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Original article

Investigating the role of *Cinnamomum verum* in zebrafish swim bladder development and anti-cancer activity in human lung cancer cell lines

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ABSTRACT

Coughs and allergies are often treated at home with cinnamon (*Cinnamomum verum*). COVID-19 patients were also benefited by the herb. Aspirating *C. verum* powder caused pulmonary toxicity, hypercapnia, and respiratory failure in humans and animals. The toxicity of *C. verum* during fetal lung development is generally unknown. *C. verum's* effects on lung development were studied in zebrafish. Zebrafish lack lungs but have a swim bladder that functions like mammalian lungs. This study examined *C. verum's* role in embryonic lung formation using zebrafish swim bladder development. *C. verum* bark was extracted in methanol, chloroform, ethyl acetate, and hexane. Zebrafish embryos received serial dilutions of these extracts. Methanol extracts of *C. verum* were not harmful, while hexane, chloroform, and ethyl acetate extracts prevented swim bladder formation and caused neurotoxicity in zebrafish embryos. Organogenesis and lung tumorigenesis share biology. The anti-cancer effect of *C. verum* against lung cancer is unclear, hence an invitro cell viability study was performed utilizing three human lung cancer cell lines. All extracts decreased lung cancer cell viability, but hexane extract was most effective, inhibiting growth at IC 50 concentrations below 50 μg/ml. The LD50 dose of hexane extract in zebrafish embryonic toxicity exceeds 100 μg/ml, indicating more activity in cancer than normal cells. The extracts also exhibited significant level of ameliorative activity against CuSO4 induced oxidative stress in live zebrafish larvae. Hexane, ethyl acetate, and chloroform fractions have high cinnamaldehyde levels according to GC–MS analyses. Thus, cinnamaldehyde may be the key element in *C. verum's* lung toxicity and anticancer properties. This study suggested that *C. verum* crude extract or pure cinnamaldehyde could treat lung cancer. The dose in pregnant women must be carefully monitored to avoid teratogenic effects on fetus lung development.

1. Introduction

Several medicinal plants and herbs, including *C. verum*, are used in folk medicine against lung inflammation (Yakhchali et al., 2021). C. *verum* bark powder is routinely used as a home remedy to suppress cough, allergic reactions, and other respiratory disorders (Ranasinghe et al., 2013). *C. verum* was reported as an effective remedy for human lung diseases, and recently it was shown to have beneficial effects against COVID-19 (Barati et al., 2020, Yakhchali et al., 2021). The essential oils and other *C. verum* components possess antibacterial, antifungal, antioxidant, and antidiabetic properties. (Chao et al., 2005, Tung et al., 2010, Lee et al., 2018).

Very recently the pulmonary toxicity of *C. verum* in human have been reported. One study has reported the hypercapnia, lower airway obstruction, and respiratory failure of a pediatric patient who aspirated *C. verum* powdered. (Peir et al., 2022). In 2013 the American Association of Poison Control Centers (AAPCC) issued a warning for possible lung poisoning by *C. verum* after they found alarming signs such as burning, inflammation in the mouth, nose, and throat and fibrosis of lungs in experimental animals (Grant-Alfieri et al., 2013). The toxicity studies conducted in adult animals have shown that the use of *C. verum* is safe (Yun et al., 2018, Abdeen et al., 2019). However, the safety profile, of *C. verum* on embryonic development and specifically on embryonic lung formation, has yet to be discovered.

Swim bladder development in zebrafish (*Danio rerio*) has been suggested as a model of lung injury (Lee et al., 2019). Researchers have used

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zebrafish swim bladders to study human mucosal and fungus infections (Gratacap et al., 2013, Voelz et al., 2015). The swim bladder elastogenesis study provided the option of using this model as an in vivo injury-repair model for lung disease (Perrin et al., 1999). Zebrafish belong to the teleost family of fish, which do not have lungs, instead, they have an organ known as swim bladder. The swim bladder is an internal gas-filled organ that helps control buoyancy in fish. The swim bladders in fish are very much similar in structure and function to mammalian lungs (Daniels et al., 2004). The lungs of higher vertebrates develop similarly to the swim bladders of fish. Both arise as outgrowths from the esophagus, with the glottis occupying the same position (Daniels et al., 2004). Zebrafish embryos were used in this study as an in-vivo lung development model to investigate the toxicity of *C. verum* on the embryonic lung development.

It is known that organogenesis (organ formation) during embryonic development and postnatal carcinogenesis (tumour development) are similar biological events. Moreover, common molecular signatures such as Sonic Hedgehog are conserved between lung cancer and lung development in many species (Powers and Mu 2008, Kugler et al., 2015). We hypothesize if *C. verum* induced lung injury, it may negatively regulate the lung cancer cell proliferation and could be used as an anti-cancer agent against lung cancer. Even though the anticancer activity of *C. verum* has been reported in many cancers (Sadeghi et al., 2019, Caserta et al., 2023). We have not come across any study, reporting the anti-cancer action of *C. verum* in human lung cancer or in lung cancer cell lines. Hence in this study, the anticancer activity of *C. verum* was investigated in three types of human lung cancer cell lines, namely; A549, H-1650 and, H-1975 and a primary non-tumor HUVEC cell was used to evaluate the cytotoxicity of *C. verum* on normal cells.

The anti-inflammatory activity of cinnamon has been reported by in-vitro assays (Abeysekera et al., 2022), however, we have not come across any study, reporting the anti-inflammatory activity of *C. verum* in experimental animals. The zebrafish copper-induced inflammation model has been used previously to investigate the anti-inflammatory action of ethanol extract of *Clerodendrum Cyrtophyllum Turcz* (Nguyen et al., 2020). In this study, the CuSO4-induced oxidative stress in zebrafish embryo was used to evaluate the in vivo anti-inflammatory activity of *C. verum* extracts.

The lung toxicity in children and adult animals due to *C. verum* aspiration or ingestion is well known, but whether *C. verum* affects lung formation during fetus development in pregnant women or animals is largely unknown. It is therefore, this this study was designed to investigate the role of *C. verum* on lung organogenesis by using zebrafish swim bladder as in-vivo lung developmental model. Additionally, the anti-cancer activity of C. *verum* against lung cancer was studied in three different types of human cancer cell lines. Moreover, Zebrafish embryos were also used to examine the in vivo anti-inflammatory effect of *C. verum*. Lastly, the chemical characterization was done to identify major phytochemicals present in various polar fractions of *C. verum to* identify the principal ingredient for its biological activity.

