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RESEARCH ARTICLE

Chemical composition, antioxidant capacity and toxicological evaluation of rhizome and leaf ethanol extracts of *Homalomena aromatica* (Spreng.) Schott in zebrafish (*Danio rerio*) embryos

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Abstract

Homalomena aromatica (Spreng.) Schott is a highly aromatic medicinal plant found in Manipur and other parts of Northeast India. Despite its varied traditional medicinal uses, there are limited reports on plant-part-specific toxicological evaluation of the rhizome and leaf ethanol extracts. *In vitro* toxicological studies may not adequately replicate complex biological interactions such as metabolism and bioavailability. Therefore, this study focused on *in vivo* evaluation to obtain a comprehensive toxicological profile. This study compared the phytochemical composition of the rhizome and leaf ethanol extracts of *H. aromatica* from Manipur, India using gas chromatography-mass spectrometry (GC-MS), antioxidant activity using ABTS and DPPH assays and toxicological profile using zebrafish as a model. The GC-MS analysis of *Homalomena aromatica* rhizome ethanol extract (HAREE) and *Homalomena aromatica* leaf ethanol extract (HALEE) revealed the presence of 31 and 25 compounds respectively. The rhizome ethanol extract had a terpenoid-rich composition with the major compounds being Linalyl acetate (monoterpenoid), Oplopanonyl acetate (sesquiterpenoid) and (1R,7S,E)-7-Isopropyl4,10-dimethylenecyclodec-5-enol (sesquiterpenoid) while the leaf ethanol extract showed a more diverse chemical composition with 2-Cyclohexen-3-ol-1-one, 2-dodecanoyl- (vinylogous acid), α- Tocopherol-β-D-mannoside (vitamin E glycoside) and n-Hexadecanoic acid (long-chain fatty acid) being the major compounds detected. The rhizome extract showed a higher 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) and 2,2´-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activity than the leaf extract with IC_{50} values of 51.23 ± 0.10 µg/mL (ABTS) and 248.81 ± 6.23 µg/mL (DPPH) respectively. However, both were less potent than the standard ascorbic acid. In contrast to the antioxidant activity, the leaf ethanol extract was found to have a higher toxic profile as compared to the rhizome ethanol extract with LC_{50} values estimated to be 198.81 (leaf) and 558.31 µg/mL (rhizome) respectively. This underscores differential distribution of secondary metabolites in the different plant parts. In conclusion, these findings highlight substantial differences between the leaf and rhizome extracts in chemical composition, antioxidant potential and safety profile.

Keywords: antioxidant capacity; GC-MS analysis; *Homalomena aromatica*; safety profile; zebrafish

Introduction

Natural products and plant-derived formulations have played an important role in traditional medicine. They are widely used in primary healthcare in many developing regions, particularly in Asia and Africa (1). The medicinal properties of plants are often attributed to the secondary metabolites they produce. Compounds such as polyphenols, flavonoids and other secondary metabolites are reported to have the ability to scavenge reactive oxygen species (ROS), thereby mitigating oxidative stress-related damage (2). The GC-MS enables detailed profiling of volatile and semi-volatile compounds in plant extracts (3). In addition, spectrophotometric assays such as 2,2´-ainobis-(3-ethylbenothiaoline-6-sulphonate) (ABTS) and 2,2-diphenyl-1-picryl-hydrayl (DPPH) radical scavenging assays are established *in vitro* approaches for determining the overall antioxidant potential of plant extracts (3, 4). However, recent studies emphasise the importance of early-stage toxicity screening of complex plant extracts (1, 5). *In vitro* toxicological studies may not adequately replicate complex biological interactions such as metabolism and bioavailability (6). Therefore, this study focused on *in vivo* evaluation to obtain a comprehensive toxicological profile. Zebrafish (*Danio rerio*) has emerged as a robust vertebrate model for assessing toxicity of plant extracts due to its rapid organogenesis, transparency of early development-stage embryos and larvae and high fecundity (5). Therefore, integration of GC-MS chemical profiling, quantitative *in vitro* antioxidant assessment and toxicity analysis in development of vertebrate embryo forms an important parameter for early safety and efficacy evaluation of plant extracts.

Homalomena aromatica (Spreng.) Schott is a highly aromatic medicinal plant belonging to the family Araceae (order: Alismatales). It is found in Manipur and other parts of Northeast India (7, 8). The plant is locally known as '*Hongu-kakla-manbi*' in Manipur (Meiteilon dialect). The Meitei and Meitei-Pangal communities of Manipur use the crushed rhizome along with *Averrhoa carambola* L. fruit juice to treat asthma (9). Different communities in Northeast India and Bangladesh use the plant for various conditions such as fever, joint pain, skin and stomach ailments (8). The different plant parts, including rhizomes, leaves and in some cases the petioles separately, are also consumed in different culinary preparations in parts of Northeast India and Bangladesh (10, 11).

Previous studies have explored and investigated the chemical composition and bioactivities of *H. aromatica* rhizome and leaf extracts and essential oil, including antioxidant, antibacterial, antifungal, larvicidal, insecticidal, neuropharmacological and thrombolytic activities (8). However, no study has reported the phytochemical profile and bioactivities of *H. aromatica* from Manipur, India. Studies have shown that environmental factors such as soil, climate, altitude and geographical location influence the composition and abundance of secondary metabolites resulting in region-specific chemical profiles (12). This in turn may influence the bioactivity shown by plants from different geographic locations. In addition, no study has reported the toxicological risk evaluation of the plant *in vivo* using zebrafish.

Therefore, the present study aims to evaluate the chemical profile of the volatile and semi-volatile compounds, quantify the *in vitro* antioxidant potential of *H. aromatica* rhizome and leaf ethanol extracts of plants collected from Manipur, India and their safety profile in zebrafish embryos. This study contributes novel region-specific phytochemical and bioactivity data along with *in vivo* safety profile in zebrafish vertebrate model. Ethanol was used as the extraction solvent in this study because of its mid-polar nature, ethanol can effectively extract a range of polar and non-polar compounds. In addition, ethanol is considered safe for use in food and pharmaceuticals as compared to other organic solvents (13).

Materials and Methods

Collection of plants and extract preparation

Plants were collected in April from Jiribam District of Manipur, India. The plant was taxonomically identified by Dr. Yumkham Sanatombi Devi, faculty at the Department of Life Sciences (Botany), Manipur University. The herbarium specimen, with accession number 001039, was deposited at the Manipur University Museum of Plants maintained at the Department of Life Sciences (Botany), Manipur University, India. The collected plants were separated into rhizomes and leaves, thoroughly washed under running tap water and finally rinsed with distilled water to remove adhering debris. The plant materials were initially shade-dried, cut into small pieces and further shade-dried until completely moisture-free. The dried samples were then pulverised into coarse powder using a laboratory grinder.

