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Hazard Assessment of *Maerua subcordata* (Gilg) DeWolf. for Selected Endpoints using a Battery of *In Vitro* Tests

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Abstract

Ethnopharmacological Relevance: *Maerua subcordata* (Gilg) DeWolf is a medicinal and wild food plant growing mainly in east Africa. Especially its root tuber is widely used in traditional medicine to treat several infectious and chronic diseases but also in some toxicity implications like use as abortifacient.

Aim of the study: the present study applied *in silico* and *in vitro* tests to identify possible hazards of *M. subcordata* (fruit, leaf, root, seed) methanol extracts focusing on developmental toxicity.

Materials and methods: Ames test, estrogen receptor alpha (ERα) assay, aryl hydrocarbon receptor (AhR) assay, embryonic stem cell test (EST), and zebrafish embryotoxicity test (ZET) were employed. Besides, a Derek Nexus toxicity prediction was performed on candidate structures obtained from metabolomics profiling of the extracts using liquid chromatography coupled to multistage mass spectroscopy (LC/MS^n) and a MAGMa software based structural annotation.

Results: Glucosinolates, which degrade to isothiocyanates, and biogenic amines were among the candidate molecules identified in the extracts by LC/MS^n - MAGMa software structural annotation. Isothiocyanates and some other candidate molecules suggested a positive mutagenicity alert in Derek toxicity predictions. All the extracts showed negative mutagenicity in the Ames test. However, the Derek predictions also identified endocrine and developmental toxicity as possible endpoints of concern. This was further assessed using *in vitro* tests. Results obtained reveal that leaf extract shows AhR and ERα agonist activities, inhibited differentiation of ES-D3 stem cells into contracting cardiomyocytes in the EST ($p<0.001$) as well as inhibited hatching ($p<0.01$) and showed acute toxicity ($p<0.01$) in the ZET. Also, the fruit extract showed toxicity ($p<0.05$) towards zebrafish embryos and both

fruit and seed extracts showed AhR agonist activities while root extract was devoid of activity in all *in vitro* assays.

Conclusion: The leaf extract tests positive in *in vitro* tests that this may point towards a developmental toxicity hazard. The current evaluations did not raise concerns of genotoxicity or developmental toxicity for the fruit, seed and root extracts. This is important given the use of especially these parts of *M. subcordata*, in traditional medicine and/or as (famine) food.

Key words: CALUX assays, *in vitro, Maerua subcordata,* hazard, embryonic stem cell test, zebrafish embryotoxicity test

1. Introduction

Human exposure to natural ingredients of botanical origin, particularly *via* the use of dietary supplements and herbal medicines, continues to rise globally (Paine and Roe,2018; Roe *et al*., 2018). The general misconception that “natural” always means “safe” together with the improved scientific data and understanding of the beneficial health effects of bioactive substances in plant foods and medicinal products have boosted worldwide use of botanicals (Mahady *et al*., 2001; Kroes and Walker, 2004; Rietjens *et al*., 2008; Fürst and Zündorf,, 2015; Onyeji *et al*., 2017; Salehi *et al*., 2018; Sharifi-Rad *et al*., 2018). Thus, both consumers and industry have a rising interest towards the development of food products, having 'functional' properties or health benefits, derived from botanicals ranging from staple food sources like cereals, fruits and vegetables, to herbals used in traditional medicine (Schilter *et al*., 2003; Rietjens *et al*., 2008). However, users may also get exposed to possible health hazards arising from inherent constituents or contaminants of botanical products that may

lead to adverse health effects (Schilter *et al*., 2003; Fürst and Zündorf, 2015) and hence, there is a need to evaluate their hazards and safety.

Toxicity studies of botanicals are challenging and complicated due to, among others, costs, time, and animal usage as well as the complex mixtures of constituents that show variability in composition, (Kroes and Walker, 2004; Little *et al*., 2017). Still, an integrative testing strategy has been suggested tailored to hazard assessment using a panel of available alternative tests for critical end points that provides optimal *in silico* and *in vitro* filters (Piersma, 2013) aimed to assess hazards of botanical products especially when safety data are insufficient and as an early screen for toxicity alerts, particularly for less commonly known botanicals (Roe *et al*., 2018). Some suggested alternative methods include an *in silico* decision tree approach (Kroes and Walker, 2004; Little *et al*.,2017), a battery of *in vitro* tests such as assays for genotoxicity, CALUX reporter gene assays, the embryonic stem cell test (EST), and the zebrafish embryotoxicity test (ZET) (Hoogenboom *et al*., 2006; Besselink *et al*., 2015; Kroese *et al*., 2015; Piersma *et al*., 2013; Haedrich *et al*., 2018; Prinsloo *et al*., 2017).

A long history of use in traditional medicine of a botanical material is generally assumed as an indicator of lack of obvious toxicity (Trease and Evans, 2002). Yet, safety issues need scientific justification if use of a botanical material is to be considered. *Maerua subcordata* (Gilg) DeWolf (Capparidaceae), a medicinal and famine food plant, has numerous traditional uses but it lacks scientific data on its potential health hazards and health benefits. Its tuber is claimed as safe antimalarial remedy for children and pregnant women in northern Ethiopia, where collection of plant material in the present study was made, while reports from Somalia show that the same plant part is used as abortifacient (Samuelsson *et al*. 1985, 1991), which, if the later claim is true, may point at a possible adverse effect. Moreover, few reports reflect

that *M. subcordata* could be potentially toxic to humans and animals. It has been marked as a noxious shrub, which is potentially toxic to wildlife and cattle (Strauch and Eby, 2012). Human safety concern could be that uncooked fruits are perceived as toxic unless well boiled to render them to be non-toxic and edible (Morgan, 1981; Wigrup, 2005). Also, possible intoxication by quaternary ammonium compounds in the root was suspected, although such a problem was not detected along the area of the Omo river where the root is commonly used as a water clarifying agent; maybe because the quantities ending up in the water were too small to cause negative health effects (Göttsch, 1992). Scientific data on *M. subcordata* are lacking. A study with brine shrimp (*Artemia salina* Leach) showed that a methanolic root extract was inactive (LC_{50} > 1000 µg/ml) against the tested organism (Gakuya et al., 2004) which could be an indication of absence of obvious toxicity, at least to this species. Therefore, considering these ethno-toxicological claims, the aim of the present study was to investigate the possible hazards related to use of parts or extracts of *M. subcordata* given that various ethno-medicinal claims on *M. subcordata* may point towards its potential uses as source of herbal medicine or as functional or famine food. A Derek Nexus toxicity prediction, performed on candidate structures obtained from metabolomics profiling of the extracts, was used to direct selection of the *in vitro* tests to be employed. Different *in vitro* tests were applied to assess especially the genotoxicity, and endocrine and developmental toxicity hazards of *M. subcordata* (fruit, leaf, root, and seed) methanol extracts.

2. Materials and Methods

2.1. Plant material: collection, authentication, and processing

The fruit, leaf, root tuber, and seed parts of *M. subcordata* (Voucher number MG001/2007) were obtained from plants collected at 'lemlem' sub-district near shiraro (14.3970° N, 37.7743° E) in Northwest Tigray, Northern Ethiopia. The fruit, leaf, and seed parts were dried at room temperature while the tuber was first chopped into small pieces and dried in an

oven at 40 ºC. The dried plant materials were packed in plastic bags, transported to the Division of Toxicology, Wageningen University & Research, the Netherlands; where they were further processed and tested. To remove moisture and facilitate powdering, each dried plant part was splashed with liquid nitrogen and then ground using an analytical electric mill, mixed well, packed in capped plastic tubes, and stored at -80 ºC until further use.