2. Material and methods

The solvents used in this study were HPLC Plus grade and were purchased from Sigma Aldrich. Methanol (646377), chloroform (650498), hexane (34859), and ethyl acetate (650528). Cell culture reagents; DMEM (Dulbecco's Modified Eagle Medium) ThermoFisher cat # 11965092, Penicillin-Streptomycin (10,000 U/mL) ThermoFisher cat # 15140122, Gibco Fetal Bovine serum Thermo Fisher cat # 10270–106, Trypsin-EDTA solution Sigma Aldrich (cat # T4174). The cell culture plates, dishes, and flask from Corning (Corning USA).

2.1. Plant material and extraction

C. verum bark was obtained from Sorrah Saudi Arabia (https://www.sorrah.sa). Sixty grams of the powder was used for each solvent extraction. The extracts were prepared by the Soxhlet extraction method described previously (Farooq Khan et al., 2021).

2.2. Zebrafish

The wild-type zebrafish were obtained from the zebrafish international resource centre in Oregon, USA, and are kept in the animal facility bioproducts research chair, college of Science, Department of Zoology Saudi Arabia. The animals are fed twice daily with the Zeigler zebrafish diet (Zeigler Bros, Inc. Gardners, PA 17324 USA). The zebrafish is maintained by following the local and international regulations regarding the use and care of laboratory animals.

2.2.1. Ethical consideration

Zebrafish embryos are obtained by natural pair-wise breeding of Adult zebrafish. The embryos are screened, and synchronous-stage embryos are used for extract screening. Zebrafish embryos and larvae less than five days post fertilization (dpf) are used in this study, which has been exempted to take the approval from institutional review board as stated in (Strahle et al., 2012, Lackmann et al., 2018).

2.3. Zebrafish embryos treatment

2.3.1. In vivo toxicity assays

The embryos were obtained by natural pair-wise breeding of the fish. The synchronous stage embryos at the shield stage were exposed to serial dilution (1, 5, 15, 50, 150, and 500 μg/ml) of *C. verum* extracts in the embryo medium. The development toxicity of zebrafish embryos was recorded the next day by screening the embryos under a Leica stereo microscope. % lethality, tail detachment, hatching, abnormal organ development and developmental staging criteria were used to record the response of the embryos toward *C. verum* extracts treatment.

2.3.2. Oxidative stress assay

The zebrafish larvae at 3dpf were exposed to CuSO4 (10 μM) by following the method described by (Singh et al., 2022). Briefly, the zebrafish larvae (3dpf) were exposed to CuSO4 (10 μM) for one hour and then co-treated with serial dilution of each extract separately overnight. The CuSO4 (10 μM) only treated embryos served as positive control and un treated embryos served as negative control. The % survival of CuSO4 and C. verum extracts co-treated was compared with CuSO4 alone treated larvae.

2.4. In vitro anti-cancer activity

Three types of human lung cancer cell lines were used to evaluate the anti-cancer potential of *C. verum* extracts on lung cancer. The cell lines are obtained from American type cell collection Human lung cancer cell line A549 (ATCC cat # CCL-185), H-1650 (ATCC cat # CRL-5883), H-1975 (ATCC cat # CRL-5908) and one type of primary cell line HUVEC (ATCC cat # PCS-100–013). Cells were grown in DMEM (Dulbecco's Modified Eagle Medium) in a humified incubator at 37 ℃ with 5% CO2. In vitro cytotoxicity assays were carried out as described previously (Alqahtani et al., 2022). Briefly Cells were seeded at 2×10^4 per well of 6 well cell culture plates, and incubated with serial dilution (0, 0.5, 1.0, 3.0, 9.0, 27.0, 100.0 μg/mL) of extracts for 24 h. Control cells were treated with solvent (methanol 0.5% v/v). Three replicates were done for all experiments. DMEM with 10% FBS and 1% penicillin–streptomycin was used to grow the cells. The anticancer activity of *C. verum* extracts was assessed in vitro by MTT assay. To each well, 10 μL of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) solution (made in PBS) was added and incubated for an additional 4 h. Finally, acidified isopropanol (0.01 N HCl) was added to dissolved MTT (formazan product). The dose–response curve was used to compute IC 50 values using Probit analysis. Cell viability was measured by using following formula.

Table 1
Crude extract yield of *C. verum* fractions.

Solvent	Quantity of *C.verum* powder used (grams)	Yield of extract (grams)	% yield
Methanol	7.1	2.46	34.64
Hexane	7.1	0.95	13.42
Chloroform	7.1	0.43	6.15
Ethyl acetate	7.1	1.00	14.08

$$\text{Cell viability}(\%) = \text{Mean}/\text{Control OD} \times 100$$

2.4.1. Gas chromatography mass spectrophotometry (GC–MS)

The extract was derivatized using Bis (trimethylsilyl)trifluoroacetamide since it contains polar and non-polar analytes. After adding a 1:1 extract-BSTFA ratio, 1 μL of the extract was injected into GC–MS QP2010 GC–MS Shimadzu (Kyoto, Japan). 250 °C GC injection port temperature was used for all samples. Split vent opened in 1.0 min. Ion source and GC–MS interface temperatures were 200 and 220 °C, respectively. The oven temperature was programmed as follows: 40 °C for 1 min, 100 °C at 10 °C/min for 2 min, 165 °C at 10 °C/min for 0 min, 190 °C at 6 °C/min for 3 min, 220 °C at 3 °C/min for 3 min, and 240 °C at 2 °C/min for 1 min. To ensure target compound retention periods for qualitative analysis, scan mode data capture was used. Similarity searches and mass spectrum data from the NIST MS Library was used to identify the compounds present in crude extracts.

2.5. Statistical analysis

All the experiments repeated for at least three times and data are represented as the mean of triplicates ± standard deviation (SD). IC_{50} for cell lines and LD_{50} for zebrafish embryo were calculated using Probit analysis (Mekapogu 2017). Using a 2-tailed Student's *t*-test (GraphPad Prism v6), statistical significance was determined. The following P-values were considered significant: **P < 0.01; ***P < 0.001; ****P < 0.0001.

