA) were used for the statistical analyses and to make graphs. Statistical significance was assigned at $p < 0.05$.

3. Results

Rotenoids were isolated from leaves of *T. Vogelii*. Flash chromatography with silica of the ethereal extract, followed by preparative HPLC, provided rotenone, deguelin and tephrosin in sufficient quantities for biological evaluation in zebrafish embryos and in RTgill-W1 cell lines. LC–MS confirmed the structures and purities of these compounds isolated by this method. The MS/MS product ion spectrums were identical to authentic specimens of rotenone, deguelin and tephrosin (Fig. S1). The CH_3Cl extract from leaves of *T. Vogelii* was characterized by the presence of rotenoids including rotenone (1), tephrosin (2) and deguelin (3) (Fig. A1 and Fig. A2).

3.1. Metabolic activity and cytotoxicity in the RTgill-W1 cell line

The RTgill-W1 cell line showed a significantly reduced metabolic activity at 24 h after exposure to ≥ 5 nM of the crude *T. vogelii* extract, as well as ≥ 50 nM of tephrosin, deguelin and rotenone (Fig. 2). At 48 h, the metabolism was approaching zero activity after exposure to 10 nM of the crude *T. vogelii* extract, 100 nM of tephrosin and rotenone, and 500 nM of deguelin. The estimated IC_{50} values are shown in Table 1.

The RTgill-W1 cell line demonstrated a concentration-dependent increase in cytotoxicity at 24 h, which was significant at ≥ 10 nM for the crude *T. vogelii* extract, at ≥ 100 nM for tephrosin and rotenone and at 500 nM for deguelin. At 48 h, the cytotoxicity was increased up to 3-fold after exposure to ≥ 10 nM for the crude *T. vogelii* extract, ≥ 50 nM for tephrosin and rotenone and 500 nM for deguelin (Fig. 3). The estimated EC_{50} values are shown in Table 1.

The IC_{50} and EC_{50} values showed that the crude *T. vogelii* extract had the highest toxic potency, followed by tephrosin, rotenone and lastly deguelin in terms of both reduction of the metabolic activity and induction of cytotoxicity in RTgill-W1 cells (Table 1). After 24 h exposure, the Alamar Blue assay was more sensitive towards the rotenoids than the cytotoxicity assay. Except for deguelin, the two assays show somewhat similar effects after 48 h exposure, suggesting that the effects observed are related to cytotoxicity rather than to inhibition of the metabolic activation or proliferation (Table 1).