2.2. Cell lines

ERα CALUX®, Cytotox CALUX®, and DR CALUX® cell lines were obtained from Bio Detection Systems BV (Amsterdam, The Netherlands). The pluripotent mouse ES-D3 cell line used for the EST was obtained from ATCC (ATCC® CRL 1934™, Wesel, Germany). The ERα CALUX cells are human bone osteosarcoma U2OS cells stably transfected with a firefly luciferase gene coupled to estrogen responsive elements (EREs) as a reporter gene to detect estrogen receptor agonists and antagonists (Besselink *et al*., 2015). The Cytotox CALUX cells are human osteosarcoma U2OS cells stably transfected with a reporter construct carrying a luciferase reporter gene under transcriptional control of a constitutive promoter. These cells have an invariant luciferase expression and were originally designed to study cytotoxicity (van der Linden *et al*., 2014). The ERα CALUX and Cytotox CALUX cells were cultured in Minimum Essential Medium alpha 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) (Gibco life technology™, Paisley, UK), supplemented with 7.5% foetal calf serum (FCS) (Gibco life technology™, Paisley, UK), and 1% nonessential amino acids (NEAA) (Invitrogen Corporation Breda, The Netherlands). The DR-CALUX cells are cells from a rat hepatoma (H4IIE) cell line, stably transfected with a construct containing the dioxin-responsive element sequence coupled to an AhR regulated luciferase reporter gene (H4I1E-*luc*) (Aarts *et al*.,1995; Bekki *et al*., 2009). The DR-CALUX cells were cultured in Minimum Essential Medium alpha (α-MEM))

148 (Gibco life technology™, Paisley, UK) supplemented with 10% FCS (Murk *et al*., 1996;
149 Bekki *et al*., 2009). The U2OS ERα, U2OS Cytotox, and H4IIE.luc (DR CALUX) cells were
150 incubated at 37ºC with 5% CO_2 in a humidified atmosphere and subcultured every 2–3 days,
151 using 0.05% trypsin-EDTA to detach the cells. The ES-D3 cell line used for the EST was
152 maintained in 25 cm^2 polystyrene cell culture flasks (Corning, the Netherlands), pre-coated
153 with 0.1% gelatine, in HyClone AdvanceSTEM™ Low Osmo Dulbecco's Modified Eagle
154 Medium (DMEM) (Fischer Scientific, Landsmeer, the Netherlands) supplemented with 15%
155 Fetal Bovine Serum (FBS) (ATCC, USA), 2 mM L-glutamine (Invitrogen, The Netherlands),
156 50 U/ml penicillin (Invitrogen) and 50 µg/ml streptomycin (Invitrogen). Cells were routinely
157 subcultured every 2–3 days using non-enzymatic cell dissociation solution (Sigma-Aldrich,
158 Schnelldorf, Germany) to detach the cells and were kept undifferentiated by the addition of
159 1000 U/ml murine Leukemia Inhibiting Factor (mLIF) (Sigma-Aldrich) (Kamelia *et al*.,
160 2017). The ES-D3 cells were incubated at 37 ºC and 5% CO_2 in a humidified atmosphere.

161 **2.3. Zebrafish eggs**

162 Eggs of zebrafish (*Danio rerio*) wild-type AB line, produced by group spawning were
163 obtained from the Animal Sciences Group of Wageningen University & Research; Carus-
164 ARF Bornse Weilanden 5, 6708 WG Wageningen, the Netherlands.

165 **2.4. Preparation of extracts from *Maerua subcordata***

166 Methanol extracts from dried powders of different parts - fruit, leaf, root tuber, and seed- of
167 *M. subcordata* were prepared by adding 3.4 ml methanol to 0.6 g of powdered plant material
168 followed by sonication for 10 min and centrifugation at 1000g for 15 min. The supernatant
169 was filtered using 0.2 µm polytetrafluoroethylene (PTFE)-filters (Whatman™, Germany) and
170 freeze-dried after the methanol was evaporated under a stream of nitrogen. Dried extracts
171 were stored at -80 ºC until used. For screening in the ERα CALUX and Cytotox CALUX

assays, the extracts were re-dissolved in DMSO:DMEM (1:2 v/v) while for screening in the DR-CALUX, EST, Ames test, and ZET, the extracts were re-dissolved in DMSO (Sigma–Aldrich).

2.5. Ames Test

The plate incorporation method of the Ames test was used to screen the mutagenicity of *M. subcordata* fruit, leaf, root, and seed extracts at concentrations of 15, 30, and 60 milligram dry weight (mgDW) per plate. The extracts were tested with and without metabolic activation as described by Ames *et al*., (1975) and with adaptions of the principles of the OECD 471 guideline (OECD, 1997). Briefly, 0.1 ml test extracts or 0.25% (v/v) DMSO (solvent control), 0.1 ml overnight bacterial (*Salmonella typhimurium,* TA98 or TA100) culture (1 x 10^8 cells/ml), and 0.3 ml S9 mixture (11-403L MUTAZYME 5% S9 from Aroclor 1254-induced male SD rat liver, reconstituted with 20 ml cold sterile water) or PBS (for tests without metabolic activation) were added into 2 ml top agar in test tubes. The contents were mixed and poured onto the surface of a glucose minimal plate. After solidification, plates were incubated upside down at 37 °C for 72 hr. At the end of the incubation, revertant colonies were counted. For each test, an average revertant colony count of three independent experiment was presented. A test plate was considered positive for mutagenicity if it produced a number of revertant colonies significantly higher (>twofold) than spontaneous revertant colonies on the solvent control plate, or otherwise considered a negative result.

2.6. Metabolomics profiling and toxicity prediction

M. subcordata methanol extracts (5 µl) were injected onto a C18 column (Phenomenex Luna 150 x 2 mm i.d., 3 µm Torrance, USA) of an Ultimate 3000 UPLC with a 60 minutes gradient and measured on a Q Exactive Orbitrap FTMS System (Thermo Scientific, Bremen) in both positive and negative mode. The raw data were converted into mzXML by the ProteoWizard

196 MSconvert software. Structural characterization was supported by 'MS Annotation based on
197 in silico Generated Metabolites' (MAGMa) (http://www.emetabolomics.org/magma) software
198 (Ridder *et al.*, 2012). Uploaded spectral data were automatically annotated with tiered trees of
199 *in silico* generated substructures of candidates, retrieved from a subset of compounds present
200 in Kegg. Candidate structures thought to be most likely present in the extracts were manually
201 selected from the MAGMa user interface. The identity of selected candidates was further
202 confirmed by LC-MS/MS (Shimadzu LC-MS 8040) based on multiple reaction monitoring
203 (MRM) using market available standard compounds such as stachydrine hydrochloride,
204 trigonelline hydrochloride (PhytoLab,Vestenbergsgreuth,Germany), glucolepidiin potassium,
205 glucobrassicin potassium (Extrasynthese,Genay Cedex,France). Likewise, although structures
206 of pyrrolizidine alkaloids (PAs) such as senecionine or senecivernine were in the tentative
207 identification, their presence in the extracts was ruled out by further LC-MS/MS analysis
208 using different standard mixture of PAs and with ≤ 1 µg/g limit of detection.
209 Toxicity prediction for query structures was performed using Derek Nexus, a software
210 program developed by Lhasa Ltd. (https://www.lhasalimited.org/) (Worth *et al.*, 2010). In
211 Derek, toxicity predictions are the result of two processes: (i) the program checks whether
212 any alerts in its knowledge base match toxicophores (substructures known or thought to be
213 responsible for a particular effect or toxicity) in the query structure and (ii) the reasoning
214 engine assesses the likelihood of a structure being toxic described by nine confidence levels
215 that were converted into three categories: active (certain, probable, plausible), equivocal, and
216 not active (doubted, improbable, impossible, open, and contradicted) (Worth *et al.*, 2010).
217 **2.7. Cell culture assays**
218 **2.7.1. DR CALUX assay (AhR assay)**
219 The AhR agonistic activities of *M. subcordata* methanol extracts were measured by the DR
220 CALUX assay (Aarts *et al.*,1995; Bekki *et al.*, 2009). H4IIE-*luc* cells were seeded in the 60

inner wells of a white 96-well view plate at a density of $3x10^4$ cells per well in 100 µL culture medium while 200 µl PBS was added to the outer 36 wells. After 24 hr incubation, the cells were inspected under a microscope and then exposed to 30 pM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Sigma–Aldrich), as positive control, 0.5%(v/v) DMSO as solvent control, and varying concentrations (0.024-12 gDW/L) of extracts in 100 µl exposure medium in the same plate. The final DMSO concentration in the incubations was 0.5% (v/v). After 6 hr or 24 hr exposure, medium was removed, cells were washed with ½ PBS (PBS half diluted with nano pure water), and exposed to 30 µl low salt lysing buffer (1.212 g Tris, 0.084 g dithiothreitol, 0.73 g 1,2-cyclohexylenedinitrilotetraacetic acid in a litre of nano pure water, pH 7.8) was added to each well to lyse the cells. Then, plates were covered with aluminium foil, placed on ice for 15 minutes and frozen overnight at -80 ^oC. For the luminescence measurement, plates were thawed at room temperature and luciferase activity per well in the lysate was measured in relative light units (RLU) using a luminometer (GloMax®-Multi Detection System–Promega) after the addition to each well of 100 µl flash mix (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4.7H_2O$, 0.1 mM EDTA, 2.0 mM dithiothreitol, 470 µM luciferine, 5.0 mM ATP; in a litre of nano pure water, pH 7.8). At least three independent experiments were conducted and results were expressed as fold induction of luciferase activity compared to the solvent control.

