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Original research paper

Untargeted metabolic analysis using LC-Q-TOF-MS and toxicity assessment of *Eryngium foetidum* in zebrafish embryos

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ABSTRACT

Toxicological studies of edible plant species are important to determine the safety of their consumption. *Eryngium foetidum* is an edible plant used in some countries for seasoning food and as a natural remedy in folk medicine. Despite this species' gastronomic and medicinal properties, the chemical composition and toxicity have been unclear. The objective of our investigation was to determine the toxic potential of *E. foetidum* in the zebrafish embryo model and identify the potential compounds involved in its toxicity by electrospray ionization liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry. Acute exposure of zebrafish embryos to *n*-hexane extract produced higher toxicity than the other extracts in a time- and concentration-dependent fashion (coagulated embryo). A 96-h median lethal concentration (LC_{50}) of 2.63 μg mL^{-1} (CI 95 % 0.58–28.5) was calculated by probit analysis. Caudal fin hypertrophy, head, yolk sac edema, caudal region, or somite malformations were observed. Secondary metabolites such as terpenes, polyphenols, and fatty acids were identified in the *n*-hexane extract. Also, pollutants such as diglycidyl resorcinol ether, diisopropyl adipate, and lauryl sulfate were found in the *n*-hexane extract. Our study revealed that chemical pollutants could be associated with the embryonic toxicity of the *n*-hexane extract of *E. foetidum*.

Keywords: *Eryngium foetidum*, metabolic profile, toxicity, zebrafish, LC-Q-TOF-MS

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INTRODUCTION

For centuries, ancient cultures have used bark, seeds, fruits, and other parts of plants to treat diseases and common illnesses (1–4). It is noteworthy that traditional medicine has gradually changed its empiric basis to a complex knowledge based upon the structure-activity of the molecules isolated from medicinal plants (5, 6). Currently, traditional medicine needs to be further assessed for non-clinical research based on pharmacology and toxicology principles by performing *in vitro* and *in vivo* studies. In addition, it is also necessary to identify new chemical entities (NCEs) as well as determine their pharmacological or toxicological properties (7, 8).

The chemical diversity of secondary metabolites in the plant kingdom is boundless (9). Chemical analysis and elucidation of the actions attributed to metabolites were previously performed by conventional analytical methods such as UV-Vis or paper chromatography for decades (10, 11). Despite these resources, sophisticated metabolomic techniques such as NMR, GC-MS, or LC-MS were employed to correlate the metabolic composition of plants with the human diet, biological responses, pharmacological treatments, and toxic effects of single molecules (12, 13), as well as the measurement of synergistic mechanisms acting on multiple targets and signaling pathways (9, 14–16).

Eryngium foetidum L. (Apiaceae), commonly named “culantro Mexicano” or “cilantro cimarron", occurs predominantly in tropical and subtropical regions around the world (17). This plant is an aromatic herb with particular features, such as fleshy, branched roots, a straight and cylindrical stem, and oblanceolate leaves with toothed margins. It grows in a basal rosette pattern. The whole plant is glabrous and strongly scented, measuring approximately 45 cm in height (18, 19). In traditional medicine, including that practiced in Mexico, *E. foetidum* has been widely used for gut disorders, hypertension, asthma, some types of cancer, heart disease, vision problems, inflammation, convulsive neurological disorders, and microbial infections, as well as being used in nutraceutical products (20–24).

The objective of this investigation was to examine the toxic effects of organic extracts derived from *E. foetidum* utilizing zebrafish (*Danio rerio*) embryos as a model and determine the metabolic profile of the most toxic extract employing a non-target screening workflow involving liquid chromatography coupled to mass spectrometry (LC-MS).

EXPERIMENTAL

Chemicals

Methanol, acetonitrile, water, and formic acid (LC-MS grade) were purchased from Tedia Company, Inc. (USA); *n*-hexane, methylene chloride, and methanol for the maceration process (technical grade) and ammonium hydroxide (LC-MS grade) were purchased from Sigma-Aldrich (USA).

Plant material and extraction

E. foetidum was collected in July 2018 in Cunducán, Tabasco (18º05'10.5" N 93º15'29.6 W) and identified by Dr. Nelly Jiménez-Pérez. The voucher specimen (No. 35776) was deposited at the herbarium of the Academic Division of Biological Sciences of the Universidad Juárez Autónoma de Tabasco, Mexico.

Dried and ground aerial parts of *E. foetidum* (101.90 g) were subjected to maceration with *n*-hexane (500 mL) three times for 72 hours at room temperature. After filtration, the extract was concentrated *in vacuo* at 40 °C and 80 rpm to obtain *n*-hexane extract (HE*Ef*). The process was repeated with the same plant material to obtain dichloromethane extract (DE*Ef*) and methanol extract (ME*Ef*) using dichloromethane and methanol, resp. (25).