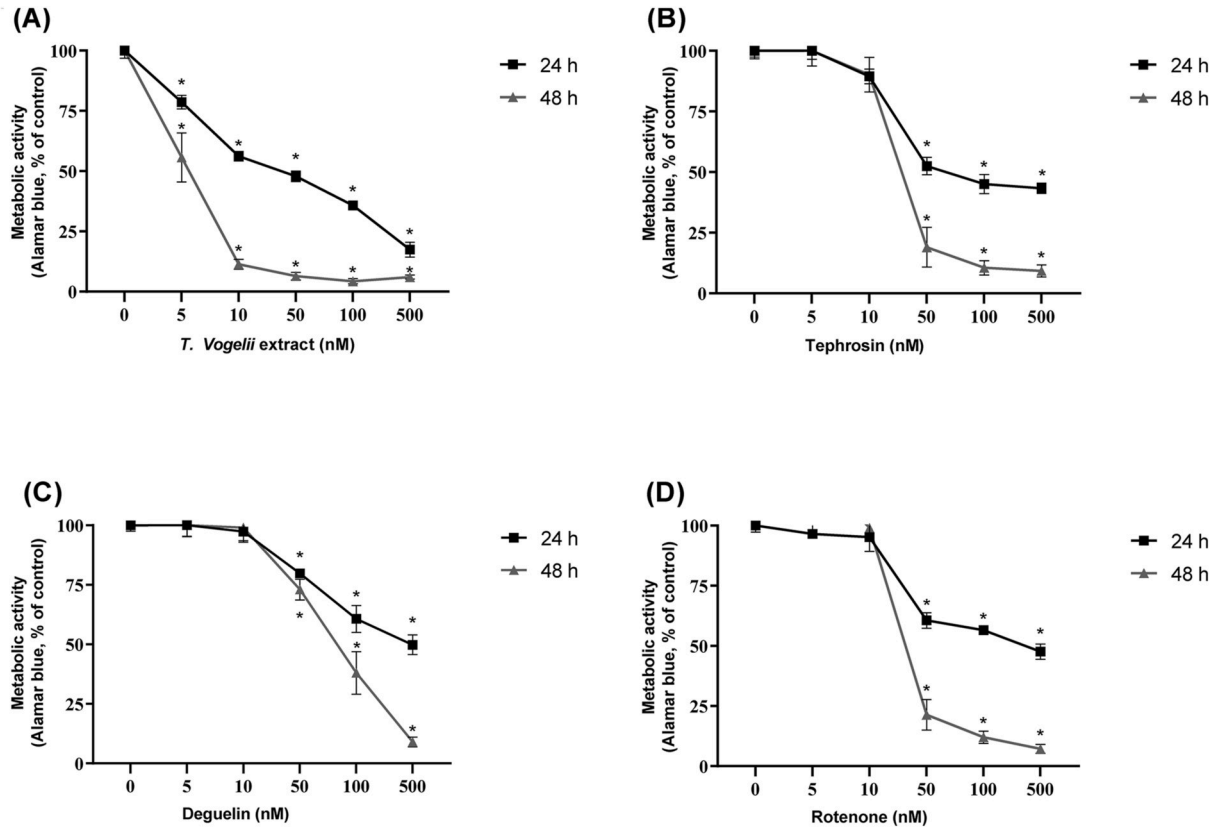


Fig. 2. Metabolic activity reduction by rotenoids in RTgill-W1 cells at concentrations and time points as indicated. The metabolic activity is measured by the Alamar Blue assay. The data represents mean ± SEM of four independent experiments. Asterisks indicate significant difference compared to control (one-way-ANOVA, Holm-Sidak's multiple comparisons test, $p < 0.05$).

3.2. *General toxicity* in vivo

Analyses of the developmental toxicity in zebrafish embryos from 24 hpf up to 72 hpf in the exposed groups showed significant differences in the higher concentrations compared to control in regard to the occurrence of mortality and deformities (Fig. 4, Table 2), while no significant differences were found for the hatching (assessed in live embryos, one-way ANOVA, $p > 0.4$).

The crude *T. Vogelii* extract showed the highest toxic potency in the zebrafish. The lowest concentration of the *T. vogelii* extract (5 nM) caused adverse health effects in 35 % embryos at 48 hpf ($p = 0.003$), but no adverse effects of this concentration were observed at 24 hpf compared to control. On the other hand, concentrations ≥ 50 nM in comparison to control caused deformities such as retardation in development at 24 hpf followed by death and 100 % mortality at 48 hpf (Figs. 4A and 5A).

Tephrosin and deguelin had the lowest toxic potency. Embryos exposed to tephrosin and deguelin in concentrations ≤ 10 nM showed no adverse health effects compared to control. Exposure to 50 nM tephrosin caused significant adverse health effects in 33 % of embryos at 48 hpf ($p = 0.03$), whereby developmental retardation and lack of hatching were most frequent and led to death at 72 hpf (Figs. 4B and 5B).

Similarly, but more severely affecting the embryos at 48 hpf compared to tephrosin, exposure to 50 nM deguelin resulted in significant deformities and mortality in 66 % of the embryos ($p < 0.0001$) compared to control, increasing with increased doses and mostly included malformations of the body axis or severe retardation in development followed by death (Figs. 4C and 5C). Although the exposure to 10 nM deguelin caused deformities, the frequency was not significantly different from control in this group. For both tephrosin and deguelin, 500 nM led to a 100 % mortality at 24 hpf.

Rotenone showed the second highest toxic potency. The exposure of zebrafish embryos to 5 nM rotenone caused no significant adverse effects compared to control. At 72 hpf, significant deformities were found after exposure to 10 nM, which were most frequently pericardial/cardiac oedema, while concentrations ≥ 50 nM caused adverse effects in more than 70 % of the embryos at 24 hpf and lead to > 90% mortality at 48 hpf (Figs. 4D and 5D).

The concentrations at the different assessment time points at which 50 % of the zebrafish embryos showed any detrimental effects of rotenoids to the development including death (EC_{50} values) were identified (Table 2).