2.7.2. ERα CALUX and Cytotox CALUX assays

The cytotox CALUX assay was done in the same way and parallel to every ERα CALUX assay because Cytotox CALUX cells serve as indicators of cytotoxicity or non-specific increases in luciferase activity. In brief, cells were seeded in the 60 inner wells of a white 96-well view plate at a density of $1x10^4$ cells per well in 100 µl assay medium: DMEM/F12 supplemented with 7.5% FCS, and 1% NEAA. The outer wells were filled with 200 µl PBS and the plates were incubated for 24 hr. The next day, the assay medium was carefully

246 removed from the wells with cells, and replaced by 100 µl of fresh assay medium. The plates
247 were incubated for another 24 hr. Then 100 µl exposure medium containing the test samples
248 was added to each well resulting in 200 µl per well assay medium. After 24 hr exposure,
249 medium was removed, cells were washed with ½ PBS, lysed with low salt buffer, frozen
250 overnight and luciferase activity was measured in the same way as described above for the
251 AhR CALUX assay.

252 **2.7.3. ES-D3 cell viability and differentiation assays**

253 ***ES-D3 cell viability assay***

254 The cytotoxicity of the test extracts to ES-D3 cells was assessed by the WST-1 assay which
255 measures the extent of conversion of tetrazolium salts into a formazan dye by mitochondrial
256 enzymes in metabolically active cells. The ES-D3 cell viability assay was done as previously
257 described by Kamelia *et al*., 2017. Briefly, ES-D3 cells were seeded in 96-well plates
258 (Greiner Bio-One, Alphen a/d Rijn, the Netherlands) at a density of 2×10^5 cells/ml (one day
259 exposure) or 10^4 cells/ml (five days exposure) in 100 µl medium (without mLIF) and
260 incubated for one day to facilitate cell adherence. Then, cells were exposed to 100 µl of
261 medium with or without test extracts (3 replicates/concentration) and incubated for one day
262 or five days at 37 °C and 5% CO_2. All extracts were tested at a range of concentrations up to
263 1500 mgDW/L. The final concentration of DMSO was kept at 0.25% (v/v). After the
264 incubation period, 20 µl of WST-1 reagent (Roche Diagnostics, Mannheim, Germany) was
265 added to each well and cells were incubated for 3 h at 37 °C and 5% CO_2. Subsequently, the
266 absorbance of the formed formazan was measured at 440 nm using a SpectraMax M2
267 (Molecular Devices, Sunnyvale, USA). Cell viability was expressed as percentage of cell
268 viability compared to the solvent control which was set at 100%. Wells containing culture
269 medium without cells plus WST-1 reagent were used as a blank (background control) and

used to define 0% viability. At least three independent experiments were done for each test extract, and results were expressed as % viability compared to the solvent control.

ES-D3 cell differentiation assay

The inhibitory potential of test extracts on differentiation of ES-D3 cells into contracting cardiomyocytes was assessed using the ES-D3 cell differentiation assay of the embryonic stem cell test (EST) as described by Kamelia *et al*. (2017). The first step of the differentiation assay is the formation of embryoid bodies (EBs) *via* hanging drop culture in medium without mLIF. On day 0, droplets of 20 µl of a cell suspension (3.75×10^4 cells/ml), with or without test sample, were placed between the well borders on the inner side of the lid of a 96-well plate. The wells of the 96-well plate were filled with 250 µl of PBSto create an optimal humidity and to prevent evaporation of the hanging drops. Sterile caps of Eppendorf tubes were placed in the corner of the plates in order to prevent direct contact of the drops with the plate and the plate was subsequently sealed with Micropore tape (3M, Neuss, Germany) to prevent evaporation of the hanging drops. The hanging drop cultures were incubated for three days at 37 °C and 5% CO_2. On day 3, the resulting EBs were transferred to 60×15 mm bacteriological petri dishes (Greiner Bio-One) containing 5 ml medium, with or without test samples. The petri dishes were incubated for another 2 days at 37 °C and 5% CO_2 in the presence or absence of the test extracts. On day 5, the EBs were transferred to a 24-well plate (Corning) (1 EB/well), containing 1 ml medium with or without test extract. The EBs in 24-well plates were then incubated for 5 days at 37 °C and 5% CO_2. On day 10, the number of wells containing contracting cardiomyocytes was determined by visual inspection using a light microscope. The concentration of solvent in the medium was kept at 0.25%(v/v) DMSO. A solvent control and positive control of 5-fluorouracil (Sigma-Aldrich), final concentration 0.065 µg/ml (0.5 µM) were included in each experiment. This assay was considered valid if the solvent control had at least 21 out of 24 wells that contained contracting cardiomyocytes.

Inhibition of differentiation by the test extracts was presented as the fraction of total EBs plated in the 24-well plate that contained beating cardiomyocytes and at least three independent experiments were done for each test extract.

2.8. Zebrafish embryotoxicity (ZET) Test

The ZET assay was performed considering the principles of the OECD 236 guideline (OECD 236, 2013) and the method described by Beekhuijzen *et al*., (2015). Zebrafish (*Danio rerio*) wild-type AB line eggs produced *via* group spawning were sorted at about 1-3 hours post fertilization (hpf) in petri dishes using a disposable plastic pipette under the microscope. Since not all the eggs were fertilized, only the fertilized eggs with normal development stages were selected and placed in the incubator until exposure at 4 hpf. The test was done in 24-well plates. Each plate was used for exposure of embryos to one concentration of test extract. In every 24-well plate, 20 wells were filled with 2 ml of exposure medium (one concentration of test sample) while 4 wells were filled with 2 ml egg water for use as internal control. One embryo of the selected fertilized eggs was transferred to every well using a disposable plastic pipette. Methanol extracts of *M. subcordata* fruit, leaf, root, and seed parts were re-dissolved in DMSO and tested at final concentrations of 150, 375, 750, 1125, and 1500 milligrams dry weight per litre (mgDW/L) added from 600 gDW/L stock solution in DMSO, the final DMSO concentration was 0.25% (v/v). Plates were then sealed with self-adhesive film cover to prevent further evaporation of test compound throughout the exposure period (up to 96 hpf). Plates were incubated at 26 ºC with a photo period of 14 hours light:10 hours dark. To ensure the validity of each of three independent experiments, positive, negative, and solvent controls were included in every experiment. 3,4-Dichloroaniline (4.0 mg/L) (Sigma–Aldrich), egg water, and DMSO (0.25%) were used as positive, negative and solvent controls, respectively. The test was done in 5 days starting from the day of exposure (Day 0) and four follow up days (Days 1-4). At the end of the exposure period, acute toxicity (OECD 236,

2013) and general morphology scores (GMS) (Beekhuijzen *et al*., 2015) were recorded every 24hr. The GMS was determined based on the scoring system with 12 developmental endpoints described by Beekhuijzen *et al*., (2015) while acute toxicity (lethality) was determined based on a positive outcome in any of four apical observations: (i) coagulation of fertilized egg, (ii) lack of somite formation, (iii) lack of detachment of the tail-bud from the yolk sac, and (iv) lack of heartbeat (OECD 236, 2013). In this GMS system, there are increasing numbers of indicators for morphology scoring at every observation time point, from 24 hpf to 96 hpf. A lower GMS score indicates a higher degree of inhibition of the zebrafish embryo development in the ZET.