800 mL of ethanol was added to 40 g each of the rhizome and leaves (solvent/plant material ratio of 20:1 v/w) and constantly agitated at 150 rpm for a total duration of 24 hr. The extract was then filtered using Whatman 1 filter paper and concentrated using a rotary vacuum evaporator. The concentrated rhizome extract (HAREE) and leaf extract (HALEE) were stored at -20 °C for further use. The percentage yield of HAREE and HALEE were respectively found to be 4.93 % and 11.90 %.

Chemicals and reagents

2,2-diphenyl-1-picryl-hydrazyl (DPPH), 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) and ascorbic acid were obtained from Sisco Research Laboratories (SRL), India. 3,4-Dichloroaniline (DCA) was obtained from Sigma-Aldrich, USA. Ethanol, methanol and dimethyl sulfoxide (DMSO) were obtained from HiMedia, India. All other reagents used are of analytical grade.

Gas chromatography-mass spectrometry analysis

Gas chromatography-mass spectrometry analysis was performed using an Agilent 8890 gas chromatograph coupled with an Agilent 5977 mass selective detector (MSD) operating in electron ionisation (EI) mode at 70 eV. The column used was an Agilent 19091S-433UI HP-5 ms Ultra Inert column (30 m × 250 µm × 0.25 µm). The carrier gas was Helium at a constant flow rate of 1.2 mL/min. The injection volume was 1 µL (split mode, split ratio 15:1) with the injector temperature set at 250 °C. The oven temperature was initially 75 °C (0.5 min hold), ramped at 5 °C/min to 180 °C (3 min hold), then at 5 °C/min to 300 °C (5 min). The total running time was 53.5 min. The MSD transfer line temperature was maintained at 280 °C, the ion source temperature at 230 °C and the quadrupole temperature at 150 °C. The mass spectrometer was operated in full scan mode over an m/z range of 50–600 with a solvent delay of 2 min. The compounds were identified by comparison with the NIST17 Mainlib database. The compounds were quantified based on only peak area percentage. No internal standard was used in this study. The compounds detected were classified using 'Classy Fire Batch' using International Chemical Identifier (InChIKey) as input (14). The InChIKeys of the compounds were retrieved from the PubChem database (15).

Antioxidant activity

Antioxidant activity was evaluated using the ABTS and DPPH radical scavenging assays by following the standard operating procedures.

ABTS radical scavenging assay

The ABTS radical cation was generated by mixing 7 mM ABTS solution (prepared in ethanol) with 2.6 mM potassium persulfate (prepared in distilled water) in equal volumes (1:1, v/v), then incubated in the dark at room temperature for 12 hr. The resulting ABTS cation stock solution was diluted with ethanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm. 1 mL of different concentrations of HAREE (20, 40, 60, 80, 100 µg/mL) and HALEE (100, 200, 300, 400, 500 µg/mL), prepared in methanol, were mixed with 2 mL of the working ABTS solution and incubated in the dark for 30 min. Absorbance was measured at 734 nm using a UV-visible spectrophotometer (16). Ascorbic acid was used as standard. 1 mL methanol mixed with 2 mL of working ABTS solution was used as the negative control. Methanol was used because the test samples were prepared in methanol.

DPPH radical scavenging assay

A 0.1 mM DPPH solution prepared in methanol was used as the working solution. 1 mL of different concentrations of HAREE (60, 120, 180, 240, 300 µg/mL) and HALEE (400, 800, 1200, 1600, 2000 µg/mL) were mixed with 2 mL of the working DPPH solution and incubated in the dark for 30 min. Absorbance was measured at 517 nm using a UV-visible spectrophotometer (17). Ascorbic acid was used as a standard. 1 mL methanol mixed with 2 mL of working

DPPH solution was used the negative control. Methanol was used because the test samples were prepared in methanol.

Calculation

The radical scavenging activity (%) was calculated using the following equation:

$$\text{Scavenging activity (\%)} = \left(\frac{A_0 - A_1}{A_0} \right) \times 100 \quad \text{(Eqn. 1)}$$

Where,

A_0 = Absorbance of the control (radical solution without sample)

A_1 = Absorbance of the test sample

IC_{50} was calculated using linear regression analysis.

All reactions were done in triplicate. The experiments were repeated thrice. The results are expressed as mean ± standard error of mean (SEM).

Zebrafish maintenance and embryo collection

Adult wild-type zebrafish were obtained from a local vendor in India and acclimatised in glass aquariums containing aerated dechlorinated water at a temperature of 28 ± 2 °C and a 14 hr light/10 hr dark photoperiod. They were fed thrice daily with commercial fish food. The fully mature adult males and females were separated one day prior to breeding. The fish were transferred to a breeding tank in the ratio of 3 males to 3 females. A net was used to protect the newly spawned eggs. The tank was maintained at a temperature of 28 ± 2 °C and 14 hr light/ 10 hr dark photoperiod. Spawning and fertilisation were instigated at the onset of the first light. The embryos were collected using a Pasteur pipette and transferred to Petri dishes containing egg water (milli-Q water containing 60 µg/mL instant ocean salt). The embryos were washed twice with egg water. Healthy 6 hr post-fertilisation (hpf) embryos were selected under a stereozoom microscope and used for treatment. Embryonic staging was performed according to established developmental stages (18).

Toxicological evaluation using zebrafish embryos

The toxicological evaluation was performed following the Organization for Economic Co-operation and Development (OECD) guideline number 236 (19). The guideline recommends 4 µg/mL of DCA as the positive control. Six hpf embryos were transferred to 6-well plates, 10 in each well. The embryos were divided into 3 groups: 0.5 % DMSO in egg water (negative control), 4 µg/mL of DCA prepared in 0.5 % DMSO in egg water (positive control) and different concentrations of HAREE and HALEE prepared in 0.5 % DMSO in egg water i.e., 100, 200, 400, 600, 800 µg/mL of HAREE and 50, 100, 150, 200, 250, 300 µg/mL of HALEE. The final volume of the treatment solution was 2 mL per well. Semi-static method was followed and the embryos were transferred to fresh solution of the same test concentration every 24 hr. The embryos were exposed for 96 hr and observations were recorded every 24 hr. Coagulated embryos and dead larvae were removed. Mortality rate (%) at 96 hpf and hatching rate (%) at 72 hpf were taken as endpoint observations. The hatched dead larvae were also considered for determining the hatching rate. Morphological deformities were also recorded.

Mortality rate (%) was calculated using the following equation:

$$\text{Mortality rate (\%)} = \frac{\text{Total number of dead embryos/larvae at 96 hpf}}{\text{Total number of embryos at the start of treatment}} \times 100 \quad \text{(Eqn. 2)}$$

Hatching rate (%) was calculated using the following equation:

$$\text{Hatching rate (\%)} = \frac{\text{Total number of hatched larvae at 72 hpf}}{\text{Total number of embryos at the start of treatment}} \times 100 \quad \text{(Eqn. 3)}$$

All treatments were done in triplicate. Each replicate consisted of 10 embryos. The results are expressed as mean ± SEM. LC_{50} was calculated using probit analysis. Zebrafish maintenance and all the experiments were conducted in accordance with the guidelines of the Institutional Animal Ethics Committee, Manipur University.