4. Discussion

4.1. *General discussion*

This study identified three rotenoids isolated from the *T. vogelii* plant from Zanzibar and compared the toxic effects of individual rotenoids (tephrosin, deguelin and rotenone) including a crude *T. vogelii* extract *in vitro* and *in vivo*. This is the first study, which reports isolation of the rotenoids in the *T. vogelii* plant from Zanzibar, Tanzania and one of the first studies, which demonstrates rotenoids in African plants. The study also assessed dose dependent toxicological effects of individual known rotenoids and a crude *T. Vogelii* extract with potential unknown bioactive compounds. The occurrence of tephrosin, deguelin and rotenone in *T. vogelii* in this study is in accordance with previous studies (Stevenson et al., 2012; Belmain et al., 2012; Chen et al., 2014).

The metabolic activity as well as cytotoxicity were tested in the RTgill-W1 cell line, while health effects and death in early life stage zebrafish embryos were used to estimate the effective doses of rotenoids and to compare the toxic effects.

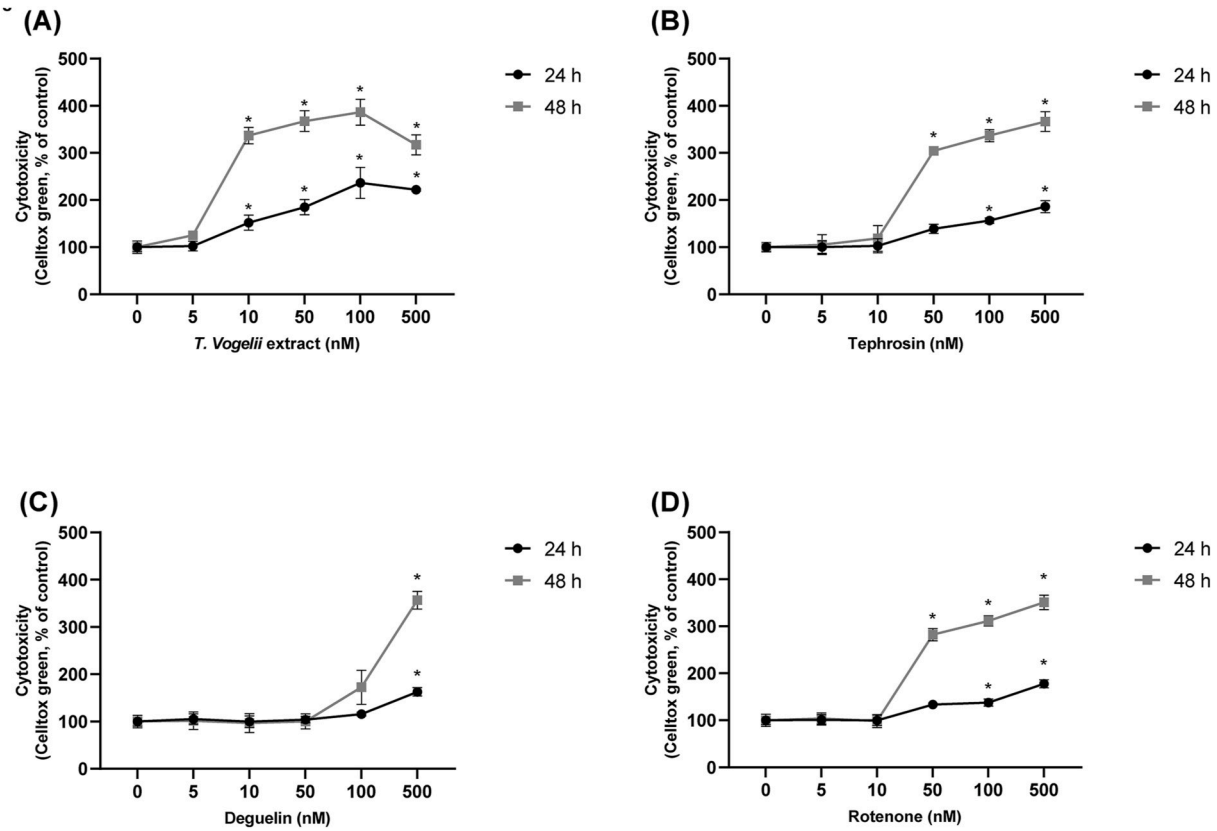


Fig. 3. Cytotoxicity induced by exposure to rotenoids in RTgill-W1 cells at concentrations and time points as indicated. Cytotoxicity; necrotic cells, were analysed by the Celltox Green assay. The data represent mean ± SEM of three independent experiments. Asterisks indicate significant difference compared to control (one-way-ANOVA, Holm-Sidak's multiple comparisons test, $p < 0.05$).