3. Results and discussion

3.1. Percent yield of different solvent extract

The cinnamon bark weighing 28.4 g was grinded to powder. Around 7 g of powder was used to prepare four types of extracts in solvents of methanol, chloroform, ethyl acetate and hexane. 500 ml of solvent was used for each extract. The percent yield for each solvent fraction is shown in Table 1. The maximum yield was from the methanol fraction (35%) while the least yield was from the chloroform fraction (6%).

3.2. In vivo toxicity of C. verum extracts in zebrafish embryos

Zebrafish embryos were treated with serial dilution of each extract. The response of the embryos varied depending on the extract's nature and the dose used. As shown in Fig. 1 and Table 2, the methanol extract (LD 50 ≥ 1 mg/ml) was safest for the embryos as compared with chloroform, hexane, and ethyl acetate extracts. The chloroform extract was the most toxic, with LD_{50} values of just 2.42 μg/ml. In this study, the chloroform and hexane extract of *C. verum* showed more toxicity as compared to methanol, and ethyl acetate extracts. The hexane and chloroform extracts induced lethality in zebrafish treated embryos at concentrations of more than at 150 μg/ml. All the extracts were safe and did not induce any noticeable phenotype when used ≤ 50 μg/ml.

3.3. Hexane, ethyl acetate, and chloroform extracts of C. Verum induced craniofacial cartilage and notochord abnormities in zebrafish embryos

To investigate whether *C. verum* extracts induced any developmental toxicity (teratogenicity) in zebrafish embryos, the embryos were treated with sublethal a dose of extracts by which the treated embryos survived up to 3 days of treatment. To see the comparative teratogenic effects of

Table 2
Toxicity of zebrafish embryos against different extracts of *C. verum*.

	Extract	LD_{50} (μg/ml)*
1	Ethyl acetate	149.7 ± 0.35
2	Hexane	113.771 ± 0,57
3	Chloroform	112.42 ± 1.03
4	Methanol	850 ± 0.55

*LD 50 is the average of three replicates ± standard deviation.

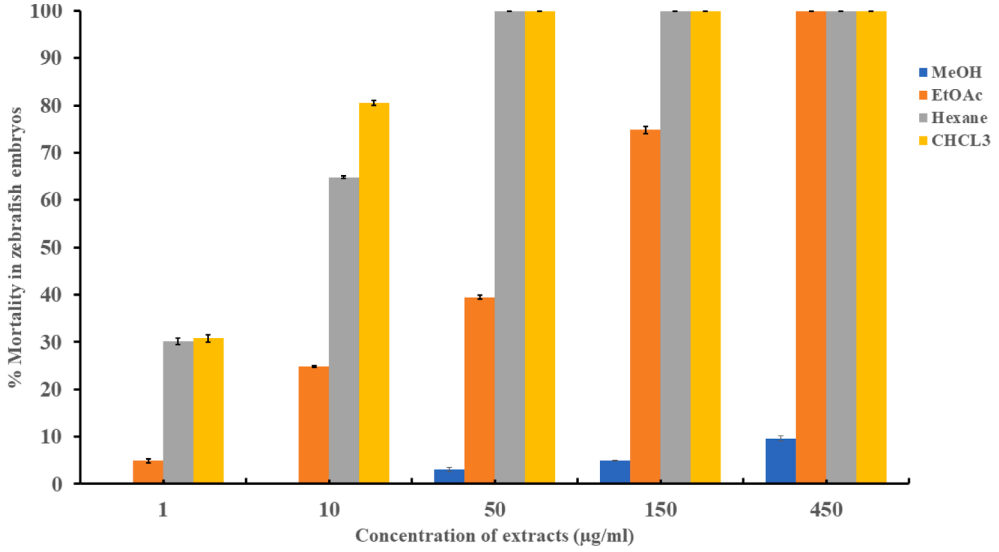


Fig. 1. The dose–response of zebrafish embryos towards different extracts of C. verum. The data presented is the mean of three replicates, and the error bars represent the standard deviation.

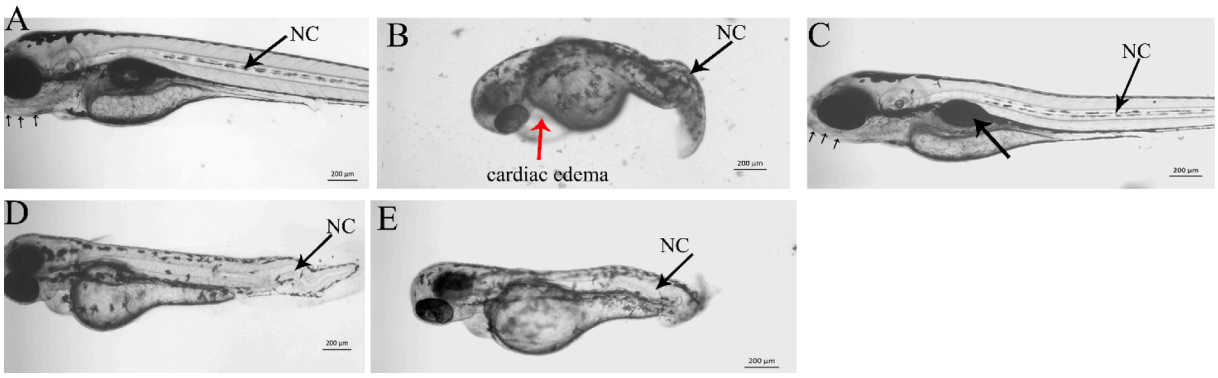


Fig. 2. Craniofacial cartilage and notochord defects induced by *C. verum* extracts in zebrafish larvae. The photomicrograph is the representative image of the live zebrafish larvae snapped at four dpf. A) Control. Small arrows under the mouth indicate the normal development of craniofacial cartilage and the black arrows indicate the notochord (NC). B) Zebrafish embryos treated with 1 μg/ml of chloroform extract. The treated embryos showed severe developmental abrnomalities, smaller as compared to control larvae at the same stage.The posterior trunk did not develop as well. The embryos had severe cardiac edema (red arrow). C) zebrafish embryos treated with 500 μg/ml of methanol extract. The methanol extract did not induce any obvious defects, and the larvae's development and size were the same as control larvae at 4dpf. D) Zebrafish embryos treated with hexane extract. Moreover, craniofacial cartilage was also absent in these embryos. The embryos were smaller size as compared to the control. E). A clear undulation of notochord can easily be visualized in zebrafish embryos treated with ethyl acetate extract. Moreover, the craniofacial cartilage did not form in these larvae. F). All images are taken by keeping the larvae anterior to the left under the same magnification. The scale bar is shown at the right lower side of each image.