Statistical analysis

Results were analyzed and visualised using GraphPad Prism 8.0.2, IBM SPSS Statistics V21.0 and MS Excel 2021. Normality of the data was assessed using the Shapiro-Wilk test. Since not all groups followed a normal distribution, non-parametric statistical analyses were performed. For the ABTS and DPPH radical scavenging activities, Kruskal-Wallis test followed by Dunn's multiple comparison test was performed at $p \leq 0.05$. The Kruskal-Wallis test was performed including the control group. For the toxicological study, Fisher's exact test at $p \leq 0.05$ was used to compare each group to the negative control group to determine the no observed adverse effect concentration (NOAEC) and lowest observed adverse effect concentration (LOAEC). The NOAEC and LOAEC were computed as the highest tested concentration that shows no statistically significant adverse effect compared with the negative control and the lowest tested concentration that shows a statistically significant adverse effect relative to the negative control.

Results

GC-MS analysis

The GC-MS analysis of HAREE and HALEE revealed the presence of 31 and 25 compounds respectively. The Total Ion Chromatograms (TICs) of HAREE and HALEE are presented in Fig. 1 and Fig. 2 respectively. The retention times (RTs), peak area percentages (%) and type/class of the compounds are summarised in Table 1 for HAREE and in Table 2 for HALEE. The reverse score, probability (%) and library ID of the compounds detected are reported in the supplementary tables (Supplementary Table 1, 2).

In case of HAREE, the major compounds identified were Linalyl acetate (19.37 %), Oplopanonyl acetate (18.97 %) and (1R,7S,E)-7-Isopropyl4,10-dimethylenecyclodec-5-enol (9.09 %). The identified compounds majorly belonged to terpenoids, accounting for the largest fraction (38.71 %) of the total number of compounds detected, followed by fatty acids and derivatives (16.13 %). In case of HALEE, the major compounds identified were 2-Cyclohexen-3-ol-1-one, 2-dodecanoyl- (20.19 %), α-Tocopherol-β-D-mannoside (12.85 %) and n-Hexadecanoic acid (12.50 %). The compounds belonged to a diverse group of compound classes with fatty acids and derivatives making up the majority fraction (12.90 %).

Antioxidant activity

The ABTS cation radical scavenging activity was higher in HAREE as compared to HALEE. The radical scavenging activity of HAREE ranged from (24.08 ± 0.22 %) at 20 µg/mL to (93.48 ± 0.10 %) at 100 µg/mL while that of HALEE ranged from (22.66 ± 0.12 %) at 100 µg/mL to (85.24 ± 0.47 %) at 500 µg/mL (Table 3). However, the radical scavenging activity of ascorbic acid was higher than both HAREE and HALEE with values ranging from (21.08 ± 0.10 %) at 2 µg/mL to (89.77 ± 0.13 %) at 8 µg/mL (Table 3).

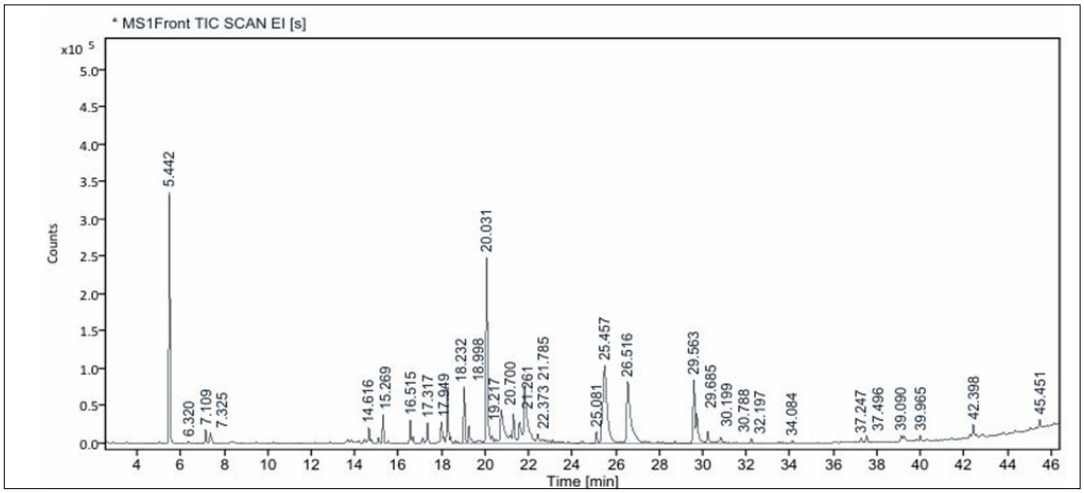

Fig. 1. Total ion chromatogram of HAREE (GC-MS analysis).

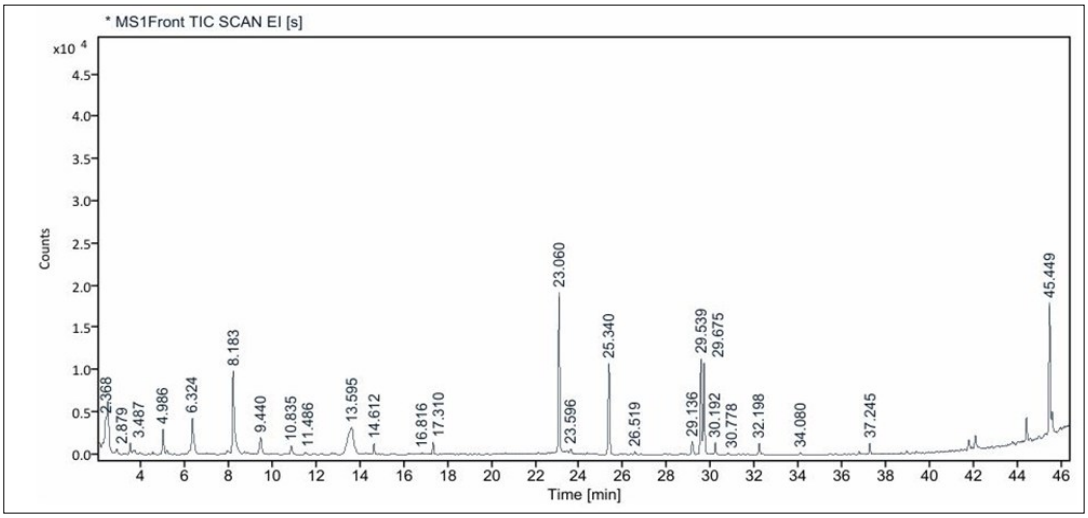

Fig. 2. Total ion chromatogram of HALEE (GC-MS analysis).