2.9. Data analysis

For each assay, at least three independent experiments were performed. Initial data analysis was done using Microsoft Excel 2016. Data from the ZET and EST assays were expressed as average scores while data from reporter gene assays were expressed as fold changes over the solvent control and for all assays, results are presented as mean values ± SEM. Statistical significance was assessed using IBM SPSS Statistics 23. Results were compared by one-way analysis of variance (ANOVA) followed by Tukey HSD Post Hoc tests. Results with $p < 0.05$ level of mean difference were considered significant. Graphing was made using GraphPad Prism software version 5.0 (California, USA). For the ZET and EST, nonlinear regression (curve fit) graphs of log(inhibitor) vs normalized response plots of mean and SEM were generated along with result sheets containing IC_{50} values (representing concentrations that decrease a given endpoint response by 50%).

3. Results

3.1. LC-MS based metabolomics profiling

LC/MS data and MAGMa software based structural annotation revealed glucosinolates, alkaloids and amines as the common secondary metabolites in all the analysed extracts

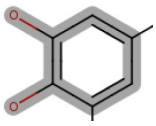
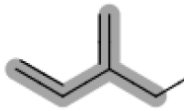
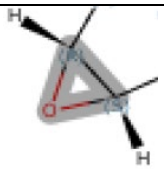
345 although other phytochemical groups such as phenolic compounds, terpenoids and iridoids
346 were also detected in the leaf extract. Further definite identification confirmed the presence of
347 glucosinolates (glucolepidin and glucobrassicin) and alkaloids or amines (stachydrine and
348 trigonelline) as well as the possible absence of pyrrolizidine alkaloids. Moreover, although
349 standard glucocapparin was not commercially available to confirm definite identification, the
350 LC-MS/MS-MRM data supported its presence in all extracts.

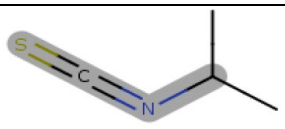
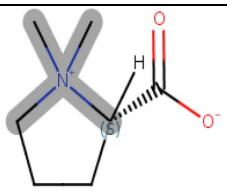
351 **3.2. Derek Nexus toxicity predictions**

352 Derek prediction on the tentatively identified candidates indicated several alerts (endpoints)
353 of toxicity hazards including genotoxicity and developmental toxicity with most of the
354 annotated candidates that reflected toxicity hazards being detected in the leaf extract. **Table**
355 **S1** (supplementary materials) provides a detailed overview of all candidate constituents that
356 fired structural alerts, **Fig S1** (supplementary materials) shows examples of candidates as
357 detected by LC/MS-MAGMa along with their toxicophores as detected by Derek predicition,
358 while **Table 1** shows some examples of target toxicophores in these toxicity predictions.
359 Candidate molecules for which their definite identification in the extracts could be confirmed,
360 such as glucosinolates and quaternary alkaloids both characterizing the Capparidaceae family
361 (Delaveau *et al*., 1973) to which the study plant belongs, are part of the Derek predictions.
362 For the glucosinolates, a carcinogenicity alert of open (no supporting or opposing evidence)
363 likelihood level was fired. Isothiocyanates which are breakdown products of glucosinolates,
364 raised a plausible alert for chromosome damage *in vitro* in mammalian systems, mutagenicity
365 *in vitro* in bacteria, and skin sensitisation in mammals. The quaternary amines were linked to
366 a plausible alert of causing irritation (of the eye and the skin) and an equivocal result for
367 causing skin sensitisation in mammals. This study foccussed on results of the Derek toxicity
368 predictions that raised concerns for genotoxicity and developmental toxicity, especially for

some candidates in the leaf extract (**Fig S1**), while in the other extracts isothiocyanates formed from glucosinolates may raise a concern for genotoxicity.

Table 1. Summary of some examples of toxicophores (shaded grey) in candidate constituents of *M. subcordata* methanol extracts

Alerts (Toxicophores)	**Description of Derek Nexus toxicity prediction**	
	Prediction ⇒Alert fired (Endpoint)	**Likelihood**
 Catechol	Carcinogenicity in mammal	Plausible
	Chromosome damage *in vitro* in mammal	Plausible
 Conjugated alkene*	Carcinogenicity in mammal	Plausible
	Hepatotoxicity in mammal	Plausible
 Epoxide*	Carcinogenicity in mammal	Plausible
	Chromosome damage *in vitro* in mammal	Plausible
	Chromosome damage *in vivo* in mammal	Plausible
	Developmental toxicity in mammal	Plausible
	Irritation (of the eye and skin) in mammal	Plausible
	Mutagenicity in vitro in bacterium	Plausible
	Mutagenicity in vitro in mammal	Plausible

	Mutagenicity in vivo in mammal	Plausible
 Isothiocyanate	Chromosome damage in vitro in mammal	Plausible
	Mutagenicity in vitro in bacterium	Plausible
	Skin sensitisation in mammal	Plausible
 Quaternary ammonium	Irritation (of the eye and the skin) in mammal	Plausible
	Skin sensitisation in mammal	Equivocal

* represent alerst from candidates detected only in the leaf extract

3.3. The Ames test

The Ames test indicated negative results (fold revertant colony count <2) (**Fig.1a-f**) for all the extracts at the tested concentrations while the positive controls, 4-nitroquinoline-N-oxide (0.01 µg/plate) and 2-aminoanthracene (2-AA, 1.0 µg/plate) (**Fig.1e&f**) show significant ($p<0.001$) positive mutagenicity. Because exposure to the higher concentrations (30 and 60 mgDW/plate) of the leaf extract with metabolic activation resulted in a fold change in colony counts of less than 1 compared to the solvent control, which may indicate cytotoxicity/ bactericidal effect, the plates were inspected under the microscope for any clearing or diminution of the background lawn (OECD, 1997) and the test for the leaf extract was repeated with lower concentrations (7.5, 15, and 30 mgDW/plate). Thus, results of the leaf extract also include a concentration of 7.5 mgDW/plate and no significant decline in fold reverentant colony count was observed for the 30 gDW/plate concentration in the repeated test (**Fig.1b**). Both the inspection and results of the repeated test showed no meaningful

difference in fold change in colony counts apart from random variation indicating that the absence of obvious mutagenicity was not due to cytotoxicity. The Ames test was first done with TA98 strain and then similar tests with TA100 at the highest non-cytotoxic concentration for all the extracts (30 gDW/plate) also resulted in negative results for mutagenicity in this strain (**Fig.1f**).

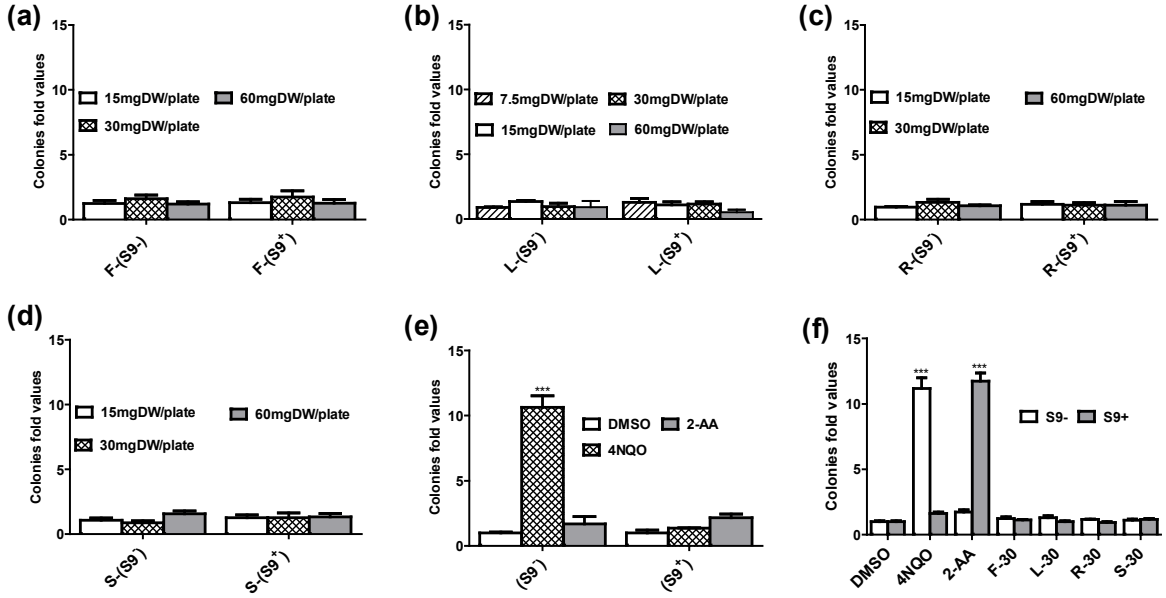


Figure 1. Ames test performed with (S9+) and without (S9-) metabolic activation, with *Salmonella typhimurium* TA98 for (a) fruit(F), (b) leaf(L), (c) root(R), and (d) seed(S) extracts of *M. subcordata*, using (e) DMSO as solvent control, 4-nitroquinoline-N-oxide (4NQO, 0.01 µg/plate) and 2-aminoanthracene (2-AA, 1.0 µg/plate) as positive controls, and (f) showing results of a similar test for the extracts (30 gDW/L) and controls done with *S. typhimurium* TA100. Results are described as fold values of average revertant colonies against the solvent control (DMSO) and are presented as mean ± SEM from three experiments. Asterisks show a significant difference from the solvent control: ***$p < 0.001$.