Zebrafish and zebrafish embryos

The wild-type zebrafish (*Danio rerio*) were purchased from a local fish farm. All experiments were reviewed and approved by our Institutional Commission on Research Ethics (CIEL-UJAT; protocol number 0884) and conducted in accordance with the Mexican Federal Regulations for Animal Experimentation and Care (SAGARPA).

Adult fish were separated into male and female groups and acclimatized to a continuous room temperature of 26 °C for at least 7 days and a 14/10 h light/dark cycle. Water was buffered with a pH ranging from 7–8, a hardness of 30–35 mg L^{-1} $CaCO_3$ supply, and bubbled with oxygen saturation. The fish were fed *Artemia salina* (Artemia Biogrow®) twice daily. Under these conditions, the night before exposure, adult fish were placed in 6-L breeding tanks in a 2:1 ratio (female-male) at 28 °C to obtain zebrafish embryos. Spawning was induced by light period initiation. The fertilized embryos obtained were transferred into a clean Petri dish and washed with bottled water for debris removal. Then, embryos were observed under an inverted microscope (Primo Vert, Carl Zeiss Iberia, S.L., Spain) to remove any unfertilized or dead embryos. After 2 hours post-fertilization (hpf), embryos were used for all toxicity tests (26, 27).

Fish embryo acute toxicity (FET) test. - The test was performed following the recommendations described in the Organization for Economic Cooperation and Development (OECD) test guidelines (28, 29). Briefly, the organic extracts were dissolved in DMSO (stock solution, 5 mg mL^{-1}). Then, a dilution process was carried out to obtain experimental dilutions: HE*Ef* were 33, 3.3, 0.33, 0.033, and 0.0033 µg mL^{-1}, whereas dilutions of DE*Ef* and ME*Ef* were 50, 5, 0.5, 0.05 and 0.005 µg mL^{-1}. The vehicle used for each concentration of the extracts was

DMSO 0.1 % in potable water. Twelve fertilized zebrafish embryos were incubated for each organic extract dilution in 24-well culture plates at 28 ± 0.5 °C in a final volume of 2 mL. The survival and morphology of the embryos were examined at 6, 12, 24, 48, 72, and 96 hours after exposure to organic extracts under an inverted microscope. Embryo mortality (coagulation) and any anatomical malformation or changes of the caudal and dorsal regions of the head were recorded each day throughout the exposure period (30).

LC-ESI-Q-TOF-MS conditions

Metabolic analysis (fingerprint) was performed using a liquid chromatography system (1290 Infinity II, Agilent Technologies, USA), equipped with a quaternary pump, coupled to an electrospray ionization with quadrupole time-of-flight (Q-TOF) mass spectrometer Agilent 6545 (Agilent Technologies). Based upon earlier reports, the HE*Ef* was dissolved in acetonitrile (1 mg mL^{-1}), diluted 1:100, and filtered by a 0.45-µm nylon filter (31, 32). Chromatographic separation was performed utilizing a Zorbax Eclipse Plus C18 column RRHD (2.1 x 50 mm, 1.8 µm) with a mobile phase A (0.1 % formic acid-water) and mobile phase B (0.1 % formic acid-acetonitrile) in positive ion mode, or mobile phase A (0.1 % ammonium hydroxide-water) and mobile phase B (0.1 % ammonium hydroxide-acetonitrile) in negative ion mode. A gradient elution method (0:100 *V/V*, 0–1 min; 25:75 *V/V*, 1–5 min; 50:50 *V/V*, 5–10 min; 75:25 *V/V,* 10–20 min and 100:0 *V/V,* 20–25 min) was conducted for chemical separation. Further, a 5-µL injection and a flow rate of 0.2 mL min^{-1} were used for this analysis. MS/MS experiments were performed to annotate metabolic profiling as well as identify fragmentation patterns by acquisition mode targeted MS2, and the fixed collision energy was 20 eV. High resolution was achieved with a reference solution added to each electrospray ionization (ESI) step: 121.0509 *m/z* (purine, $C_5H_5N_4$) and 922.0098 *m/z* (hexakis(1*H*,1*H*,3*H*-tetrafluoropropoxy)phosphazine, $C_{18}H_{19}O_6N_3P_3F_{24}$). The instrument parameters were as follows: capillary voltage 3500 V, nozzle voltage 1000 V, nebulizer pressure 241,316.5 Pa, drying gas flow rate 8 L min^{-1}, gas temperature 325 °C, fragmentor 175 V.