Table 1. List of compounds detected in GC-MS analysis of HAREE

Sl. No.	Compound name	RT[a] (min)	Area (%)	Type/class
1	Linalyl acetate	5.444	19.37	Monoterpenoid
2	Oplopanonyl acetate	20.034	18.97	Sesquiterpenoid
3	(1R,7S,E)-7-Isopropyl4,10- dimethylenecyclodec-5- enol	26.517	9.09	Sesquiterpenoid
4	2,2,6-Trimethyl-1-(3-methylbuta-1,3-dienyl)-7-oxabicyclo[4.1.0]heptan-3-ol	25.454	7.31	Oxepane
5	9(E),11(E)-Conjugated linoleic acid	29.561	5.07	Linoleic acid/derivative
6	Isospathulenol	21.785	4.97	Sesquiterpenoid
7	.tau.-Cadinol	18.234	4.70	Sesquiterpenoid
8	Aromadendrene oxide-(2)	19.003	4.38	Sesquiterpenoid
9	Diepicedrene-1-oxide	20.703	4.12	Oxepane
10	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	15.271	2.37	Sesquiterpenoid
11	(-)-Spathulenol	16.515	1.96	Sesquiterpenoid
12	12-Methyl-E,E-2,13- octadecadien-1-ol	21.260	1.91	Long-chain fatty alcohol
13	.tau.-Muurolol	17.953	1.62	Sesquiterpenoid
14	Neointermedeol	17.315	1.51	Sesquiterpenoid
15	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	29.686	1.32	Linoleic acid/derivative
16	N- (Deshydroxyethyl)dasatinib	19.222	1.28	Aromatic anilide
17	(±)-11-Hydroxy-.DELTA.9- tetrahydrocannabinol	25.079	1.20	Benzopyran
18	(9Z,12Z)-(E)-3,7- Dimethylocta-2,6-dien-1- yl octadeca-9,12-dienoate	42.401	1.14	Wax ester
19	1H-Cyclopropa[a]naphthalene, decahydro-1,1,3a-trimethyl-7-methylene-, [1aS-(1aα,3aα,7aβ,7bα)]-	14.615	1.06	Sesquiterpenoid
20	Terpinen-4-ol	7.113	1.02	Monoterpenoid
21	Octadecanoic acid	30.199	0.89	Long-chain fatty acid
22	Peiminine	45.451	0.87	Cerveratrum-type alkaloid
23	Benzoic acid, 2-[(2- bromophenyl)methoxy]-	22.372	0.59	Benzoic acid
24	3H-1,4-Benzodiazepin-2- amine, 7-chloro-Nmethyl-5-phenyl-	37.494	0.57	Benzodiazepine
25	(E)-3,7-Dimethyl-2,6- octadien-1-yl propionate	39.963	0.49	Fatty alcohol ester
26	1,6-Octadiene, 2,6- dimethyl-	7.326	0.47	Unsaturated hydrocarbon
27	Nordazepam	37.244	0.39	Benzodiazepine
28	Lucidin	32.199	0.38	Hydroxyanthraquinone
29	1-Tetradecen-3-yne	30.786	0.28	Enyne
30	3-Methyl-2-nitrobenzyl alcohol	34.081	0.18	Nitrobenzene
31	4H-Pyran-4-one, 2,3- dihydro-3,5-dihydroxy-6- methyl-	6.319	0.14	Dihydropyranone

RT[a]: Retention time in minutes

Table 2. List of compounds detected in GC-MS analysis of HALEE

Sl. No.	Compound name	RT[a] (min)	Area (%)	Type/class
1	2-Cyclohexen-3-ol-1-one, 2-dodecanoyl-	23.060	20.19	Vinylogous acid
2	α-Tocopherol-β-D-mannoside	45.451	12.85	Vitamin E glycoside
3	n-Hexadecanoic acid	25.341	12.50	Long-chain fatty acid
4	Linoelaidic acid	29.542	11.20	Linoleic acid /derivative
5	5-Hydroxymethylfurfural	8.188	9.80	Aryl-aldehyde
6	cis,cis,cis-7,10,13- Hexadecatrienal	29.679	9.06	Fatty aldehyde
7	1-Cyclopentyl-2,2-dimethyl-1-propanol	13.595	3.81	Secondary alcohol
8	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	6.325	3.59	Dihydropyranone
9	D-Glucosamine-1-phosphate	9.438	2.81	Monosaccharide phosphate
10	6-Azathymine	4.988	2.46	Triazine
11	10-Chloro-1-decanol	29.136	1.63	Primary alcohol
12	Nordazepam	37.243	1.26	Benzodiazepine
13	Ethane, 1,2-dibromo-1,2- dichloro-	32.199	1.20	Organochloride
14	Ethyl trans-caffeate	17.308	1.17	Primary amine (Coumaric acid /derivative)
15	2-Ketobutyric acid	10.832	1.02	Short-chain keto acid/ derivative
16	Fumaronitrile	30.780	1.02	Nitrile
17	(-)-Perillyl alcohol	14.614	0.96	Menthane monoterpenoid
18	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	3.487	0.86	Furanone
19	Thiophene, tetrahydro-3-methyl-	2.368	0.73	Thiolane
20	Enniatin B	23.591	0.52	Depsipeptide
21	N-Methyl-L-glutamic acid	2.881	0.43	Alpha amino acid/derivative
22	Benzoic acid, 4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]	26.516	0.26	Diarylthioether
23	Oxygen difluoride	11.482	0.25	Non-metal halide
24	Bicyclo[3.2.0]hepta-2,6- diene	34.087	0.19	Polycyclic hydrocarbon
25	Isobutylamine	16.815	0.10	Monoalkylamine

RT[a]: Retention time in minutes

Table 3. ABTS radical scavenging activity (%) of varying concentrations of ascorbic acid, HAREE and HALEE

Ascorbic acid (µg/mL)	Radical scavenging activity (%)	HAREE (µg/mL)	Radical scavenging activity (%)	HALEE (µg/mL)	Radical scavenging activity (%)
2	21.08 ± 0.10	20	24.08 ± 0.22	100	22.66 ± 0.12
4	39.27 ± 0.15	40	43.69 ± 0.48	200	42.61 ± 0.18
6	65.47 ± 0.26	60	60.99 ± 0.27	300	59.66 ± 0.94
8	89.77 ± 0.13	80	77.96 ± 0.29	400	67.87 ± 0.78
-	-	100	93.48 ± 0.10	500	85.24 ± 0.47

Data are presented as mean ± SEM of 3 different experiments performed in triplicate.

Similarly, the DPPH radical scavenging activity was also found to be higher in HAREE as compared to HALEE. The radical scavenging activity of HAREE ranged from (15.09 ± 0.31 %) at 60 µg/mL to (57.55 ± 1.31 %) at 300 µg/mL while that of HALEE ranged from (23.03 ± 0.55 %) at 400 µg/mL to (74.23 ± 0.19 %) at 2000 µg/mL (Table 4). Similar to the ABTS assay, the radical scavenging activity of ascorbic acid was found to be higher than both HAREE and HALEE with values ranging from (7.76 ± 1.27 %) at 2 µg/mL to (53.96 ± 0.62 %) at 12 µg/mL (Table 4). Therefore, ascorbic acid recorded the lowest IC_{50} value in both the assays as compared to HAREE and HALEE (Table 5).