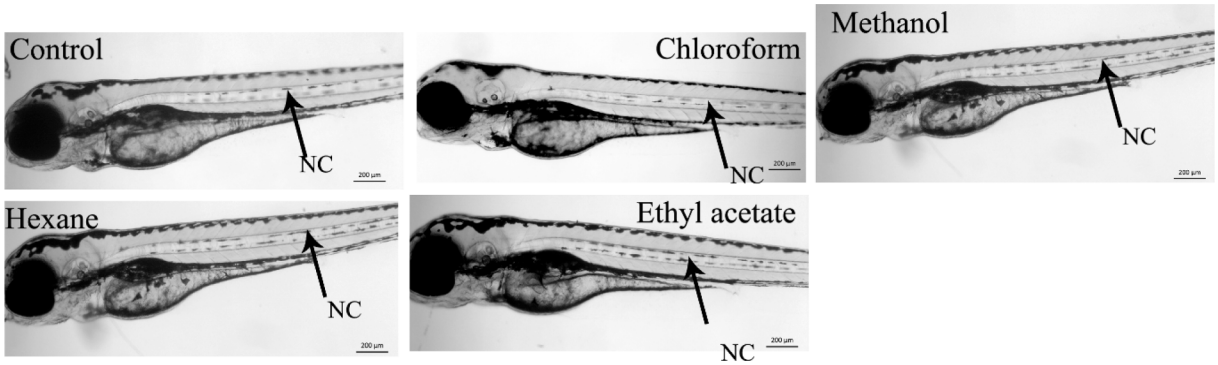


Fig. 3. Solvent toxicity profiling in zebrafish embryos. Representative live images of zebrafish larvae at 3 days post fertilization. Control (no treatment), chloroform, methanol, hexane and ethyl acetate treated with 1% (V/V) solvents alone. The embryos were treated at shield stage (6 h post fertilization) and images were recorded at 3 days post fertilization.

different extracts, the zebrafish embryos were treated with an equal amount (100 μg/ml) of extracts. Each of the extracts was dissolved in methanol to prepare the final working solution, and hence the methanol (0.5% V/V) was used as mock control. As shown in Fig. 2A, no teratogenic effect was observed in mock treated embryos. The notochord in control embryos was straight and connected (NC pointed by black arrow). Similarly, no deformities were noted in craniofacial cartilage in mock-treated embryos (small black arrows under the mouth). The embryos which were treated with methanol extract of *C. verum* did not show any observable embryonic abnormalities (Fig. 2C). The highest level of embryonic abnormalities were observed in zebrafish embryos treated with *C. verum* chloroform extract. As shown in Fig. 2B, comparing to control embryos, these embryos are developmentally retarded and also cardiac edema is quite prominent in these embryos (Fig. 2B white arrow). The posterior trunk did not develop in 100% (n = 150) of chloroform treated embryos resulting in shortened bodies. The hexane and ethyl acetate extracts showed similar types of embryonic abnormalities as observed by chloroform extracts. Fig. 2D and E show the zebrafish embryos treated with hexane and ethyl acetate extract respectively. The craniofacial cartilage did not form in treated embryos and they showed an undulated notochord (black arrow NC).

To evaluate whether the toxic effects were due to the extracts or solvents, the zebrafish embryos were treated with relevant solvents (as mock control for each solvent) which were used for the extraction. The concentration of the solvent was kept equal to the final highest working concentrations of the solvent (50 μL in 5 ml). As shown in Fig. 3, we have not detected any lethality and embryonic abnormalities in solvent alone treated embryos, which means that the teratogenic effects which were observed in zebrafish embryos were specific to the *C. verum*.

C. verum has been extensively studied to investigate its various level of biological activities in many in-vitro and in-vivo systems. However, its safety profile on embryonic development is largely not known. Many studies have reported that *C. verum* did not induce toxicity when tested in adult experimental animals (mostly rodents). One study has reported normal body weight in rats after ingestion of *C. verum* by a 13-week repeat-dose oral toxicity assay. The same study has shown that *C. verum* extract was not mutagenic or clastogenic by in-vitro mammalian cell, and in vivo, bone marrow micronucleus assays (Yun et al., 2018). The aqueous extract of *C. verum* was used to treat female Sprague Dawley rats, and the authors suggested that the *C. verum* extract is safe when used below 0.5 g/kg dose (Abdeen et al., 2019, Hussain et al., 2019). Similarly, the ameliorative activity of *C. verum* essential oil has been demonstrated in rats against carbon tetrachloride-induced hepatoxicity (Bellassoued et al., 2019).

The chloroform extract of hexane in this study induced severe teratogenic phenotype at sublethal dose (100 μg/ml) in zebrafish embryos. Developmental delay was one of the most noticeable effects of chloroform extracts of *C. verum* at this concentration.