3.4. DR CALUX assay (AhR CALUX assay)

AhR agonist activities of *M. subcordata* extracts were evaluated by the *in vitro* DR CALUX assay as these endpoints were previously reported to play a useful role in an *in vitro* battery of tests to detect developmental toxicity (Kamelia *et al*., 2017; Kamelia *et al*., 2018a,b). Results show that upon 6 hr exposure, the fruit, leaf, and seed extracts increased luciferase activity in a concentration dependent manner while the root extract was almost lacking activity in the concentration range tested (**Fig.2a**). A second set of experiments was performed in which luciferase activity from cells exposed for different time periods (6 hr and 24 hr) was quantified in order to check if the AhR induction was transient or persistent. The results from the 6 hr exposure reproduced the previous results while results from the 24 hr exposure showed that all extracts had little or no effect on luciferase activity (**Fig.2b&c**) although the extent of fold induction by TCDD from 24 hr was increased nearly twofold as compared to the 6 hr exposure.

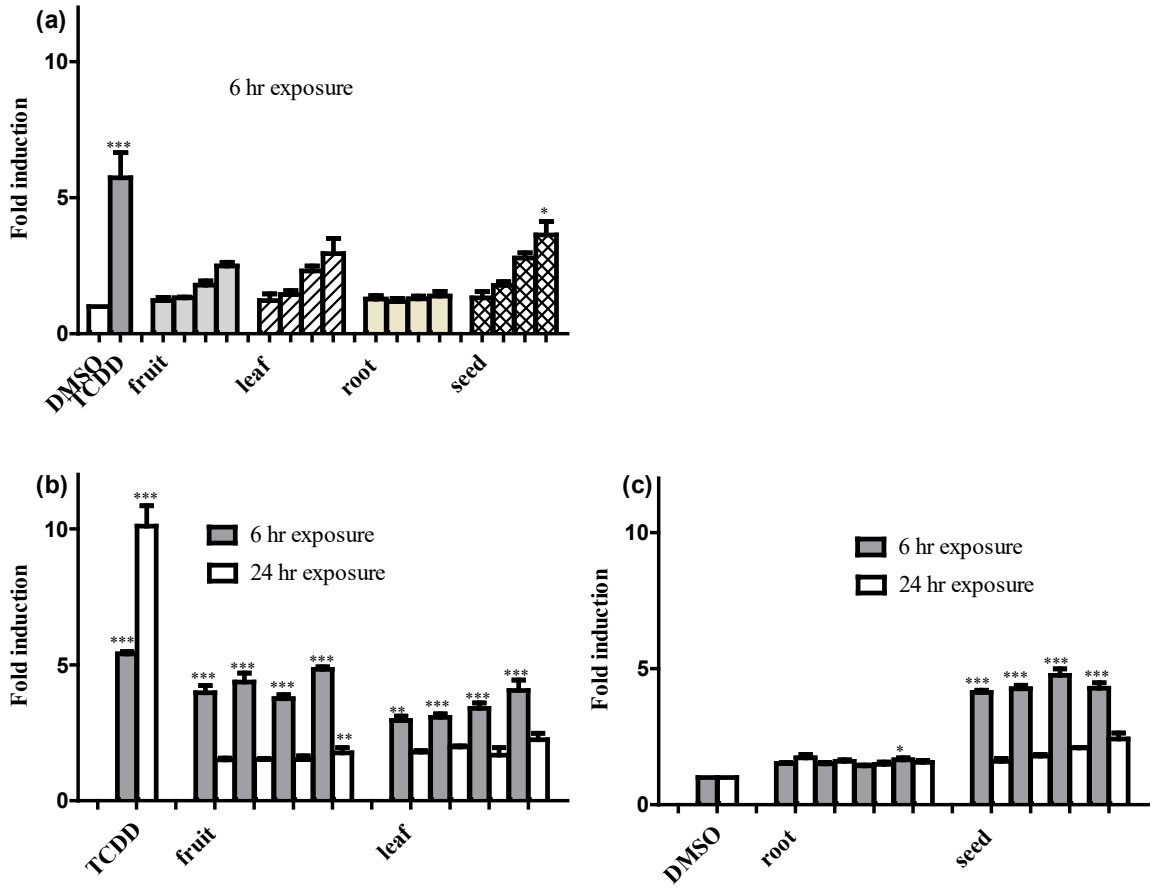


Figure 2. Induction of luciferase activity in rat hepatoma H4IIE-*luc* cells by TCDD at 30 pM (positive control) and different concentrations of fruit, leaf, root, and seed extracts of *M. subcordata* after (a) 6 hr exposure to the extracts at concentrations of 24, 120, 600, and 3000 mgDW/L; (b and c) after 6 hr and 24 hr exposures to the extracts at concentrations of 1.5, 3, 6, and 12 gDW/L. Results are described as fold induction of luciferase activity compared to 0.5% (v/v) DMSO as a solvent control and data are presented as mean ± SEM from at least three independent experiments. Asterisks show a significant difference from the solvent control: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.5. ERα CALUX and Cytotox CALUX assays

ERα CALUX assay results (**Fig.3**) for *M. subcordata* methanol extracts show that the leaf extract at a concentration of 30 gDW/L increased ERα mediated induction of luciferase

activity with an average fold induction of 4.5±0.1 compared to the solvent control. This effect was biologically relevant (fold induction ≥2) (Yun and DasGupta, 2014) but statistically not significant ($p>0.05$) (Martı´nez-Abraın, 2008). In the same experiment, 17-β-estradiol (E2) at 5 pM displayed an average fold induction of 27.5±2.5 ($p<0.0001$). The fruit, the root, and the seed extracts did not induce luciferase activity in the U2OS ERα assay. **Fig.3** also shows results of parallel screens in the cytotox CALUX assay which reflect luciferase activity similar to the solvent control implying the absence of any cytotoxicity or non-specific changes in luciferase activity as a result of exposure to the tested samples.

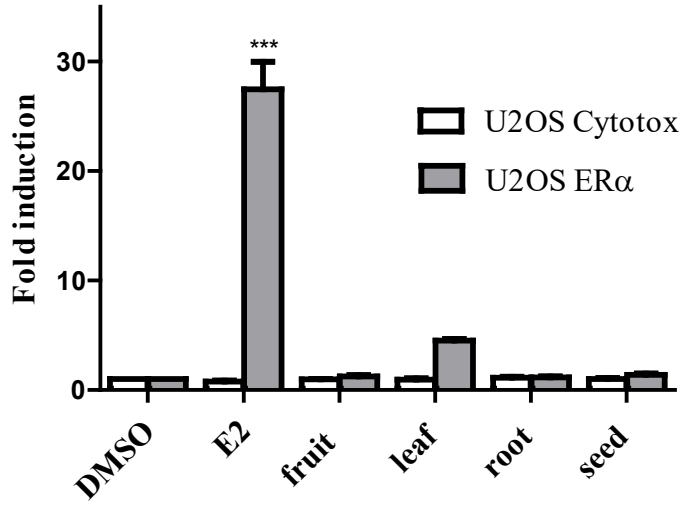


Figure 3. Induction of luciferase activity in U2OS ERα (shaded bars) and U2OS cytotox cells (white bars) cells after 24 hr exposure to 5 pM 17-β-estradiol (E2) (positive control) and *M. subcordata* fruit, leaf, root, and seed extracts at 30 gDW/L. Results are expressed as fold induction of luciferase activity compared to 0.5%(v/v) DMSO as solvent control. Data are presented as mean ± SEM from three independent experiments. Asterisks show a significant difference from the solvent control: ***$p < 0.001$.