The Kruskal-Wallis test revealed a significant difference among the different concentrations in all the tested samples and standard (ascorbic acid, HAREE and HALEE) in both the ABTS and DPPH scavenging activities. The Kruskal-Wallis statistic (H), degrees of freedom (df) and the *p* values are reported in Table 6. However, the difference did not reach statistical significance in the Dunn's post hoc test. This may be due to the small sample size (n = 3).

Table 5. IC_{50} values of ascorbic acid, HAREE and HALEE in the ABTS and DPPH assays

IC_{50} (µg/mL)	Ascorbic acid	HAREE	HALEE
ABTS	4.58 ± 0.01	51.23 ± 0.10	279.37 ± 2.42
DPPH	11.32 ± 0.15	248.81 ± 6.23	1189.58 ± 3.23

Data are presented as mean ± SEM of 3 different experiments performed in triplicate.

Table 6. Kruskal-Wallis test statistics of ascorbic acid, HAREE and HALEE in the ABTS and DPPH assays

Assay	Group	Kruskal-Wallis statistic (H)	Degrees of freedom (df)	*p* value
ABTS	Ascorbic acid	13.60	4	<0.0001
	HAREE	16.65	5	0.0052
	HALEE	16.65	5	0.0052
	IC_{50}	7.200	2	0.0036
DPPH	Ascorbic acid	19.69	6	0.0031
	HAREE	16.65	5	0.0052
	HALEE	16.65	5	0.0052
	IC_{50}	7.200	2	0.0036

Table 4. DPPH radical scavenging activity (%) of varying concentrations of ascorbic acid, HAREE and HALEE

Ascorbic acid (µg/mL)	Radical scavenging activity (%)	HAREE (µg/mL)	Radical scavenging activity (%)	HALEE (µg/mL)	Radical scavenging activity (%)
2	7.76 ± 1.27	60	15.09 ± 0.31	400	23.03 ± 0.55
4	17.49 ± 0.57	120	27.03 ± 0.55	800	37.56 ± 0.15
6	25.81 ± 0.74	180	38.65 ± 1.26	1200	52.49 ± 0.08
8	34.48 ± 0.65	240	47.79 ± 1.14	1600	64.32 ± 0.11
10	44.39 ± 0.51	300	57.55 ± 1.31	2000	74.23 ± 0.19
12	53.96 ± 0.62	-	-	-	-

Data are presented as mean ± SEM of 3 different experiments performed in triplicate.

While comparing the different IC_{50} values shown by ascorbic acid, HAREE and HALEE, in both the ABTS and DPPH assays, only ascorbic acid and HALEE showed significant difference in the Dunn's post hoc test ($p = 0.0219$).

Toxicological evaluation using zebrafish embryos

The HAREE was found to be less toxic to zebrafish embryos as compared to HALEE. In case of HAREE, at 96 hpf, the embryos treated with 100 and 200 µg/mL showed no mortality while those treated with 400 µg/mL showed a low mortality percentage of (6.67 ± 6.67 %) which did not significantly differ from that of the negative control. The embryos treated with 4 µg/mL DCA showed a mortality percentage of (93.33 ± 3.33 %) at 96 hpf. A 100 % mortality was observed at the highest tested dose, i.e., 800 µg/mL (Fig. 3A). Despite the low mortality, a significant hatching delay was observed at 72 hpf in the embryos treated with 400 µg/mL HAREE showing a hatching rate of (36.67 ± 8.82 %) which was significantly lower than that of the negative control (100 ± 0 % hatching rate) (Fig. 3B).

In case of HALEE, at 96 hpf, the embryos treated with 200 µg/mL showed a significantly higher mortality rate with a value of (43.33 ± 3.33 %) as compared to that of the negative control. A 100 % mortality was observed in the embryos treated with 300 µg/mL (Fig. 3C). A significant hatching delay was observed in the embryos treated with 150, 200 and 250 µg/mL while embryos treated with 300 µg/mL coagulated before hatching (Fig. 3D).

The embryos treated with 4 µg/mL DCA showed distinct morphological deformities such as yolk-sac edema (YSE) and pericardial edema (PE) as compared to the control embryos (Fig. 4A–D). The embryos treated with 800 µg/mL HAREE showed a distinct developmental delay (Fig. 4E). The embryos treated with 200 µg/mL HALEE showed distinct morphological deformities including upward tail curvature (TC), lordosis (spine curvature (SC)), YSE and PE (Fig. 4F).

The lethal concentration 50 % (LC_{50}) was lower in the case of HALEE as compared to HAREE (Table 7 & 8). The NOAEC (mortality and hatching rate) were respectively 400 and 200 µg/mL for HAREE and those of HALEE were 150 and 100 µg/mL respectively. The LOAEC (mortality and hatching rate) were 600 and 400 µg/mL for HAREE and 200 and 150 µg/mL for HALEE, respectively (Table 9 & 10).