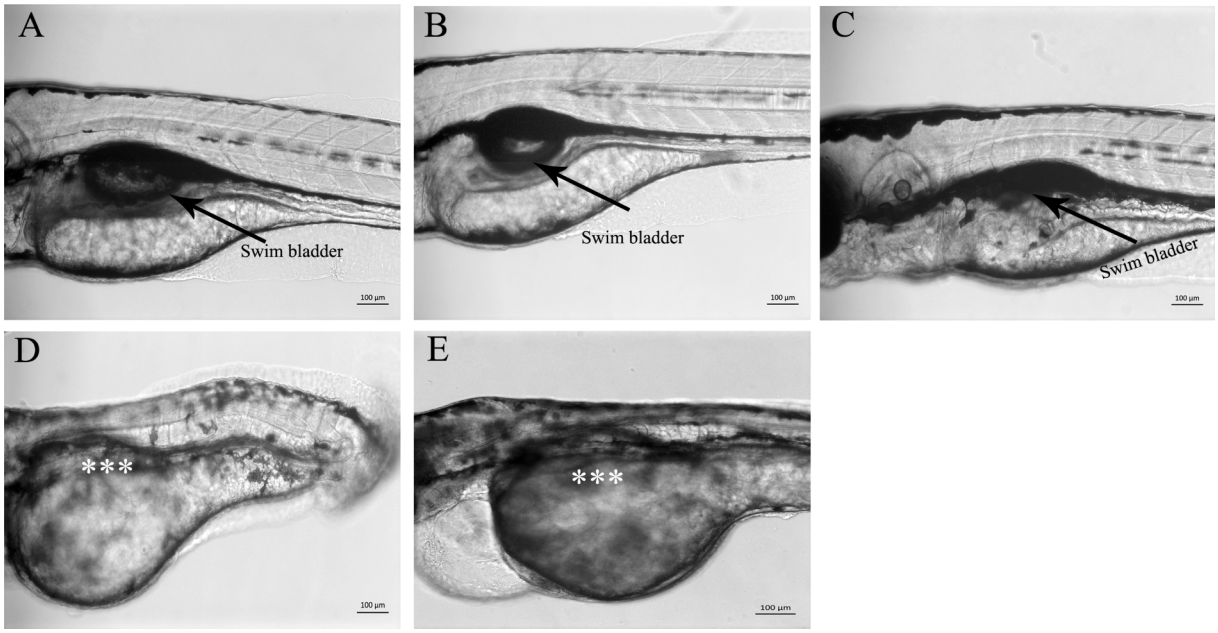


Fig. 4. C. *verum* extracts blocked the formation and growth of swim bladder in zebrafish embryos. Representative images of live zebrafish larvae at 4dpf. Zebrafish embryos were treated with sub-lethal doses of C. verum extracts starting from the shield stage (6 hpf), and swim bladder development in control and treated larvae were recorded at 4dpf. A) Mock (0.5% methanol) treated control larvae with fully developed and inflated swim bladder (black arrow) B) Zebrafish larvae treated with methanol extract of *C. verum* (500 μg/ml). The methanol extract also did not affect the formation and growth of the swim bladder. C) Zebrafish larvae treated with *C. verum* ethyl acetate extract (10 μg/ml) show un-inflated and small-size swim bladder (back arrow) D). Hexane extract (5 μg/ml) treated larvae show the absence of swim bladder at 4dpf (white asterisk). E) Chloroform extract (2 μg/ml) treated larvae shows absence of swim bladder (white asterisk). All images were taken under the same magnification. The scale bar is shown at the bottom right corner.

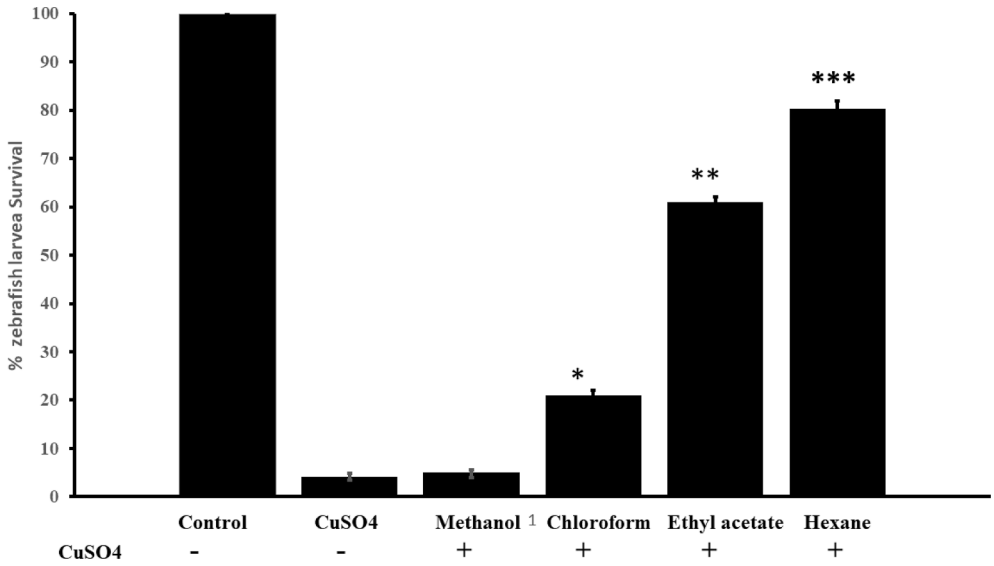


Fig. 5. Organic extract of *C. verum* exhibited the highest anti-inflammatory activity in zebrafish larvae. Zebrafish larvae were exposed to 10 μM of CuSO4 for one hour and then co-exposed to C. verum extracts equal concentration (1 μg/ml) for 24 h. As indicated by bar graph, only 4% of larvae survived after 24 h treatment with CuSO4 alone. 100% larval survival was observed in negative control larvae (not exposed to CuSO4 or C. verum extracts). Methanol extract of *C. verum* showed the weakest anti-inflammatory activity, and only 2% of larvae survived, which were treated with CuSO4 and methanol extract. Hexane extract of *C. verum* showed strong ameliorated activity against CuSO4-induced toxicity with an 80% survival rate. The ethyl acetate showed moderate anti-inflammatory activity against CuSO4-induced toxicity. The data presented are the average of three independent experiments. *: indicates degree of statistical significance (*** p-value 0.0001, ** p value 0.001, * p value 0,01) between the CuSO4 alone and the larvae treated with CuSO4 and *C. verum* extracts. Error bars represent the ± standard deviation between three replicates.

3.4. C. verum extracts perturbed the formation of the swim bladder in treated zebrafish embryos

Zebrafish embryos were treated with an equal concentration (100 μg/ml) of different extracts. The extracts were added at the shield stage (6 hpf), and the experiment lasted for four days, and swim bladder development was evaluated at 4dpf. The Mock (0.05% methanol V/V) treated embryos served as control. The black arrow in Fig. 4A, shows a fully developed and inflated swim bladder (SB) in control larvae. Similarly, methanol extract of *C. verum* did not cause toxicity and swim

Table 3
Cytotoxicity of *C. verum* extracts against human lung and primary cell lines.