Data analysis

Data were recorded as the mean of survived zebrafish embryos from a basal count of 12 zebrafish embryos per group after exposure to organic extract concentrations. The median lethal concentration (LC_{50}) was calculated by probit analysis after a 96-hour exposure using the

USDA-ARS PriProbit analysis program (version 1.63). Survival plots were constructed by the Prism 7 software (GraphPad. Inc., Boston, MA, USA) (33).

Data acquisition and analysis of the experiments in LC-ESI-Q-TOF-MS were performed by Agilent MassHunter Workstation software (version B.07.00). The ions were annotated using the Molecular Feature Extraction (MFE) algorithm and Molecular Formula Generation software (Agilent Technologies) to compare with the METLIN Metabolite Database (www.metlin.scripps.edu; METLIN | Scripps Research, La Jolla, CA, USA). Finally, MS fragmentations at each retention time were reviewed using the fragmentation tool of the ChemDraw Pro 12.0 software (Supplementary data) (31).

RESULTS AND DISCUSSION

Organic extract yields and fish embryo acute toxicity

The maceration process obtained a yield of 0.11, 0.10, and 0.78 % for HE*Ef*, DE*Ef*, and ME*Ef*, resp. Fig. 1a shows the percentage of fish embryos that survived after exposure to HE*Ef*. At 24 h, a concentration- and time-dependent survival was observed, whereas a minimal survival % was observed after 96 h (HE*Ef* 33 µg mL^{-1}). The calculated LC_{50} was 2.63 µg mL^{-1} (95 % CI 0.58-28.5 µg mL^{-1}). Interestingly, a high survival rate was noted when zebrafish embryos were exposed to DE*Ef* (Fig. 1b). Treatment with ME*Ef* did not produce any mortality in the zebrafish embryo population exposed to this extract.

It is noteworthy that embryos exposed to the vehicle (DMSO 0.1 %), DE*Ef,* or ME*Ef* presented normal embryonic development. Figs. 2a and 2b illustrate representative micrographs of the zebrafish zygote and normal developing zebrafish embryo; Figs. 2c–g shows the phenotypic defects induced by HE*Ef* exposure, such as somite formation and head malformation, yolk sac edema, and caudal region; and Fig. 2h shows a coagulated embryo (dead state). Fish embryos showed morphological malformations when exposed to HE*Ef* 33 µg mL^{-1} after 72 h.

Metabolic profiling by LC-ESI-QTOF-MS

As HE*Ef* was the most toxic extract in the zebrafish embryo preparations, it was of interest to determine its chemical composition by LC-Q-TOF-MS *via* the application of gradient

elution. The total compound chromatograms (TCC) of the total HEEf content and acetonitrile in a positive and negative ion mode were then obtained (Fig. 3).

Thirteen compounds from the base peak chromatograms were annotated. The peak number was assigned by increasing order regarding retention time. The identification, molecular formula, ion type, experimental *m/z*, and MS/MS fragments of 9 compounds shown in positive ion mode are listed in Table I: **1**) absindiol, **2**) 5,8,12-trihydroxy-9-octadecenoic acid, **3**) diglycidyl resorcinol ether, **4**) eplerenone, **5**) diisopropyl adipate, **6**) anethole, **7**) 3"-hydroxy-geranylhydroquinone, **8**) 2,4,8-eicosatrienoic acid isobutylamide, and **9**) 25-azacholesterol. Their chemical structures are illustrated in Fig. 4. Ion fragments in positive ion mode were included in Supplementary Data 1.

Four compounds were also annotated by negative ion mode and are listed in Table II: **10**) 5,8,12-trihydroxy-9-octadecenoic acid, **11**) lauryl sulfate **12**) 6-octadecenyl valerate and **13**) (*R*)-3,4-dihydro-2-(4,8,12-trimethyl-3,7,11-tridecatrienyl)-2*H*-1-benzopyran-6-ol. Their chemical structures are presented in Fig. 5. Ion fragments in negative ion mode are included in Supplementary Data 2.

Our study showed that HE*Ef* had a higher yield compared with dichloromethane and methanol extracts, indicating that aerial parts contain more non-polar metabolites than hydrophilic molecules (34). In fact, data suggest that the medicinal properties of the plant may be generated predominantly by these types of molecules compared to others with different chemical properties. However, this suggestion remains to be reconsidered and elaborated.

In accordance with OEDC, an acute zebrafish embryotoxicity test is a required component in the environmental hazard assessment of industrial chemicals, plant products, biocides, pharmaceuticals, and feedstuffs (29, 35, 36). In the FET test performed here, exposure of zebrafish embryos to HE*Ef* decreased survival in a concentration- and time-dependent manner. Unfortunately, it was not possible to determine the non-observed adverse effect level (NOAEL), a measure that indicates the greatest concentration or amount of a substance that initiates no detectable adverse alterations in morphology, growth, development, or lifespan of an organism, as the lower concentration tested (3.3×10^{-3} μg mL^{-1}) still produced a 10 % mortality in zebrafish embryos. Interestingly, the embryos exposed to HE*Ef* at 33 μg mL^{-1} (Fig 1a) showed a non-concentration- and time-dependent behavior, probably due to the biological variability of

the embryos, although they are from the same progenitors; genetic variability and the high fecundity of the fishes can influence their phenotype and therefore the biological responses to xenobiotics and the environment (37).