4.2. Rotenoids isolated from T. Vogelii *cause toxic effects in the RTgill-W1 cell line and zebrafish embryos, with the crude plant extract being the most potent*

The rotenoids isolated from *T. Vogelii* reduced the metabolic activity and were cytotoxic to the RTgill-W1 cell line in a time and concentration-dependent manner (Figs. 2 and 3). The IC_{50} and EC_{50} values showed that the crude *T. vogelii* extract was the most toxically potent compared to the other rotenoids in both the *in vitro* and *in vivo* tests.

After 24 and 48 h of exposure, the crude *T. Vogelii* extract had the highest inhibitory effect on the metabolic activity and was the most potent inductor of cytotoxicity in RTgill-W1 cells, followed by tephrosin, rotenone and lastly deguelin (Table 1).

The zebrafish embryo exposures demonstrated that the rotenoids caused no significant differences in the hatching time of zebrafish at lower concentrations and at early time points ($\leq$ 48 hpf), but the crude *T. Vogelii* extract and the individual rotenoids induced concentration-dependent adverse health effects, such as deformities leading to mortality at 72 hpf (Table 2, Figs. 4 and 5). Significant adverse developmental defects were observed at concentrations $\geq$ 5 nM for the crude *T. vogelii* extract, 10 nM for rotenone, and 50 nM for tephrosin and deguelin, concluding that the crude *T. vogelii* extract is most potent followed by rotenone, and then deguelin and tephrosin. The zebrafish embryo test results confirmed the test results in the RTgill-W1 cell line, that the crude *T. Vogelii* extract is the most toxically potent.

Compared to the test of metabolic activity and cytotoxicity in RTgill-W1, tephrosin was found to be more toxically potent than rotenone, while in the zebrafish embryo test, rotenone was more potent to induce developmental defects. The mechanisms behind these differences are unclear. The RTgill-W1 culture direct and continuous exposure could alter the cellular response, inducing cytotoxicity due to the lack of the continuous detoxification and clearance of compounds, which for example occurs in the whole body of the zebrafish. Therefore, the concentrations which might be less toxic *in vivo* could induce cytotoxicity *in vitro*. On the other hand, due to the fact that cytotoxicity plays an important role in a number of pathological processes in the body, including disrupted signalling pathways such as reported for deguelin (Wu et al., 2016), carcinogenesis, inflammation, modulation of the activity of free radicals, irritants, and genotoxins (Di Nunzio et al., 2017), the occurring subcellular mechanisms of action in lower concentrations might be more effective in causing damage in specific cell types in the embryonic fish. Further studies are warranted in order to determine why rotenone was more toxically potent in zebrafish embryos and if this could be attributed to differences in the metabolization of the rotenoids in other cells apart from gills, such as the liver, which is another toxicant target tissue (Melo et al., 2015).

In a toxicity study on *T. vogelii* rotenoids in beetles (*Callosobruchus maculatus*), it was demonstrated that rotenone had the highest toxic potency, followed by deguelin, which was significantly more active than tephrosin (Belmain et al., 2012). In a another experiment, zebrafish were exposed to doses of 30–100 nM rotenone, and the 100 nM dose arrested embryonic development between late gastrula and the tail-bud stage, while the 30–40 nM doses caused less severe effects with a higher fraction of nearly normally developing larvae (Byrnes et al., 2018). In our study, however, we demonstrated that a rotenone dose as low as 10 nM was highly toxic and caused visible deformities in more than 95 % of the larvae. The most frequent rotenone induced deformity observed was pericardial oedema. This deformity was reported in another study, which also demonstrated inhibition of antioxidant enzymes in zebrafish larvae (Melo et al., 2015). Apart from acute toxicity, chronic exposure of zebrafish to rotenone at 2 μg/L (5 nM) was reported to induce