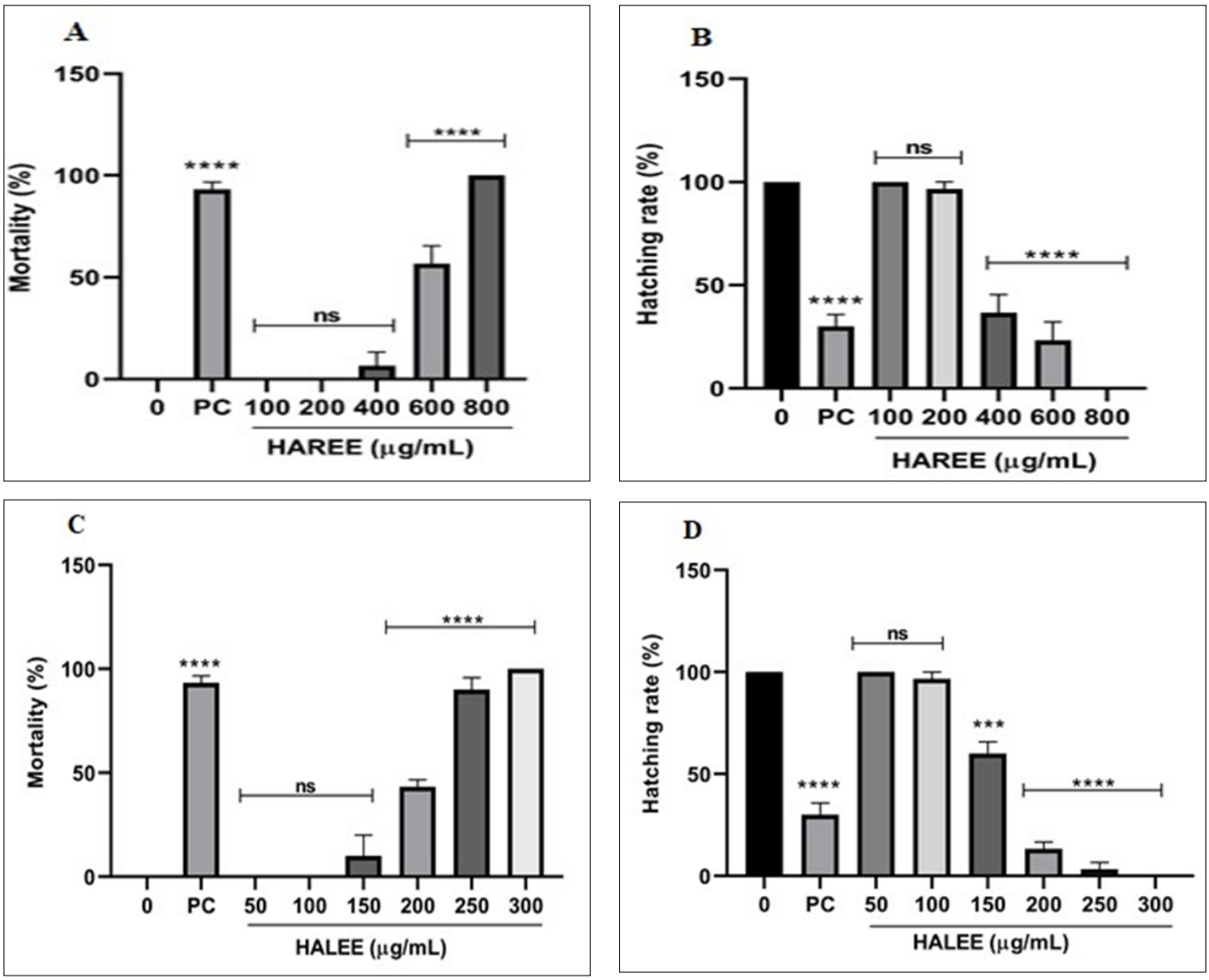

Fig. 3. Embryo and developmental toxicity of HAREE and HALEE in zebrafish embryos.

(A) mortality (%) at 96 hpf; (B) hatching rate (%) at 72 hpf respectively of zebrafish embryos treated with HAREE; (C) mortality (%) at 96 hpf; (D) hatching rate (%) at 72 hpf respectively of zebrafish embryos treated with HALEE. Each bar represents the mean ± SEM. Statistical differences between groups were analysed using Fisher's exact test, $p \leq 0.05$. Significance levels in the figure represent the difference of each column from the negative control column.

*** = $p < 0.001$; **** = $p < 0.0001$; ns = not significant; hpf: hours post fertilisation; PC: positive control (4 µg/mL DCA)

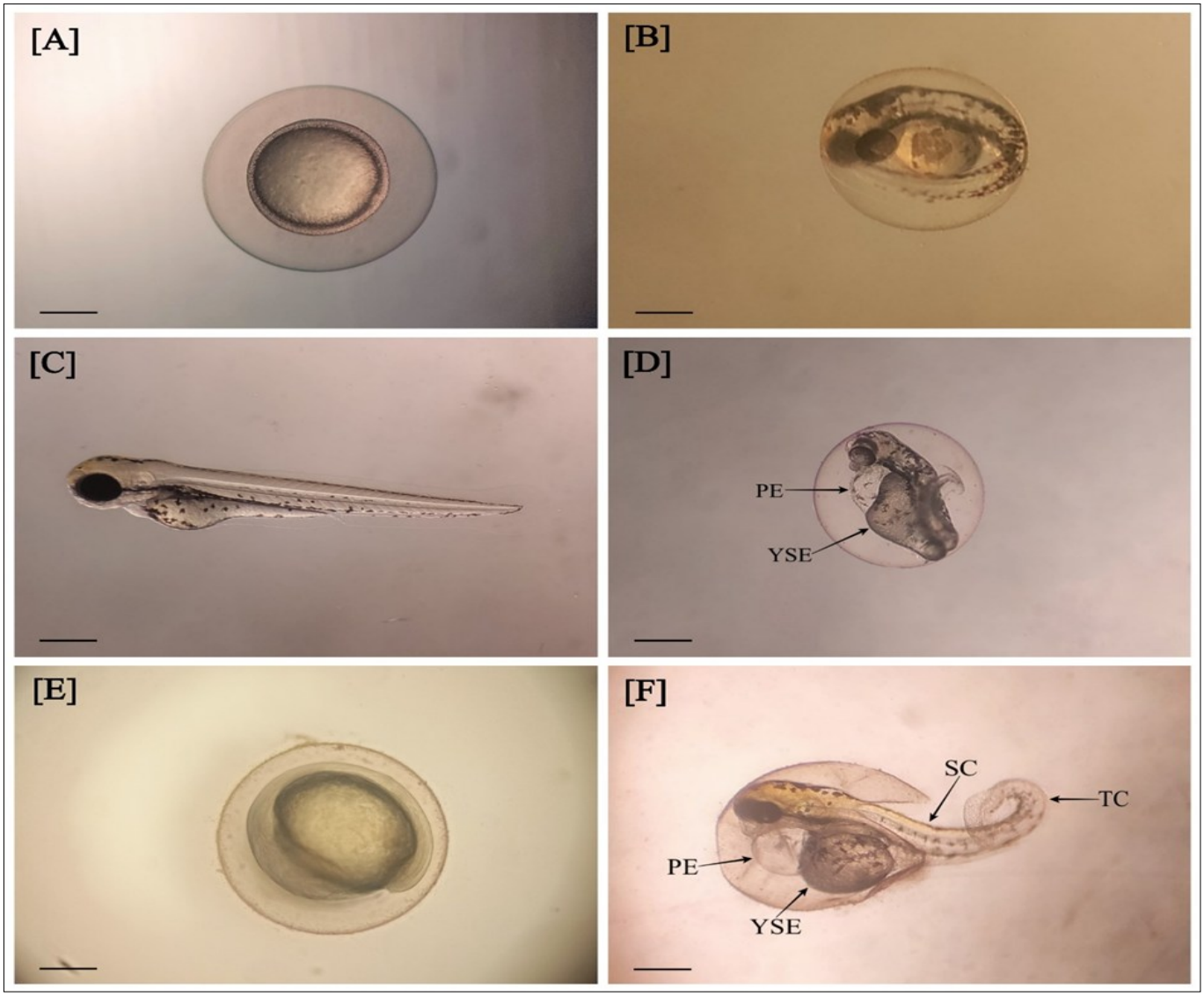

Fig. 4. Representative images of toxicological evaluation of HAREE and HALEE in zebrafish.