The compounds annotated by the LC-ESI-QTOF-MS analysis that might be associated with mortality and altered morphogenesis of some organs are the diglycidyl resorcinol ether, diisopropyl adipate, and lauryl sulfate shown in Tables I and II. In this context, diglycidyl resorcinol ether is an additive that demonstrated *in vitro* genotoxic effects in mammalian cells, mutagenicity by the Ames test (38), and carcinogenic effects (39), and due to its higher relative abundance than the other compounds, it may be mainly responsible for the toxic effects observed in the embryos. In addition, diisopropyl adipate, an emollient and permeation enhancer ingredient for pharmaceuticals, has not been examined comprehensively regarding toxicity yet (40, 41). Further, lauryl sulfate, a surfactant used in pharmaceuticals, cosmetics, and foods, is a previously reported recognized human and environmental toxic agent (42–44).

Environmental pollutants interfere not only with plant metabolism by modifying enzymatic and genetic activity, cellular detoxification, and biological interaction with the environment, producing biotic stress and susceptibility (45–48), but these pollutants might also be part of a biomagnification chain that consequently results in toxic effects on human health in different ways (47, 49, 50). More investigations on this topic are recommended since it is not known whether all edible plants that consumers are exposed to may produce toxic responses.

Similar to this investigation, Castro and colleagues (51) reported that the toxicity of *E. foetidum* on zebrafish embryos and larvae was dependent upon the extraction solvent, the extract concentration used, and exposure duration. They found that *E. foetidum* polar extracts (aqueous, ethanol, and methanol) increased mortality rates, delayed hatching time, and produced changes in enzymatic activities. In addition, these organic extracts delayed the development of zebrafish embryos and elevated glutathione-*S*-transferase activity, free radical production, and oxidative damage. They concluded that concentrations above 0.625 $\mu g\ mL^{-1}$ might exert adverse effects in the early stages of zebrafish embryonic development. The above findings are in agreement with our data showing that ME*Ef* adversely altered fish embryo development; however, our highest concentration of 33 $\mu g\ mL^{-1}$ was still much lower. More investigation is required to examine this issue.

Finally, complementary investigations were published on animal models. Janwitthayanuchit and colleagues (52) reported a 24-week chronic toxicity study where mice were fed with a rodent diet supplemented with ground freeze-dried *E. foetidum* leaves at 0.8, 1.6, or 3.2 %. Data demonstrated that the modified diet produced tubulonephrosis and chronic interstitial nephritis in animals fed with 1.6 or 3.2 % preparations. These toxic effects showed that the malformations observed in the zebrafish embryos can also induce damage in rodents and, probably, higher animals.

CONCLUSIONS

The current study provided a metabolic profile of *E. foetidum* by LC-ESI-QTOF-MS. The environmental pollutants found were diglycidyl resorcinol ether, diisopropyl adipate, and lauryl sulfate, possibly related to the observed anatomic malformations and elevated mortality rates noted in zebrafish embryos after exposure to HE*Ef*. Therefore, further investigation needs to be performed on edible plant species to examine various exposure scenarios to xenobiotic pollutants, since these may represent a source of toxic substances that might exert adverse effects on human health.

Abbreviations, acronyms, codes. – DE*Ef* - dichloromethane extract of *E. foetidum*, FET – fish embryo acute toxicity, LC-ESI-QTOF-MS – liquid chromatography coupled with electrospray ionization-quadrupole time of flight mass spectrometry, LC_{50} – median lethal concentration, ME*Ef* – methanol extract of *E. foetidum*, HE*Ef* - *n*-hexane extract of *E. foetidum*, NOAEL – non-observed adverse effect level, OECD – Organization for Economic Co-operation and Development (Paris, France), TCC - total compound chromatogram

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Conflict of interest. – The authors have no conflicts of interest to declare.

Data availability. – Supplemental material for this article is available online. Additional data will be made available on request.