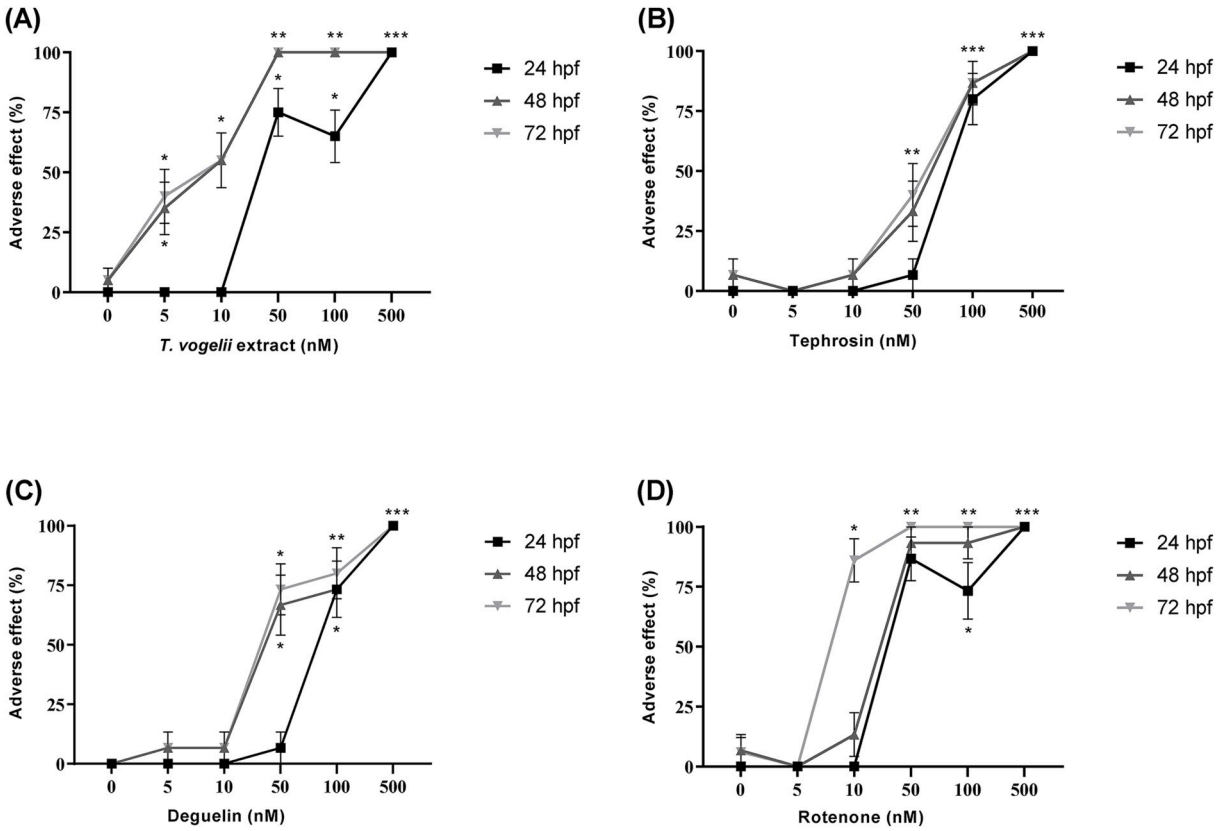


Fig. 4. Adverse health effects in zebrafish embryos exposed to *T. vogelii* extract (**A**), tephrosin (**B**), deguelin (**C**) and rotenone (**D**); i.e. occurrence of mortality and deformities, which hindered normal development up to 72 hpf. Data presented as mean + SEM and significance denoted with asterisks (one-way ANOVA, Holm-Sidak's multiple comparisons test, $p < 0.05$).

Table 2
Summary of estimated EC_{50} values for adverse health effects for zebrafish embryo development (exposed at the blastula stage) at the different assessment time points (n = 15–20 embryos for each exposure concentration and control).

	EC_{50} (nM)		
Time point	**24 hpf**	**48 hpf**	**72 hpf**
***T. vogelii* extract**	38.6	8.0	7.5
Tephrosin	85.5	53.2	48.5
Deguelin	93.4	37.5	31.7
Rotenone	31.8	21.7	7.6

neurotoxicity, as it is associated with acquiring Parkinson's disease in humans (Wang et al., 2017).