449 **3.6. ES-D3 cell viability and cell differentiation assays**

450 Results (**Fig.4a-d**) on the effects of *M. subcordata* extracts on the viability of ES-D3 cells
451 showed that all extracts exhibited relatively similar effects (>89% cell viability) as the
452 solvent control 0.25%(v/v) DMSO, upon both one day and five days exposure, up to the
453 highest tested concentration (1500 mgDW/L). As concentrations showing viability above
454 80% are generally accepted for further assays (Li *et al*., 2016; Queiroz *et al*., 2017),
455 concentrations up to 1500 mgDW/L of all extracts were considered acceptable in the present
456 study to further characterise effects on ES-D3 cell differentiation. **Fig.4a-d** also show the
457 effects of the extracts on differentiation of ES-D3 cells into contracting cardiomyocytes,
458 revealing that only the leaf extract significantly ($p<0.001$) inhibited ES-D3 cells
459 differentiation at concentrations ≥750 mgDW/L. 5-fluorouracil (0.5 μM), used as a positive
460 control, showed 85.42±5.51% inhibition of ES-D3 cells differentiation. Conversely, no
461 inhibitory effect was shown by the root extract while the fruit and the seed extracts caused
462 slight effects (26.7% and 16.7% affected fractions, respectively) of inhibition at the highest
463 concentration. The effect was statistically significant ($p<0.05$) for the seed extract but not for
464 the fruit extract although the later seem to show more effect may be because results of
465 repeated experiments of the fruit extract had relatively higher variability compared to those
466 from the seed extract. Only for the leaf extract an IC_{50} value (209 mgDW/L) could be derived
467 from these data, representing the concentration that inhibits ES-D3 cell differentiation into
468 beating cardiomyocytes by 50%. As the extract was non-cytotoxic at the concentrations
469 tested, cytotoxicity may not be expected to contribute to this inhibition of ES-D3 cell
470 differentiation.

471

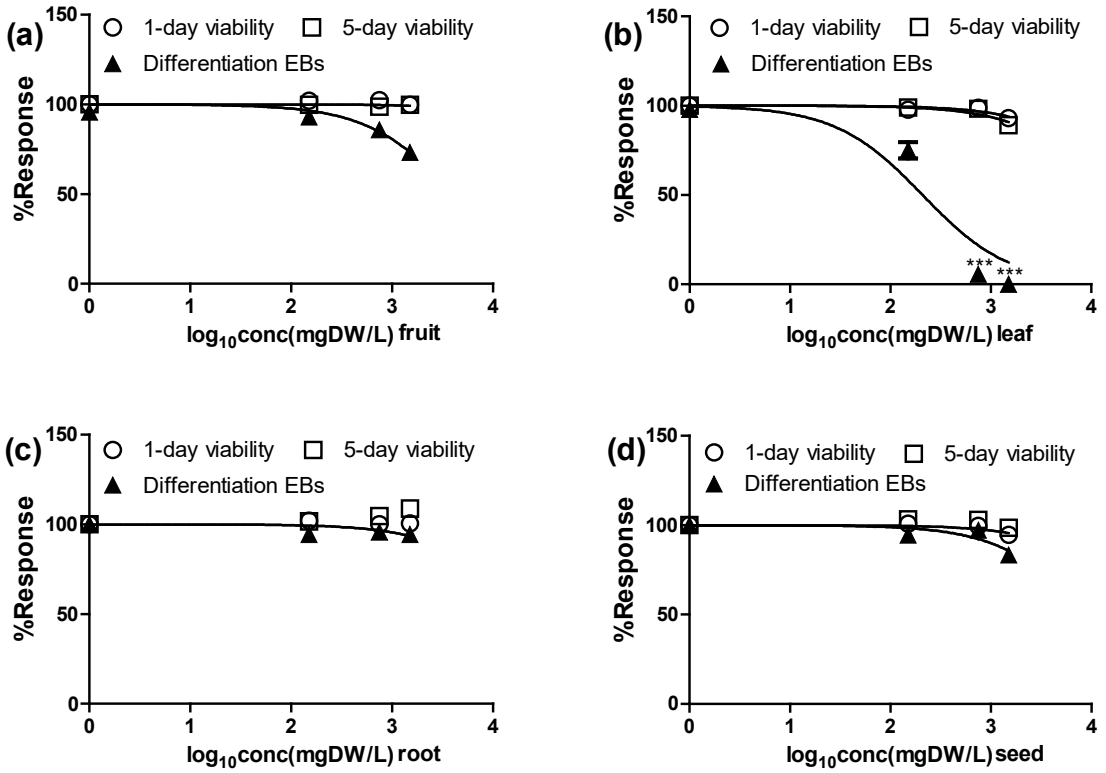


Figure 4. Effects of increasing concentrations (150-1500 mgDW/L) of *M. subcordata* fruit, leaf, root, and seed extracts on ES-D3 cells viability upon 1 day (black circles) and 5 days (black squares) exposures and on inhibition of ES-D3 cells differentiation into contracting cardiomyocytes upon a 10 days exposure (filled black triangles). Results represent mean ± SEM from at least three independent experiments. Inhibition of ES-D3 cell differentitaion at concentrations where cytotoxicity is not yet observed indicated *in vitro* developmental toxicity. Asterisks show a significant difference from the solvent control: ***$p<0.001$.

3.7. Zebrafish embryotoxicity test (ZET)

To further assess the *in vitro* acute toxicity and/or developmental toxicity potential of *M. subcordata* extracts, the effects of the extracts on lethality and/or on the general morphology of developing zebrafish embryos were determined by the ZET. Results (**Fig.5a-d**) show that for all concentrations of the tested extracts, the lethality and GMS records were almost

overlapping and that in both records, the fruit extract at the highest concentration (1500 mgDW/L, $p<0.05$) and the leaf extract at 750 mgDW/L($p<0.05$) and 1500 mgDW/L($p<0.01$) induced statistically significant effects as compared to the internal control (IC) or negative control (egg water) and the solvent control (0.25%DMSO) whereas the root and the seed extracts did not show statistically significant effects. 3,4-dichloroaniline (4.0 µg/ml), used as a positive control, induced a lethality of 98 ±1.67%. Death of the zebrafish embryos occured either before 24 hpf or immediately after hatching. Besides, occasional cases of malformation of the heart (abdominal and/or pericardial oedema, irregular shape due to edema or aplasia, abnormal heartbeat) (Beekhuijzen *et al*., 2015) in embryos exposed to all test extracts (0 to 5% incidence) but also to 0.25%(v/v)DMSO (3.33% incidence) as well as few curved body/tail in dead embryos exposed to the fruit (1.67% incidence) and the seed (3.33% incidence) extracts were observed. **Fig.6a-g** show examples of normal and abnormal body morphology of some zebrafish embryos exposed to extracts of M. *subcordata*.

Because Selderslaghs *et al*., (2009) reported that DMSO, even at 0.5% (v/v), caused adverse effects on the development of zebrafish embryos and proposed 0.25% (v/v) DMSO (mean% larvae affected <10; not significantly different from controls) as the no observed effect concentration of DMSO, the ≤5% sub-lethal abnormalities (signs of malformation of the heart) (**Fig.6c-d**) observed in the present study were considered not significantly different from the control (0.25% DMSO) and not biologically relevant. Besides, upon exposure to the fruit and seed extracts, some of the embryos that hatched during the time of observation were suffering from stiffness and staggered mobility. Few of the embryos that died immediately after hatching appeared to retain body shape similar to what they were inside the chorion while those that survived maintained normal body shape. Thus, the few cases of curved body/tail (**Fig.6e-g**) in dead embryos exposed to the fruit and seed extracts may be considered as symptoms of acute toxicity rather than a sign of abnormality since survivors

did not show these effects. Also, despite that the fruit, root, and seed extracts had little or no influence on hatchability, the leaf extract showed a significant ($p<0.01$) effect at concentrations ≥750 mgDW/L (**Fig.5b**). An IC_{50} value (249 mgDW/L) could be derived from these data only for the leaf extract, representing the concentration that inhibit hatching by 50% and implying that the IC_{50} values of the other extracts would be beyond the tested concentration range and hence reflecting little or no inhibitory effects on hatching.