(A) 6 hpf embryo; (B) 48 hpf control embryo; (C) 72 hpf control larva; (D) Embryo treated with 4 µg/mL DCA (72 hpf) showing yolk-sac edema, pericardial edema and hatching delay; (E) Embryo treated with 800 µg/mL HAREE (24 hpf) showing developmental delay; (F) Embryo treated with 200 µg/mL HALEE (72 hpf) showing yolk-sac edema, pericardial edema and lordosis with an upwardly curved tail. Scale bar represents 100 µm.

hpf: hours post fertilisation; PE: pericardial edema; SC: spine curvature; TC: tail curvature; YSE: yolk-sac edema.

Table 7. LC_{10}, LC_{50} and LC_{90} values of HAREE in zebrafish embryos

HAREE	Estimated LC value	Lower bound	Upper bound
LC_{10}	435.31	372.39	477.04
LC_{50}	558.31	516.69	599.97
LC_{90}	716.07	658.39	821.64

Table 8. LC_{10}, LC_{50} and LC_{90} values of HALEE in zebrafish embryos

HALEE	Estimated LC value	Lower bound	Upper bound
LC_{10}	156.31	137.81	169.10
LC_{50}	198.81	186.70	210.71
LC_{90}	252.87	235.66	281.81

Table 9. NOAEC and LOAEC of HAREE in zebrafish embryos with respect to mortality and hatching rate

HAREE	NOAEC[b] (µg/mL)	LOAEC[c] (µg/mL)
Mortality	400	600
Hatching	200	400

NOAEC[b]: No observed adverse effect concentration; LOAEC[c]: Lowest observed adverse effect concentration.

Table 10. NOAEC and LOAEC of HALEE in zebrafish embryos with respect to mortality and hatching rate

HALEE	NOAEC[b] (µg/mL)	LOAEC[c] (µg/mL)
Mortality	150	200
Hatching	100	150

NOAEC[b]: No observed adverse effect concentration; LOAEC[c]: Lowest observed adverse effect concentration.

Discussion

The chemical composition of the HAREE was predominantly terpenoid in nature, with linalyl acetate (monoterpenoid), oplopanonyl acetate and (1R,7S,E)-7-isopropyl-4,10-dimethylenecyclodec-5-enol (sesquiterpenoids) identified as the major constituents. Previous studies on the essential oil composition of *H. aromatica* rhizome have also reported the presence of linalyl acetate (20). Linalyl acetate is an acetate ester derived from linalool (21) which is the major constituent of the *H. aromatica* rhizome essential oil (8, 20, 22). The rhizome essential oil has also been reported to be rich in terpenoids such as terpen-4-ol, δ-cadinene, t-muurolol, α-cadinol, viridiflorol, α-selinene, M-cymene, spatulenol and γ-muurolene (20). Similarly, the present study also detected the presence of terpen-4-ol, τ-muurolol, (-)-spathulenol, isospathulenol and τ-cadinol. The certain similarities and differences between the chemical composition of the rhizome essential oil reported in previous studies and the ethanol rhizome extract reported in the present study may be attributed to the method of extraction and nature of solvent used. Essential oil extraction by distillation majorly extracts volatile components while ethanol, being a mid-polar solvent, extracts both polar and non-polar phytochemicals, including less volatile constituents such as fatty acids and esters (13, 23, 24). In contrast, the HALEE exhibited a more chemically diverse profile. The major constituents included 2-cyclohexen-3-ol-1-one, 2-dodecanoyl- (vinylogous acid), α-tocopherol-β-D-mannoside and n-hexadecanoic acid. The heterogeneous nature of the compounds identified in the leaf extract suggests a broader spectrum of phytochemical classes, which may contribute to diverse biological activities.

HAREE showed a higher antioxidant activity in both the ABTS and DPPH assays as compared to HALEE. The IC_{50} values of HAREE and HALEE in the ABTS assay were respectively 51.23 ± 0.10 and 279.37 ± 2.42 µg/mL while the values in the DPPH assay were 248.81 ± 6.23 and 1189.58 ± 3.23 µg/mL, respectively. This might be due to the presence of linalyl acetate in HAREE. Previous studies have reported positive bioactivities, including antioxidant properties, of linalyl acetate. For instance, oral administration of different doses of linalyl acetate markedly mitigated ethanol-induced gastric damage in rats. In addition, pre-treatment with linalyl acetate lessened gastric injury by lowering both gastric fluid volume and total antioxidant status (25). It has also been suggested that linalyl acetate may help in delaying or preventing mild cognitive impairment owing to its antioxidant and anti-inflammatory properties (26). This also aligns with the traditional use of the plant for stomach ailments and inflammatory conditions such as joint pain and asthma.

However, HAREE showed a markedly lower antioxidant potential as compared to the standard ascorbic acid which showed IC_{50} values of 4.58 ± 0.01 µg/mL in the ABTS assay and 11.32 ± 0.15 µg/mL in the DPPH assay. The moderate antioxidant potential observed is consistent with the predominantly terpenoid nature of HAREE. This is in line with previous reports that compared the antioxidant potential of phenolic-rich and terpenoid-rich fractions of essential oils and found that terpenoid-rich fractions had weaker antioxidant properties (27). In addition, it was observed that in all the 3 cases, i.e., ascorbic acid, HAREE and HALEE, the ABTS scavenging activity was higher than that of DPPH. This may suggest that the chemical profile of all 3 tested samples may be more sensitive towards the ABTS cation radical as compared to the DPPH radical. This aligns with previous reports that suggested that the ABTS assay is more flexible because the ABTS cation is sensitive to both hydrophilic and lipophilic antioxidants while the DPPH assay is more suited for hydrophobic antioxidants (28–30). The IC_{50} values of HAREE and HALEE in the ABTS and DPPH assays markedly differs from previous reports. A study assessing the DPPH scavenging activity of *H. aromatica* leaf methanol extract reported an IC_{50} value of 199.51 µg/mL (31). Another study reported significant antioxidant activity of the rhizome ethanol extract at 20 µg/mL in the ABTS and DPPH assays (32). This may be due to the differences in the time and place of collection, method of extraction and type of solvent used. Numerous studies have reported that these factors could greatly influence the chemical composition and antioxidant activity of plant extracts (33–36).

The potential toxicity of HAREE and HALEE was assessed across a range of concentrations maximising at 800 µg/mL for HAREE and 300 µg/mL for HALEE. The higher concentrations were included in this study to determine the thresholds for adverse effects, including the upper toxicity thresholds. In contrast to the antioxidant activity, HALEE was found to have a higher toxic profile as compared to HAREE with LC_{50} values estimated to be 198.81 (HALEE) and 558.31 µg/mL (HAREE) respectively. This may suggest a differential distribution of secondary metabolites between the different plant parts. The more diverse composition of HALEE may contain potential teratogenic compounds that could induce developmental and acute toxicity in zebrafish embryos. A dose-dependent increase in mortality and decrease in hatching rate was observed in both HAREE and HALEE. In case of both HAREE and HALEE, embryos treated with 400 and 150 µg/mL respectively showed a low mortality rate, however, there was a marked delay in hatching rate, indicating sublethal developmental toxicity. Hatching rate is attributed as one of the most important parameters for assessing developmental toxicity (37). Hatching delay might be because of two reasons. Firstly, delayed embryonic development or secondly, inability of the embryos to break through the chorion (38). These observations suggest that the plant extracts could either contain some phytocompounds that could interfere with the normal embryonic development or hinder neuromuscular functions of the embryo, thereby preventing it from breaking through the chorion. Embryos treated with 200 µg/mL HALEE showed YSE, PE and lordosis with an upwardly curved tail. Studies have reported that YSE and PE are due to failures in early osmoregulatory and cardiovascular homeostasis while SC and TCs are due to disruptions in notochord integrity, extracellular matrix and pathways that co-ordinate axial straightening (39–41). This could suggest that HALEE may contain one or more compounds that hinder early-stage homeostasis and normal axial development.