Authors contributions. – S.R.M., I.S.L., and O.H.A. conceived and designed the experiments. R.V.C., O.A.P.M., A.Y.H., and L.C.R. performed the experiments. N.J.P., C.A.S., N.R.C. and O.H.A.

analyzed the data. S.R.M., R.V.C., O.A.P.M., L.C.R., and O.H.A. wrote or contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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Table 1. Compounds annotated by LC-ESI-QTOF-MS in HEEf by positive ion mode

Peak No.	t_R (min)	Identification	Molecular formula	Ion type	Experimental (*m/z*)	MS/MS fragments	*AUC* (RIA min)
1	8.86	Absindiol	$C_{15}H_{22}O_4$	$(M+Na)^+$	289.1406	249.1469	62,385.2
2	9.67	5,8,12-Trihydroxy-9-octadecenoic acid	$C_{18}H_{34}O_5$	$(M+Na)^+$	353.2298	337.1979, 281.0992, 244.2632, 188.9178	129,815.0
3	11.36	Diglycidyl resorcinol ether	$C_{12}H_{14}O_4$	$(M+Na)^+$	245.0782	214.9172	3,566,561.0
4	12.25	Eplerenone	$C_{24}H_{30}O_6$	$(M+Na)^+$	437.1935	415.2113, 304.2848, 261.1318, 175.0178	979,151.8
5	12.91	Diisopropyl adipate	$C_{12}H_{22}O_4$	$(M+Na)^+$	253.1403	230.8895, 214.9172, 143.0015	293,754.8
6	14.25	Anethole	$C_{10}H_{12}O$	$(M+H)^+$	149.0961	121.0509	105,659.7
7	14.57	3"-Hydroxy-geranylhydroquinone	$C_{16}H_{22}O_3$	$(M+Na)^+$	285.1459	244.2634	198,920.8
8	14.99	2,4,8-Eicosatrienoic acid isobutylamide	$C_{24}H_{43}NO$	$(M+H)^+$	362.3419	304.2852	118,957.0
9	17.24	25-Azacholesterol	$C_{26}H_{45}NO$	$(M+H)^+$	388.3581	301.1409, 272.2946, 231.9069	56,464.36

AUC – area under curve; RIA – relative ion abundance.

Table II. Compounds annotated by LC/QTOF-MS in HEEf by negative ion mode

Peak number	t_R (min)	Identification	Molecular formula	Ion type	Experimental (*m/z*)	MS/MS fragments	*AUC* (RIA min)
10	0.780	5,8,12-Trihydroxy-9-octadecenoic acid	$C_{18}H_{34}O_5$	$(M-H)^-$	329.2334	201.1135, 187.0978 154.9475	21,362.4
11	9.888	Lauryl sulfate	$C_{12}H_{26}O_4S$	$(M-H)^-$	265.1484	209.0667, 194.9061	67,236.0
12	17.594	6-Octadecenyl valerate	$C_{23}H_{46}O_2$	$(M-H)^-$	353.3427	325.1840 311.1689	7,025.0
13	24.978	(*R*)-3,4-dihydro-2-(4,8,12-trimethyl-3,7,11-tridecatrienyl)-2*H*-1-benzopyran-6-ol	$C_{25}H_{36}O_2$	$(M-H)^-$	367.2638	325.1840, 188.9390, 136.9367	17,226.62

AUC – area under curve; RIA – relative ion abundance.