The results obtained for deguelin in the *in vivo* test, correspond to a previous study whereby 200 nM deguelin inhibited zebrafish embryonic development at ~19 hpf, while 500 nM caused a complete developmental arrest at 12 hpf, targeting fibroblast growth factor receptor 4 (FGFR4) (Wu et al., 2016). Deguelin has been suggested to have an anticancer effect and the capacity to trigger apoptosis may be the mechanism behind this trait (Wu et al., 2016; Fang and Casida, 1998). Together with deguelin, tephrosin, which is reported to be less toxic than deguelin *in vivo,* was reported to be the most abundant in the leaves of the *T. Vogelii* plant, however the exact mechanism of action of tephrosine is not clear (Stevenson et al., 2012; Gersdorff, 1931).

As previously reported for various other plants, combined rotenoids with a possible additive effect could be contributing to the greater toxic potency of the *T. Vogelii* plant (Nirma et al., 2012; Deharo and Ginsburg, 2011; Rasoanaivo et al., 2011; Mikami et al., 2018). This study shows that the rotenoids have different toxic potency and that they may also exert toxicity with different modes of action. Differences in biological activity among the rotenoids may be of importance for the identification of which rotenoid to apply for different treatments such as eradication of non-native species and to control pests and parasites as well cancer treatment.

5. Conclusion

Tephrosia vogelli originating from Zanzibar is a plant species with a potential to be used as a natural pesticide, in soil improvement therapy and an antiparasitic for animal diseases. In this study, the crude *T. vogelii* plant extract showed the highest toxic potency in both the trout gill epithelial cell line (RTgill-W1) and the zebrafish embryo test. The *T. Vogelii* extract decreased cellular metabolic activity, and induced adverse health effects at 5 nM, while rotenone showed these effects at concentrations ≥ 10 nM, followed by tephrosin and deguelin (≥ 50 nM). This result suggests potential additivity of the individual rotenoids, or presence of other bioactive compounds contributing to the toxic activity of the *T. Vogelii* plant extract and warrants further studies. The rotenoid concentrations needed to reduce the metabolic activity and induce cell death in the RTgill-W1 cell line corresponded very well to the concentrations that induced adverse health effects in the zebrafish embryo test. This is in accordance with several other studies were the RTgill-W1 cell line is considered to be a useful tool to investigate acute toxicity (Fischer et al., 2019). However, there were some differences when comparing the order of potency of the different rotenoids; *T. vogelii* extract > tephrosin > rotenone > deguelin in the RTgill-W1 cells and *T. vogelii* extract > rotenone > deguelin > tephrosin in the zebrafish embryo test. This further supports that the RTgill-W1 is a good model for acute toxicity, but other models are still needed to evaluate effects that involve more complex intracellular signalling and the whole organism. In the present