Extract	Cytotoxicity IC_{50} (µg/ml)			
	HUVEC	A549	H-1650	H-1975
Methanol	NA*	NA	NA	NA
Chloroform	143.43 ± 0.58	116.72 ± 0.57	134.10 ± 0.58	145.71 ± 0.57
Ethyl acetate	107.69 ± 1.00	114.09 ± 1.52	114.92 ± 1.00	129.62 ± 0.58
Hexane	75.85 ± 0.57	44.30 ± 0.58	42.12 ± 057	30.33 ± 0.57

NA: Not active, Data are presented as the mean of three replication ± standard deviation.

bladder formation in the zebrafish larvae. As the methanol extract did not cause swim bladder formation defects at 100 µg/ml, the concentration of methanol extract was increased up to 1000 µg/ml to see whether it could affect swim bladder development at higher concentration but it was well tolerated by zebrafish larvae and did not cause any obvious developmental defects in 10 times more concentration than other solvents extract. Zebrafish larvae treated with *C. verum* ethyl acetate extract (100 µg/ml) exhibited an inflated but small swim bladder (back arrow Fig. 4C). As shown in Fig. 4D, the swim bladder did not form in zebrafish embryos treated with C. verum hexane extract (100 µg/ml) at 4dpf, (absence of swim bladder is indicated by a white asterisk.) Similarly, the swim bladder did not form in zebrafish embryos treated with chloroform C. verum extract (100 (Fig. 4E).

Teleost (bony) fish do not have lungs, but they have a swim bladder, an organ that functions like the lungs. The swim bladders of fish develop similarly to the lungs of higher vertebrates. The anatomical structure, morphological development, and transcriptional patterns of the zebrafish swim bladder, which functions as a variable buoyancy device, are similar to those of the lung (Zheng et al., 2011, Cass et al., 2013). Both arise as outgrowths from the esophagus, with the glottis occupying the same position (M. 2023). We have not come across any report in which the effect of *C. verum* on embryonic lung development was studied in any animal model. Hence this is the first report demonstrating the role of *C. verum* on lung formation using zebrafish.

3.5. Ani-inflammatory activity of C. Verum extracts in CuSO4 induced oxidative stress in zebrafish embryos

The ameliorative function of *C. verum* against CuSO4 induced toxicity in zebrafish larvae was used as in-vivo model to measure the anti-inflammatory activity of extracts. Zebrafish larvae (3dpf) were exposed to 10 µM of CuSO4 for one hour and then co-treated with C. *verum* extracts with LD_{50} concentration (Table 1) for 12 to 15 h. Untreated larvae were negative control, and CuSO4 alone treated larvae as a positive control. The protective effect of *C. verum* extracts is shown in Fig. 5. Exposure to CuSO4 at a concentration of 10 µM induced 96% mortality in zebrafish larvae in 24 h. In contrast, no mortality was found in the negative control. The methanol extract (500 µg/ml) showed the weakest level of anti-inflammatory activity against CuSO4-induced oxidative stress and only 5% of zebrafish larvae survived by co-administration of methanol extract. Twenty one percent (21%) of zebrafish larvae survived, which were treated with 10 µM of CuSO4 and 110 µg/ml chloroform extract. Ethyl acetate (150 µg/ml) and hexane extract (110 µg/ml) showed strong protective activity against CuSO4-induced toxicity with 61 and 80% larvae survival. The protective action of ethyl acetate and hexane against CuSO4-induced toxicity was statistically significant, with a p-value of 0.05 compared with CuSO4 alone treated larvae.

Many in vitro and in vivo studies have documented *C verum* extracts or their components, especially cinnamaldehyde, as an anti-inflammatory agent (Abeysekera et al., 2022, Chen et al., 2022). Gunawardena *et al.* also reported that the highest level of anti-inflammatory activity was present in the organic fractions than the methanol and water extract *of C. zeylanicum and C. cassia* (Gunawardena et al., 2015). However, there is no report in which the anti-inflammatory activity of *C. verum* has been checked in live animals. Zebrafish possess an excellent in vivo model of inflammation as it shares several inflammatory genetic signatures with mammals (Zanandrea et al., 2020). Copper adversely affects organisms' development, physiology, reproduction, and survival and has immunosuppressive effects on certain fish species (Schink et al., 2018, Hong et al., 2020). Ethanol extract of leaves of C. *cyrtophyllum* showed protective activity against CuSO4 toxicity in 3dpf zebrafish larvae, and researchers proved that the protective activity was due to the high content of flavonoids in ethanol extract of leaves of *C. cyrtophyllum* which resulted in a high level of anti-oxidant (IC_{50} of 16.45 µg/mL) and anti-inflammatory. The co-administration of ethanol extract of leaves of *C. cyrtophyllum* and CuSO4 downregulated inflammatory response genes in zebrafish larvae (Nguyen et al., 2020). Similarly, the protective effect of acacetin against CuSO4-induced toxicity was also attributed to the anti-inflammatory activity of acacetin. The co-administration of acacetin resulted in upregulation of anti-oxidant genes and suppressing pro-inflammatory response genes in CuSO4-exposed zebrafish larvae (Singh et al., 2022).

Table 4
Methanol fraction.

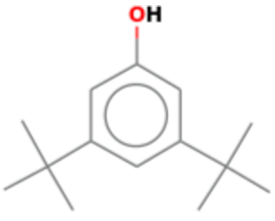
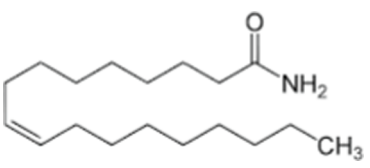
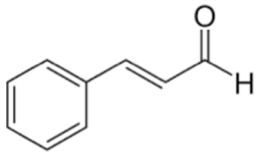
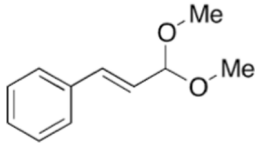
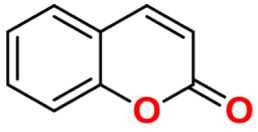
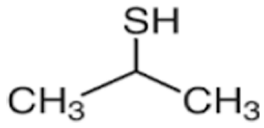
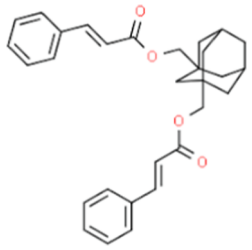
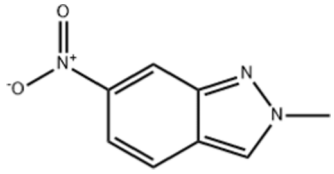
No	Retention Time	Compound in %	Compound Name	Formula	Structure
1	15.82	9.74	Phenol, 3,5-bis(1,1-dimethylethyl)	C14H22O	
2	29.697	90.26	9-octadecenamide	C18H35NO	

Table 5
Chloroform fraction.