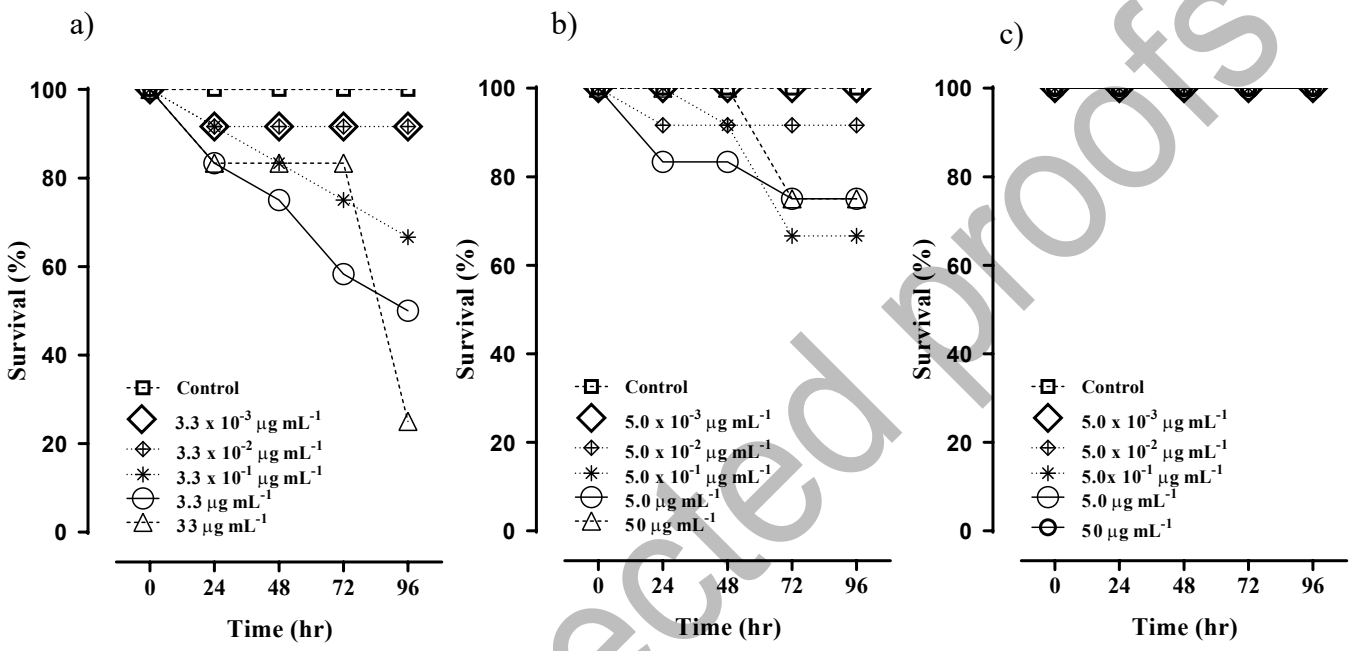
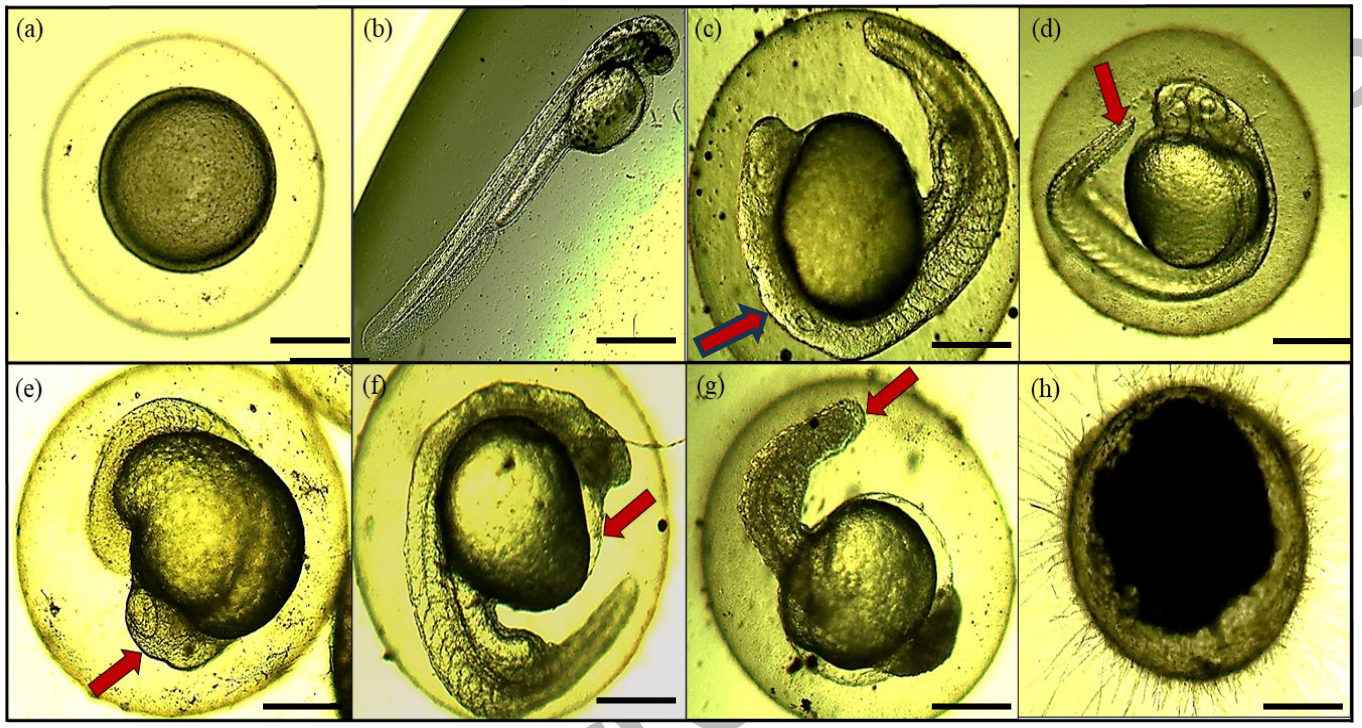


Fig. 1. Effect of dilutions of: a) HE*Ef*; b) DE*Ef* and c) ME*Ef*, on survival of zebrafish embryos. Data plotted are means calculated from twelve replicates.