The embryos treated with positive control, i.e., 4 µg/mL DCA showed a mortality percentage of (93.33 ± 3.33 %) at 96 hpf. They also showed distinct morphological deformities such as YSE and PE and hatching delay at 72 hpf as compared to the control embryos. This might be due to disruption of normal developmental pathways. Proteomics studies have revealed that exposure of zebrafish embryos and larvae to DCA alters proteins involved in metabolic processes, hormone metabolism and developmental processes and organogenesis (42). At the molecular and cellular levels, DCA exposure triggers a stress response as evidenced by elevated heat shock protein (Hsp70) levels, thereby reflecting generalised proteotoxic and cellular stress (43). In addition, in

Javanese medaka, exposure to DCA interfered with hormonal activity, thereby suggesting that it could act as an endocrine disruptor, affecting normal development (44). These studies, in addition to the recommendation of the OECD FET test guideline 236, validate the use of DCA as a positive control in the toxicological analysis.

The LOAEC of HALEE with respect to mortality and hatching rate were respectively 200 and 150 µg/mL and those of HAREE were 600 and 400 µg/mL all of which are above the safety limit dose of 100 µg/mL given by OECD (19). However, as observed from these findings, even though the LOAEC for mortality are 200 and 600 µg/mL for HALEE and HAREE, the next lower tested dose of 150 and 400 µg/mL shows sublethal toxicity in both cases. In case of HALEE, the hatching LOAEC is very close to the limit dose assigned by OECD. Therefore, there is a need for chronic and organ-specific toxicological studies to fully ascertain the safety profile of the plant extracts. The higher toxicity shown by HALEE may be attributed to the presence of compounds such as linoelaidic acid (11.20 %) and 5-Hydroxymethylfurfural (5-HMF) (9.80 %). A study in nerve growth factor differentiated PC12 cells has shown that linoelaidic acid increases ROS production, decreases mitochondrial membrane potential and ATP, reduces antioxidant defenses and promotes caspase activity and DNA damage markers thereby leading to apoptosis (45). In a mouse study involving repeated oral gavage, linoelaidic acid has been shown to significantly increase serum triglycerides and LDL-cholesterol, alter gut microbiota profiles, increase oxidative stress and liver inflammation and activate pro-inflammatory signaling pathways (46). In a study using human endothelial cells, linoelaidic acid has been shown to increase superoxide production and NF-κB activation and reduce vascular nitric oxide production leading to endothelial dysfunction and vascular disorders (47).

Previous studies have shown that 5-HMF exhibits toxic effects in zebrafish. Embryonic exposure to 5-HMF led to dose-dependent developmental toxicity including increased mortality, hatching delay, reduced body length, YSE, PE and spine curvature. Behavioural assessment showed reduced locomotor activity. Skeletal staining revealed that 5-HMF significantly inhibited cartilage development and reduced bone mineralisation, particularly in craniofacial structures and axial skeleton. Molecular analyses demonstrated downregulation of key chondrogenic and osteogenic genes. In addition, exposure to 5-HMF led to elevated ROS levels and altered expression of antioxidant defense genes (48). Another study also demonstrated that exposure to 5-HMF resulted in dose-dependent cardiac abnormalities including PE, reduced heart rate, impaired blood circulation and defective vascular development. Histological and fluorescent vascular imaging showed disrupted angiogenesis and abnormal heart morphology. In addition, 5-HMF significantly increased ROS production, induced cardiomyocyte apoptosis. Mechanistic investigation revealed that 5-HMF suppressed the Wnt/β-catenin signaling pathway which is crucial for embryonic cardiogenesis (49).

Therefore, the presence of linoelaidic acid and 5-HMF in HALEE may have contributed to the observed toxic effects through overlapping mechanisms involving oxidative stress, mitochondrial dysfunction and apoptosis, particularly ROS-mediated suppression of expression of genes involved in cartilage formation and bone mineralisation as well as inhibition of Wnt/β-catenin signaling pathway. The disruption of these pathways is a potential mechanistic basis for the toxic effects observed including the morphological deformities such as spine and tail curvatures and PE.

Overall, the chemical composition of HAREE and HALEE differ significantly. The terpenoid-rich composition of HAREE may have contributed to its higher antioxidant potential while the presence of fatty acids such as linoelaidic acid and furan-derived aldehyde 5-HMF may have been responsible for the higher toxic profile shown by HALEE.

Conclusion

The study presented an integrated investigation comprising of GC-MS profiling of phytochemicals, assessment of antioxidant activity and evaluation of toxic potential of *H. aromatica* rhizome and leaf ethanol extracts using zebrafish as model. It has been observed that the different plant parts showed remarkably varying phytochemical composition, antioxidant capacity and toxic properties. The rhizome ethanol extract showed a higher antioxidant activity in both the ABTS and DPPH assays as compared to the leaf ethanol extract. In contrast to the antioxidant activity, the leaf ethanol extract was found to have a higher toxic profile as compared to the rhizome ethanol extract. This finding suggests that the secondary metabolites present in the plant extracts might be responsible for the varying biological activities observed in the plant extracts. In addition, the study reinforces that *in vitro* antioxidant studies such as ABTS and DPPH scavenging activities do not predict *in vivo* safety. In other words, a relatively high *in vitro* antioxidant activity is not synonymous with *in vivo* safety. Therefore, toxicological evaluation in *in vivo* systems is crucial while studying the biological activities of plant extracts. To the best of our knowledge, this study is the first that reports toxicological assessment of *H. aromatica* rhizome and leaf ethanol extracts using zebrafish. In addition, this study also reported the GC-MS profiling and *in vitro* radical scavenging activities of the rhizome and leaf ethanol extracts of *H. aromatica* collected from Manipur, India. These findings are important for comparative analyses of plants collected from different regions with varying micro-climates, soil physico-chemical properties and the rhizosphere microbiome.

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Authors' contributions

SY is responsible for funding acquisition and conceived, designed, conducted the study, analysed and visualised the results and wrote the original draft. BSW contributed to laboratory analysis and writing the original draft. RK contributed to laboratory analysis and critically reviewed the original draft. WWC, ST and OK contributed to data analysis and visualisation. TY and MDD supervised the study, critically reviewed the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Grok to improve language and sentence structure. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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