No	Retention Time	Compound in %	Compound Name	Formula	Structure
1	10.767	9.10	Cinnamaldehyde	C9H8O	
2	13.535	69.31	Cinnamaldehyde dimethyl acetal	C11H14O2	
3	14.290	4.33	2H-1-Benzopyran-2-one	C10H9NO2	
4	14.4870	1.55	2-Thiapropane	C3H8S	
5	16.618	3.50	1,3-Bis(cinnamoyloxymethyl)adamantane	C30H32O	
6	18.082	12.2	2-METHYL-6-NITRO-2H-INDAZOLE	C8H7N3O2	

3.6. The anticancer activity of C. Verum against human lung cancer cell lines

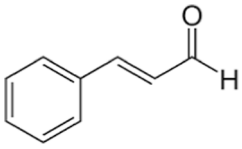
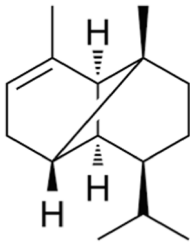
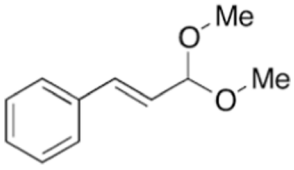
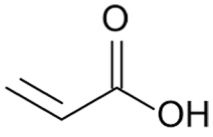
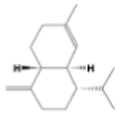
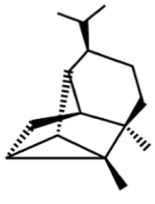
As presented in the previous section, most of the *C. verum* extracts affected the swim bladder formation in zebrafish embryos. We next tested whether these extracts could induce cytotoxicity in human lung carcinoma cell lines? Three types of human lung carcinoma cell lines, namely A549, H-1650, and, H-1975, and a primary cell (non-cancer) human umbilical vein endothelial cell (HUVEC), were selected for such investigation.

The 50% inhibitory concentration (IC_{50}) of the extract against lung cancer cell and primary cell lines are presented in Table 3. The hexane extract of *C. verum* was most active against A549, H-1650, and H-1975 with IC_{50} values of 44.30 ± 0.58, 42.12 ± 057, 30.33 ± 0.57 μg/ml respectively. The methanol extract was not active in all three types of lung cancer cell line and HUVEC. The cytotoxicity *C. verum* extract in human lung cancer cell lines indicates that the co– related with the in vivo toxicity profile of these extracts in zebrafish embryos. The hexane extract showed maximum level of cytotoxicity with minim concentration as compared to chloroform, ethyl acetate and methanol. Its LD_{50} values in zebrafish embryos and IC_{50} concentration in HUVEC cell were more than almost double as compared its IC50 values in cancer cell lines, which shows that hexane extract showed higher toxicity towards cancer cell the normal cell.

The anti-cancer activity of cinnamon against human cancer cell lines and xenograft cancer models has been reported previously. *Cinnamomum cassia* essential oil (CEO) infused chitosan nanoparticles suppressed the growth of 4T1 breast cancer cells in a mice xenograft model via

inhibiting the expression of the Ki-67 protein (Xu et al., 2023). Cinnamaldehyde, the main ingredient has been suggested for the treatment of breast Cancer in women (Liu et al., 2020). The aqueous extract of cinnamon has been shown to inhibit the proliferation of bladder cancer 5637 cells by inhibiting glycolysis and induction of apoptosis (Aminzadeh et al., 2022). Proteasome inhibition by aromatic monophenols (procyanidin-B2), from cinnamon induced the death of prostate cancer cells by autophagy-dependent apoptosis (Gopalakrishnan and Ismail 2021). The anti-proliferative function of the cinnamon extract was also reported in three hematological cancer cell lines (Schoene et al., 2005).

Table 6
Hexane fraction.

No	Retention Time	Compound in %	Compound Name	Formula	Structure
1	10.743	11.6	Cinnamaldehyde	C9H8O	
2	13.059	8.50	Copaene	C15H24	
3	13.535	64.50	Cinnamaldehyde dimethyl acetal	C11H14O2	
4	14.833	2.20	2-Propenoic acid	C3H4O2	
5	15.144	3.23	1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.beta.,8a.alpha.)-	C15H24	
6	15.619	3.01	(+)-Cyclosativene	C15H24	

(*continued on next page*)

Table 6 (*continued*)

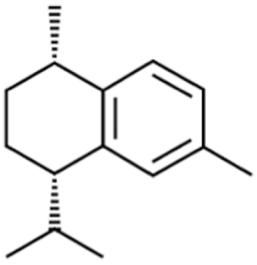
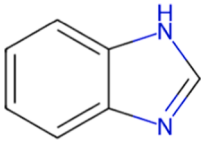
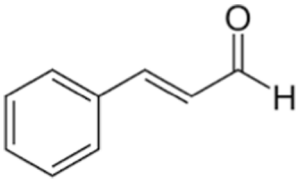
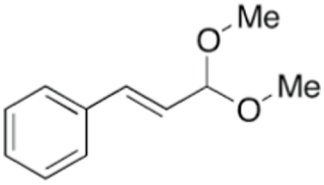
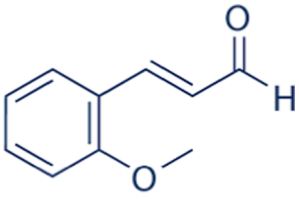
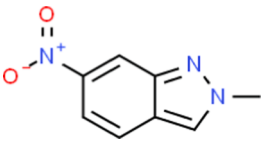
No	Retention Time	Compound in %	Compound Name	Formula	Structure
7	16.06	6.6	1 S-CIS-CALAMENENE	C15H22	
8	18.094	4.73	1H-Benzimidazole	C7H6N2	

Table 7
Ethyl acetate fraction.