Scale bar= 250 µm

Fig. 2. Representative micrographs of: a) zygote in vehicle; b) embryonic development without malformations; c) somite formation; d) caudal fin hypertrophy malformation; e) head malformation; f) yolk sac edema malformation; g) caudal region malformation; h) coagulated embryo (dead).

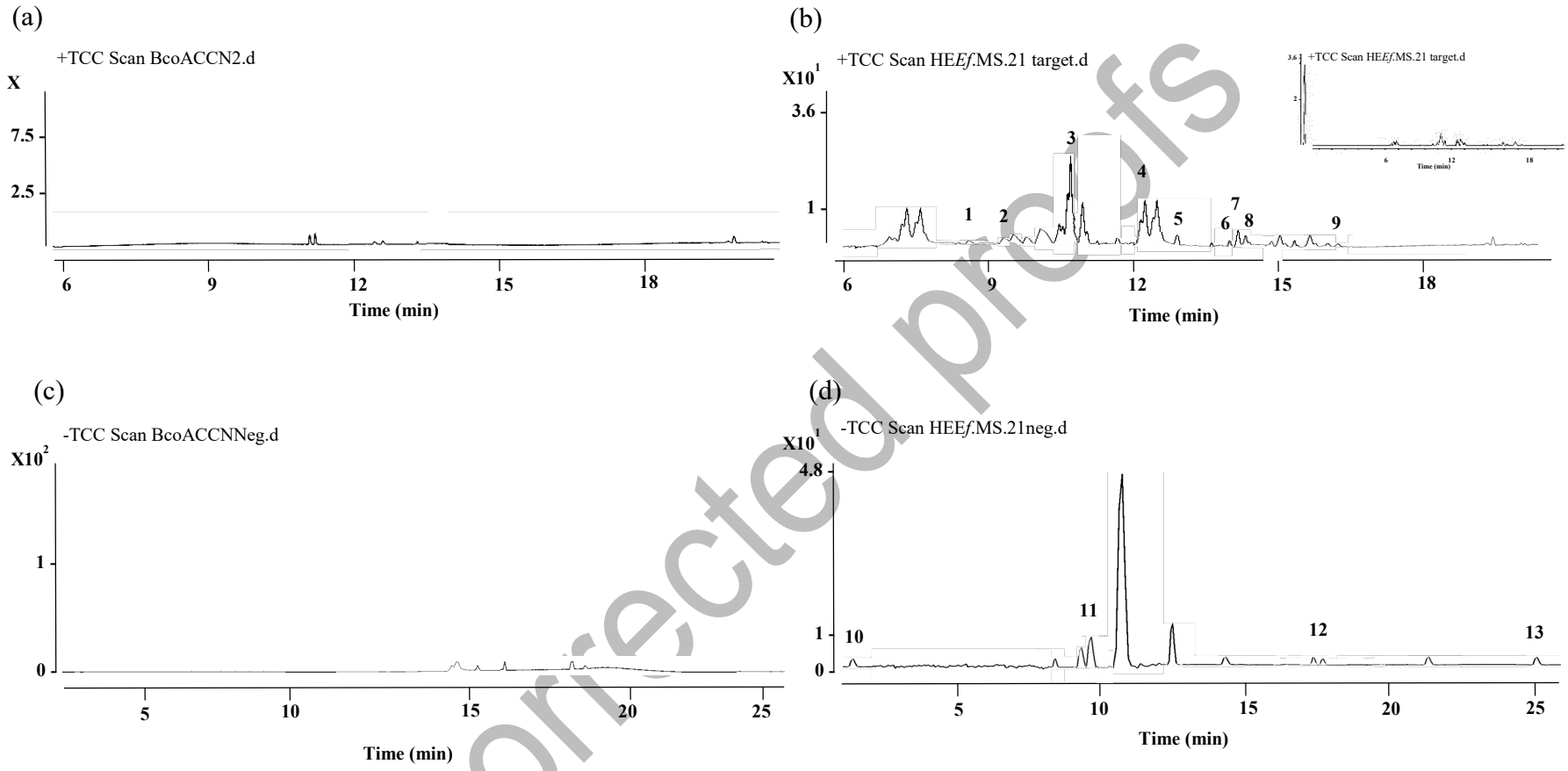


Fig. 3. The total compound chromatograms (TCC) of the following samples: a) acetonitrile and b) *n*-hexane extract of *E. foetidum* in positive ion mode; c) acetonitrile and d) *n*-hexane extract of *E. foetidum* in negative ion mode.