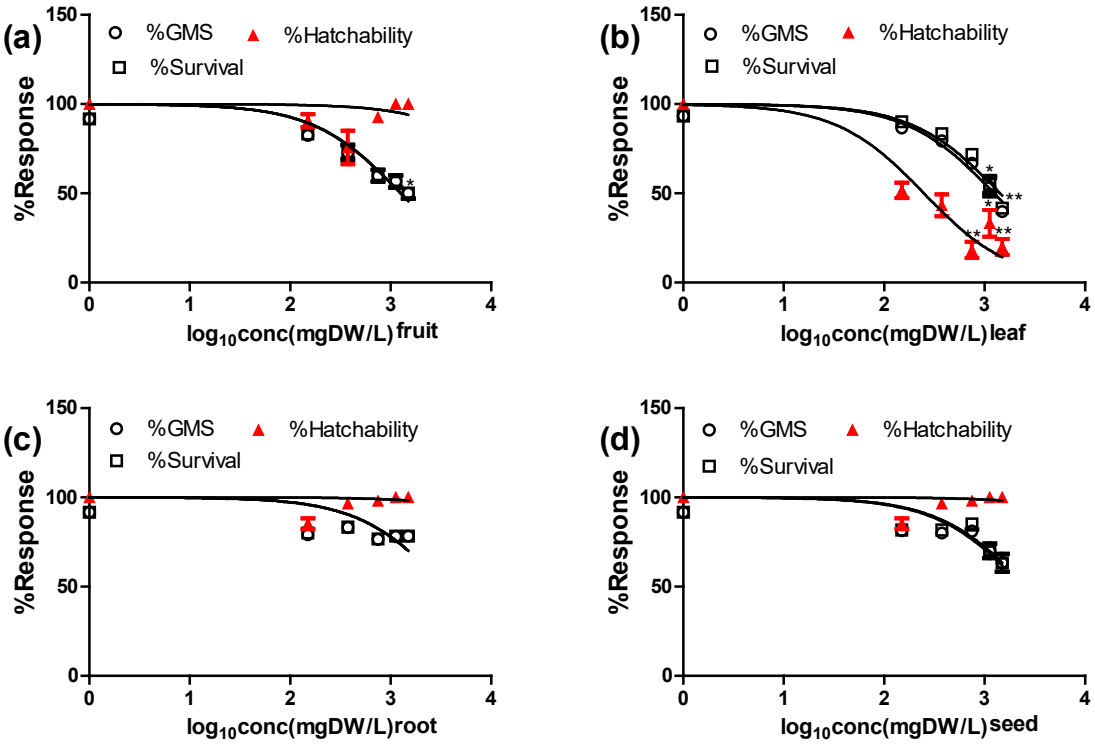


Figure 5. Effects of increasing concentrations (150, 375, 750, 1125, and 1500 mgDW/L) of *M. subcordata* fruit, leaf, root, and seed extracts on zebrafish embryos as compared to solvent control on lethality (described as %survival), general morphology scores (described as %GMS), and %hatchability. Results are described as mean ± SEM of three independent experiments. Asterisks show a significant difference from the solvent control: *$p < 0.05$; **$p <0.01$.

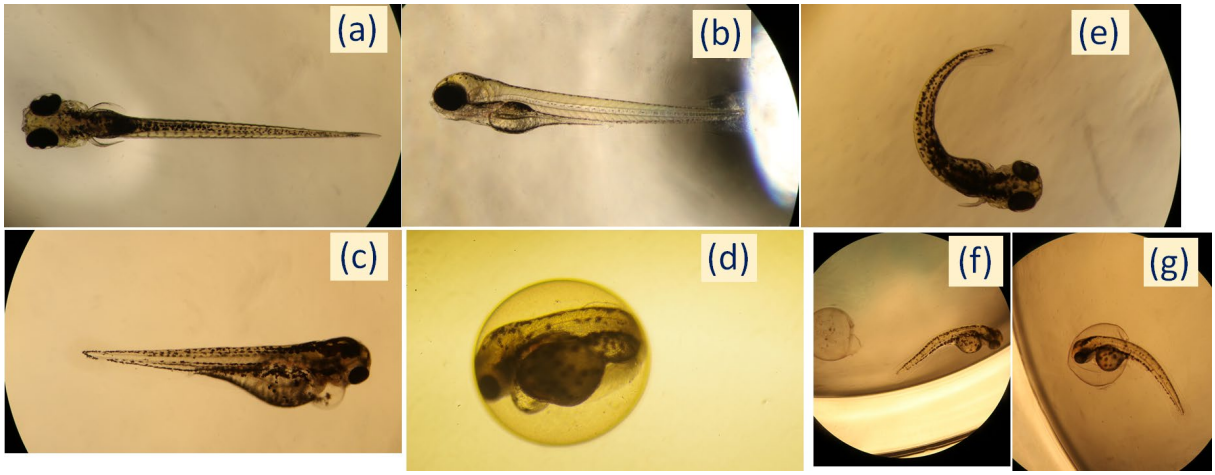


Figure 6. Examples of different morphologies of zebrafish embryos exposed to extracts of *M. subcordata*. (a-b) normal embryos at 96 hpf, (c-d) embryos with signs of malformation of the heart at 96 hpf, and (e-g) curved body/tail in dead embryos between 72-96 hpf.

4. Discussion

The present study applied different *in vitro* tests to assess the genotoxicity and developmental toxicity hazards of methanol extracts of the fruit, leaf, root, and seed parts of *M. subcordata*, a medicinal and (famine) food plant. These endpoints were selected based on the outcomes of a Derek Nexus toxicity prediction, performed on candidate structures obtained from metabolomics profiling of the extracts, and the fact that parts of the plants were reported to be used as abortifacient (Samuelsson *et al*. 1985, 1991).

Although plants are often thought to have anti-mutagenic effects against chemicals and environmental factors, they may also have mutagenic and cytotoxic effects (Erena and Özata, 2014). LC/MS-MAGMa metabolomics profiling plus Derek toxicity predictions showed that candidate constituents for especially the leaf extracts of *M. subcordata* point at genotoxicity

hazards. In addition the glucosinolates, detected in all the extracts, may raise a concern because they usually degrade to isothiocyanates (ITCs) and ITCs are suspected of potential genotoxicity in some studies (Kassie *et al*., 2001) while they were also flagged as potentially genotoxic in the Derek predictions. Results of the present study, however, showed that *M. subcordata* extracts tested negative for mutagenicity in the Ames test, which overrules these concerns. The Ames test has been widely used to assess the mutagenicity of herbal products (Ouedraogo *et al*., 2012; Erena and Özata, 2014). Although a single test, i.e. the Ames test, cannot cover all genotoxic endpoints and especially does not exclude concerns in relation to chromosomal damage, *in vitro* bacterial reverse mutation test systems are likely to cover the majority of "critical" endpoints, i.e. DNA-reactivity, of herbal substances (EMEA, 2007). Thus, as the EU guidelines for herbal products define the Ames test as the primary endpoint to judge the genotoxicity (EMEA, 2007; Ouedraogo *et al*., 2012; Kelber *et al*., 2014), the results of the present study can be used to support the conclusion that these extracts may not raise a concern with respect to the genotoxicity of *M. subcordata*.

The current study also assessed a second endpoint of concern being developmental toxicity. Herbal remedies, being considered as 'harmless' and 'natural', are often used during pregnancy (Mohammed *et al*., 2016). Although there are generally insufficient data on the potential embryotoxicity of herbal remedies, some data indicate that their use during the early stages of pregnancy may not be safe for fetal development (Li *et al*., 2015; Mohammed *et al*., 2016). Therefore, the extracts were tested in a series of *in vitro* assays from a battery of tests developed to assess the *in vitro* developmental toxicity of complex substances (Kamelia *et al*., 2017; Kamelia *et al*., 2018a,b). These tests included the AhR CALUX assay, the ERα CALUX assay, the EST, and the ZET.