No	Retention Time	Compound in %	Compound Name	Formula	Structure
1	10.767	4.819	Cinnamaldehyde	C9H8O	
2	13.528	78.734	Cinnamaldehyde dimethyl acetal	C11H14O2	
3	16.174	1.755	ORTHO METHOXY CINNAMIC ALDEHYDE	C10H10O2	
4	18.094	14.692	2-METHYL-6-NITRO-2H-INDAZOLE	C8H7N3O2	

3.7. Chemical composition of C. verum in different solvent fractions

The biological activity of any crude extract is due to the presence of one or many phytochemicals in the crude extract. So, it is important to know what kind of phytochemicals are present in the crude extract. Gas chromatography coupled with mass spectrophotometry (GC–MS) is any easy technique to identify the different active compounds especially the volatile compounds in crude extract. The four solvent fractions (methanol, chloroform, ethyl acetate and hexane) of *C. verum* were analyzed by GC–MS and results are presented in Tables 4–7. We detected only two peaks in the methanol fraction and the identified compounds are Phenol, 3,5-bis(1,1-dimethylethyl), and 9-octadecenamide. Both of these compounds have been reported previously from the *C. verum* bark. The Phenol, 3,5-bis(1,1-dimethylethyl) from the bark of specie *Cinnamomum Zeylanicum* (Hameed et al., 2016), and 9-octadecenamide was identified in small quantity from the bark of *C. cambodianum* and *C. caryophyllus* (Son et al., 2014).

The Cinnamaldehyde was found in abundance in chloroform, hexane and ethyl acetate fractions. These fractions have Cinnamaldehyde mostly in cinnamaldehyde dimethyl acetal form. Cinnamaldehyde converted to Cinnamaldehyde dimethyl while using methanol for GC–MS which is a common phenomenon also has been reported previously (He et al., 2021). That mean that cinnamaldehyde dimethyl acetal is actually Cinnamaldehyde itself and is present from 60 to 80% in these fractions.

Several studies have reported cinnamaldehyde as the major ingredient (present as 65.00 to 80.00% in bark) and most of the biological properties of C. *verum* are attributed towards cinnamaldehyde (Singh et al., 2007, Rao and Gan 2014, Lu et al., 2022). We have observed the craniofacial cartilage and notochord deformities in zebrafish embryos exposed to 100 μg/ml of hexane, ethyl acetate and chloroform crude extract of *C. verum.* Surprisingly such type of deformities in zebrafish larvae were observed when zebrafish embryos were treated with pure cinnamaldehyde ((Bhattacharya et al., 2021)., which means that these toxicities are mainly due to cinnamaldehyde, and thus care should be taken by pregnant mothers, while using C. *verum* as whole or pure cinnamaldehyde. The neurotoxicity in zebrafish embryos upon exposure to cinnamaldehyde is also reported. The treated embryos had damaged ventricular structures, abnormal eyes, shortened body length, undulated trunk, and pericardial edema in zebrafish (Chang et al., 2022). We also have observed similar kind of neurotoxicity in treated embryos by hexane extracts. We have performed an online program (http://www.swisstargetprediction.ch/) to identify the protein targets in humans using the chemical structure of Cinnamaldehyde. The results have been shown as Supplementary Table S1. The Swiss protein target prediction shows the high probability of "Transient receptor potential cation channel subfamily A member 1 (TRPA1)" being a specific target of cinnamaldehyde. TRPA1 channel plays a vital role in regulating brain development and the physiological function of astrocytes (Shigetomi et al., 2011). Hence, it could be postulated that cinnamaldehyde present in *C. verum* could have blocked the activity of TRPA1 which resulted the neurotoxicity in treated zebrafish larvae. One study also reported the inhibition of angiogenesis blood vessels in transgenic zebrafish line (*flk1:GFP*) embryos by high doses of *C. verum* extract (Bansode et al., 2013). We have not observed any defects in angiogenesis blood vessels by exposure of *C. verum* extracts to TG (*fli; EGFP*) zebrafish embryos (data not shown). This could be due to the nature of the solvent used, as we have not used the DMSO, or the angiogenesis defects could be due to secondary affect due to the high dose of *C. verum*. The neuro and cardiovascular toxicity with high dose of *C. verum* in zebrafish embryos has been reported by other studies, but we have not found any study which reported the effect of *C. verum* on lung toxicity in embryos. This study is unique in the sense that the effect of *C verum* on zebrafish lung (swim bladder) development was being reported for the first time an also its anticancer potential against lung cancer.

4. Conclusion

Cinnamon (C. verum) is a popular home remedy for cough and allergies, and has been reported to have beneficial effects against COVID-19. However, the toxicity of C. verum during fetus lung development is not well understood. Zebrafish were used as an in vivo animal model to investigate the role of C. verum in embryonic lung development. *C. verum* extracts of variable polarity were Mreapted using methanol, chloroform, ethyl acetate, and hexane solvents. Methanol extracts did not show any toxicity towards zebrafish. However, hexane, chloroform, and ethyl acetate extracts blocked the formation of swim bladder and induced neurotoxicity in treated zebrafish embryos.. The invitro cell viability data from this study shows that hexane, chlorforom and ethyl acetate fractions inhibited the cell viability of three lung cancer cell lines. The GC–MS analysis showed the abundance of cinnamaldehyde compound in hexane, ethyl acetate, and chloroform fractions, suggesting that cinnamaldehyde could be the main ingredient responsible for lung toxicity and anticancer activity of *C. verum*. The hexane, chloroform, and ethyl acetate extract of C. verum also showed significant level of ameliorative activity against CuSO4-inducedd oxidative stress representing strong anti-anti-inflammatory action of these extracts.

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6. Institutional review board statement

Zebrafish embryos and larvae that are used in this study are younger than five days post fertilization (dpf), which does not need approval from the institutional review board (IRB) as stated [19, 20].

Data availability statement

All data reported in this study has been included in the manuscript. Original images can be obtained from the corresponding author by reasonable request at any time.

Declaration of competing interest

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

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2023.105361.

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