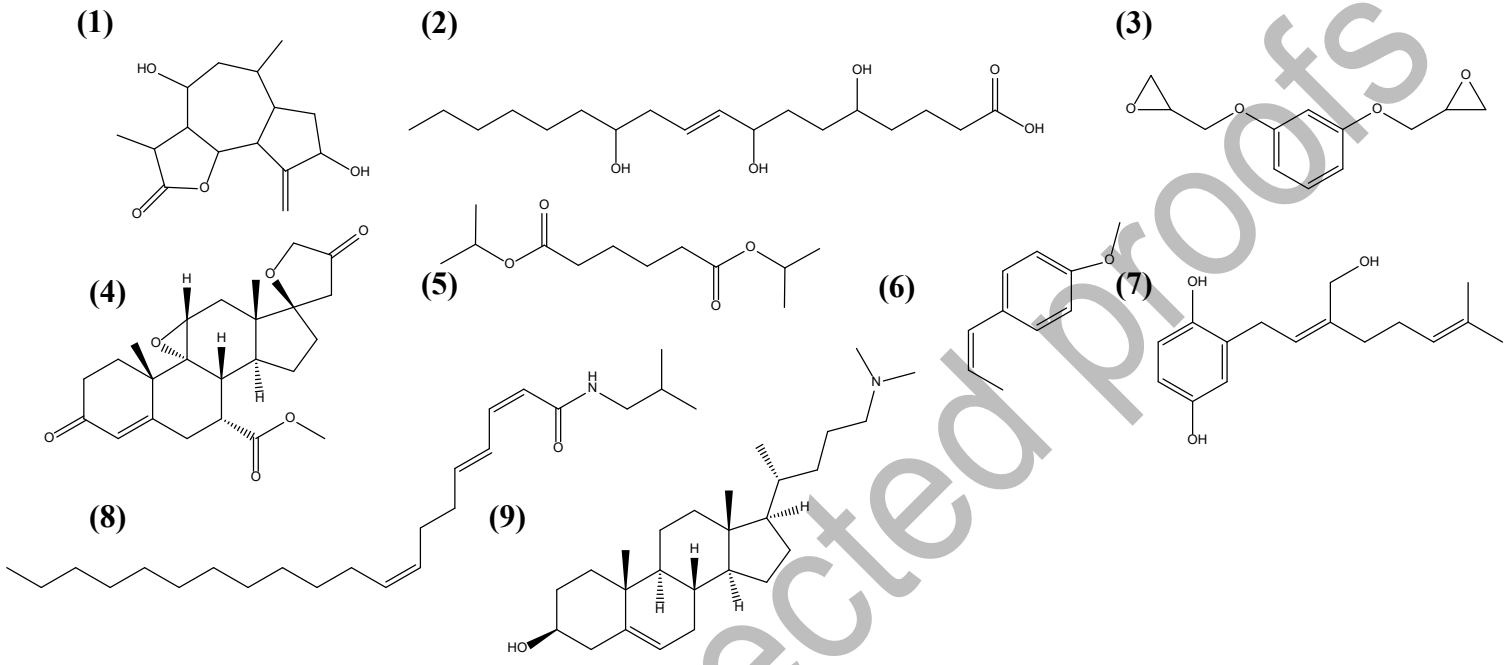


Fig. 4. Chemical structures of compounds annotated in HE*Ef* by positive ion mode.

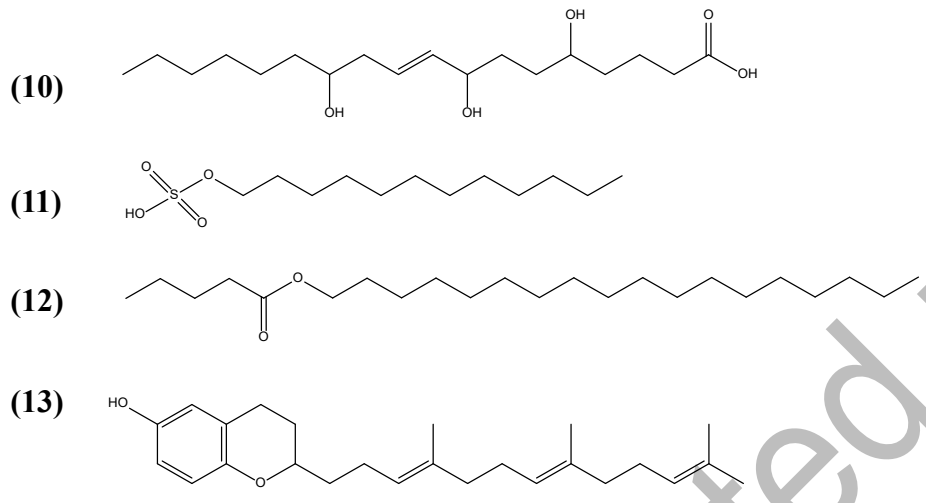


Fig. 5. Chemical structures of compounds annotated in HE*Ef* by negative ion mode.