AhR, being responsible for the induction of genes that contain a xenobiotic responsive element (XRE, sometimes called a dioxin responsive element) in their promoter regions

(Hayes *et al*., 2009), was implicated in both drug metabolism as well as carcinogenic and toxicological responses against environmental contaminants such as TCDD, but was also shown to be of use in an alternative testing strategy for developmental toxicity (Kamelia *et al*., 2018). The AhR agonist activities of *M. subcordata* extracts, assessed by the rapid and sensitive *in vitro* CALUX assay (Aarts et al., 1995; Murk *et al*., 1996), showed time of exposure dependent variation. The AhR regulates both adaptive and toxic responses (Mitchell and Elferinka, 2009). Reports state that transient AhR activation by molecules like 6-formylindolo[3,2-b]carbazole (FICZ), an endogenous ligand with greater binding affinity than TCDD, may be vital for the putative role of the AhR in cell homeostasis (Bock *et al*., 2013; Farmahin *et al*., 2016) while sustained AhR activation by molecules like TCDD culminates in toxic responses (Mitchell and Elferinka, 2009). Unlike TCDD mediated AhR activation, the AhR activation by *M. subcordata* extracts was of short duration resulting in a transient effect indicating that dioxin like toxicity by the extracts may be limited if not absent. However, AhR activation upregulates transcription of many genes, including those encoding members of the xenobiotic - metabolizing cytochrome P450 1 family of enzymes (CYP1s) (Bengtsson,, 2016) and induction or inhibition of CYP enzyme catalysed drug metabolism is among the most common pharmacokinetic interactions responsible for herb drug interactions (HDIs) that result in several significant HDI related adverse health effects (Koe *et al*., 2014; Wanwimolruk and Prachayasittikul, 2014; Onyeji *et al*., 2017). Therefore, possible adverse HDIs arising from modulation of the function of the AhR CYP system may be of relevance in cases of concurrent uses of certain drugs and the fruit, leaf, and seed extracts of *M. subcordata*.

Botanical related developmental toxicity may also arise from the endocrine-disrupting (ED) potential of certain plant metabolites, called phytoestrogens (Kristanc and Kreft, 2016). Many

591 ED chemicals adversely impact estrogenic signalling by interacting with estrogen receptors
592 (Shanle and Xu, 2011). Although various beneficial health effects have been ascribed to
593 phytoestrogens, their (anti)oestrogenic properties have also raised concerns since they might
594 act as endocrine disruptors, indicating a potential to cause adverse health effects including
595 developmental toxicity (Rietjens *et al*., 2017). In this regard, only the leaf extract of *M.*
596 *subcordata* induced some increase in luciferase activity (**Fig.3**) in the ERα CALUX assay,
597 implying possible beneficial/adverse health effects while the activity of fruit, root, and seed
598 extracts was similar to that of the solvent control implying a possible absence of agonistic
599 estrogen activity.
600 In the EST assay, only the leaf extract of *M. subcordata* indicated possible developmental
601 toxicity since it exhibited significant ($p<0.001$) inhibition of differentiation of ES-D3 cells
602 into contracting cardiomyocytes at non cytotoxic concentrations (**Fig.4b**). In the ZET test, the
603 concentration response curves for lethality and GMS were quite similar (**Fig.5a-d**) suggesting
604 that the GMS scores mostly came from acute toxicity (lethality) effects of the extracts rather
605 than from abnormality endpoints. Malformation of the heart may indicate a teratogenicity
606 endpoint (Beekhuijzen *et al*., 2015) and this effect was observed in a very few (≤5%)
607 embryos upon exposure to all the tested extracts but also upon exposure to the DMSO solvent
608 control, signifying the absence of a significant difference from the control (Selderslaghs *et*
609 *al*., 2009). However, the leaf extract significantly ($p<0.01$) inhibited hatching (**Fig.5b**) which
610 was a major sub-lethal effect by the leaf extract but not induced by the fruit, root, and seed
611 extracts or the solvent control. Hatching is part of the GMS but it is not an endpoint used to
612 measure lethality. It ensures exposure of the embryo without a potential barrier function of
613 the chorion, and as such may help data interpretation (OECD 236, 2013). Although David *et*
614 *al*., (2016) reported that delay or failure to hatch may indicate teratogenic effects, this effect
615 was not part of teratogenic endpoints as described by (Beekhuijzen *et al*. (2015) to assess

developmental toxicity. Accordingly, the fruit, root, and seed extracts did not show any obvious developmental toxicity (Beekhuijzen *et al*., 2015) apart from acute toxicity (OECD 236, 2013) but the leaf extract may be suspected of posing such a hazard. Besides, the failure to hatch of the embryos exposed to the leaf extract might have compromised complete scoring of some endpoints such as deformed body shape which were scored on only the small fraction of hatched embryos.

To sum up, various ethno-medicinal claims on *M. subcordata* may point towards its potential uses as source of herbal medicine or functional or famine food. The different *in vitro* tests of the present study, summarized in **Table 2**, show that all extracts test negative in the Ames test with strain TA98 and TA100, both with and without metabolic activation and hence do not raise a concern for genotoxic hazard (EMEA, 2007); the root extract was apparently devoid of activity in all tests suggesting that further studies may consider the root as potential food item; the fruit and seed extracts seem to have minor toxicity effects except for the fact that they cause strong but transient AhR activation implying caution of, at least, HDI related adverse effects; the leaf extract showed effects in almost all tests except the Ames test indicating its potential multiple biological activities. Hence further studies considering its development and use as potential herbal medicine should also include an adequate safety and risk assessment focussing on at least its potential developmental toxicity. The overall results of the present study do not provide adverse-health effect based counter indications for the ethno-medicinal claim in the plant collection area, northern Ethiopia, that the root tuber part of the plant is a preferred antimalarial remedy for children and pregnant women when there is a fear that other herbal remedies may harm children or pregnant women. This is also in line with the general assumption that a long history of use of a botanical material in traditional medicine is an indicator of lack of obvious toxicity (Trease and Evans, 2002).

641 **Table 2**. Summary of negative (-) or positive (+) responses to a battery of toxicity tests
642 applied in the present study to assess possible genotoxicity and developmental toxicity
643 hazards of *M. subcordata* extracts.

Assays/ Tests	**Endpoints**	**Response**			
		Fruit	**Leaf**	**Root**	**Seed**
Ames	Mutagenicity	-	-	-	-
ERα	Estrogenic agonist activity	-	+	-	-
AhR	AhR agonist activity	+	+	-	+
EST	Inhibition of embryonic stem cell differentiation	-	+	-	-
ZET	Acute toxicity	+	+	-	-
	Hatching inhibition	-	+	-	-

644
645

646 ## 5. Conclusion

647 In conclusion, *M. subcordata* leaf extract showed a potential of multiple biological activities
648 including activation of the aryl hydrocarbon receptor, activation of estrogen receptor alpha
649 mediated gene expression, inhibition of embryonic ES-D3 stem cell differentiation into
650 contracting cardiomyocytes, inhibition of hatching of zebrafish embryos, and acute toxicity to
651 zebrafish embryos. These activities may imply potential pharmacological and/or toxicological
652 consequences including developmental toxicity by the leaf extract. The fruit extract showed
653 some cytotoxicity to zebrafish embryos and both the fruit and seed extracts showed induction
654 of the aryl hydrocarbon receptor while the root extract was almost devoid of activity. Safe use
655 of the fruit, root and seed extracts may be expected because hazards of genotoxicity and
656 developmental toxicity as detected in the series of *in vitro* assays applied in the present study

appeared limited if not absent. Yet, caution of herbal drug interactions arising from aryl hydrocarbon receptor activation may be suggested in cases of concurrent uses of the fruit, leaf, and seed extracts with certain drugs.

6. Acknowledgments

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7. Conflicts of Interest

The authors declare no conflict of interest.

8. Authors contributions

MGH did and handled all experimental activities, performed data interpretation, and wrote the manuscript. IMCMR supervised and helped the study starting from design of tests up to write up and editing of the manuscript. LK did the EST and helped in other *in vitro* tests, involved in reviewing and editing of the manuscript. SW and JV handled the LC-MS related tasks and edited the manuscript LH and BS assisted all experimental techniques, read and contributed to improve this manuscript.

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