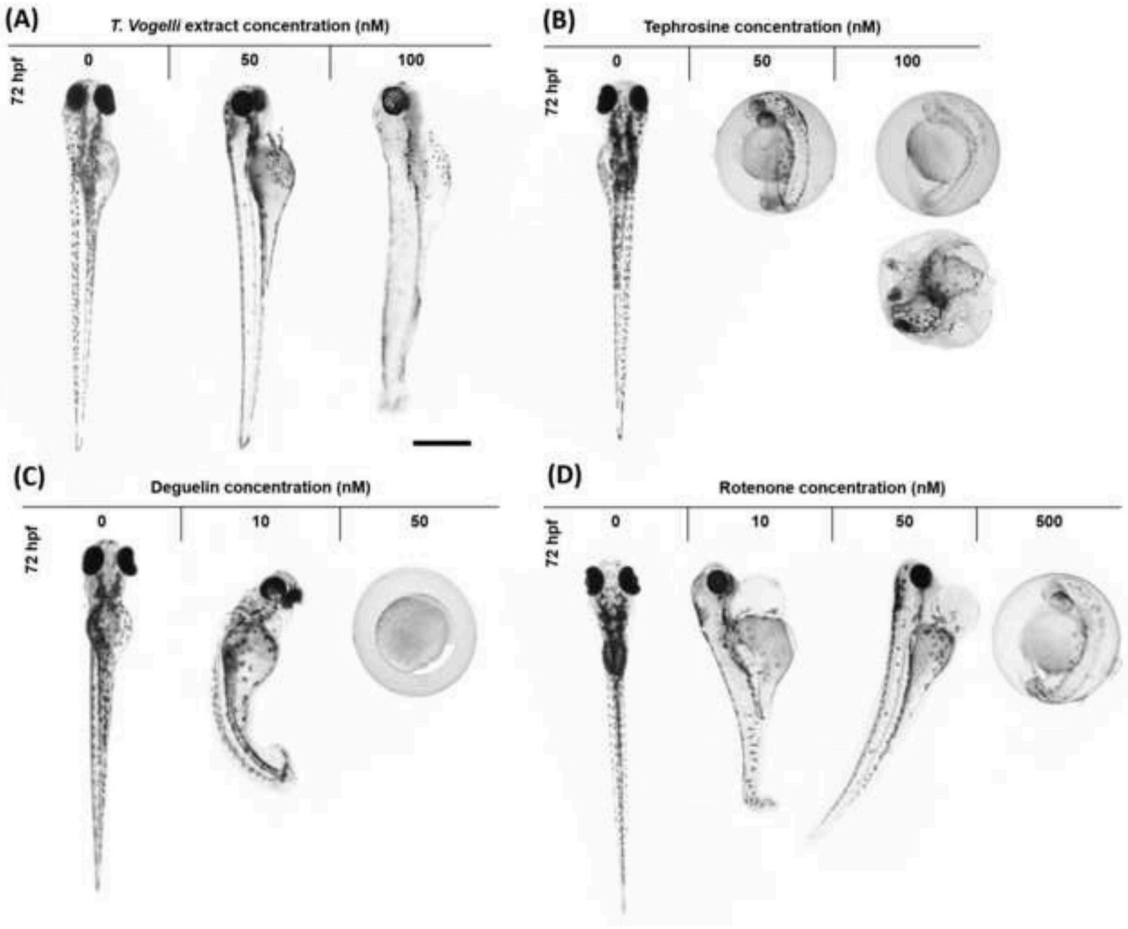


Fig. 5. Examples of deformities at 72 h post fertilization (hpf) in zebrafish exposed to different concentrations of (**A**) *T. vogelii* extract, (**B**) tephrosin, (**C**) deguelin and (**D**) rotenone; Scale bar = 500 μm.

study, we showed that the crude *T. Vogelii* plant extract had significantly higher toxic effects than the individual rotenoids, suggesting that the crude extract or any unknown compound in the extract, may be most efficient for pest and parasite control or for the eradication non-native species. Together, these results provide information of the toxicity of the *T. Vogelii* plant and rotenoids to aquatic biota and could further support regulations and decisions for their utilization in aquatic ecosystems.

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.

CRediT authorship contribution statement

Aziza H. Said: Formal analysis, Investigation, Writing - review & editing. **Anita Solhaug:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization, Supervision. **Morten Sandvik:** Methodology, Formal analysis, Investigation, Writing - review & editing, Visualization. **Flower E. Msuya:** Conceptualization. **Margareth S. Kyewalyanga:** Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition, Writing - review & editing, Project administration, Funding acquisition. **Aviti J. Mmochi:** Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Selma Hurem:** Conceptualization, Validation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Acknowledgements

This work is a part of the project TRAHESA (Capacity Building for Training and Research in Aquatic and Environmental Health in Eastern and Southern Africa) and was supported by the Norwegian Research Council funded through the Norwegian Agency for Development Cooperation (NORAD) and the Norwegian Programme for Capacity Building in Higher Education and Research for Development (NORHED) (project number Tan-13/0027). We also acknowledge the opportunity for presentation of this work Western Indian Ocean Marine sciences association (WIOMSA) during the 11th conferences as well as in the African Network for the Chemical Analysis of Pesticides (ANCAP) conference.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2020.05.013.

Ethical statement

The study was performed at the zebrafish laboratory of the Norwegian University of Life Sciences, Oslo, Norway. The unit is licensed by the Norwegian Animal Research Authority (NARA) (www.mattilsynet.no). The study was carried out under the regulations approved by the unit's animal ethics committee (Institutional Animal Care and Use

Committee/IACUC) following Norwegian laws and regulations controlling experiments and procedures on live animals in Norway. The animal experiments in this study were performed in accordance with the Norwegian Animal Protection Act (implemented EU Directive, 2010/63/EU). The larvae were euthanized at 96 hpf using an overdose of tricaine (MS-222, Sigma Aldrich), followed by rapid freezing at (−70 °C).

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