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Ecotoxicological evaluation of an aqueous phytoextract of *Melia azedarach* L.

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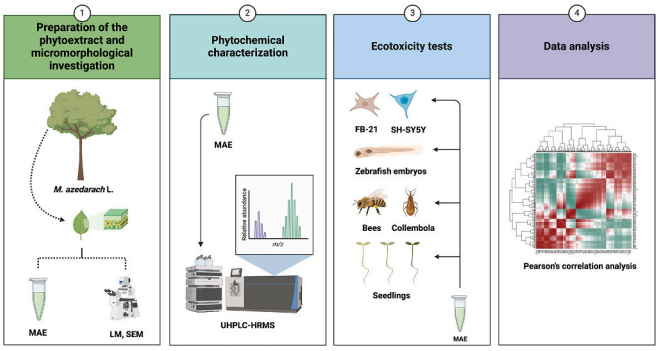
HIGHLIGHTS

- *Melia azedarach* extract (MAE) demonstrated cytotoxic effects on both human fibroblasts and neuroblastoma cells.
- *Danio rerio* embryos exposed to high concentrations of MAE exhibited mortality and significant alterations.
- MAE significantly inhibited seed germination and root elongation in three plant species: *Brassica rapa*, *Sorghum vulgare*, and *Cucumis sativus*.
- No toxic effects were observed in *Apis mellifera* and *Folsomia candida* subjected to chronic exposure to MAE.

GRAPHICAL ABSTRACT



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ABSTRACT

Melia azedarach L. is a Meliaceae that has shown important insecticidal activities. However, few researchers have extensively studied the toxicology of aqueous extracts of *M. azedarach* (MAE). Therefore, the main objective of

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this study was to characterize the phyto-eco-toxicological profile of MAE. First, a botanical and phytochemical characterization of MAE was performed using a histological, and metabolomic multi-analytical approach. Second, the toxicological effects on pollinating insects (*Apis mellifera ligustica*) and soil collembola (*Folsomia candida*) were evaluated. In addition, acute toxicity was evaluated in zebrafish (*Danio rerio*) to assess effects on aquatic fauna, and toxicity was determined in human neuroblastoma (SH-SY5Y) and fibroblast (FB-21) cell models. Finally, phytotoxic effects on germination of *Cucumis sativus* L., *Brassica rapa* L. and *Sorghum vulgare* L. were considered.

Metabolomic analyses revealed the presence of not only limonoids but also numerous alkaloids, flavonoids and terpenoids in MAE. Histological analyses allowed us to better localize the areas of leaf deposition of the identified secondary metabolites. Regarding the ecotoxicological data, no significant toxicity was observed in bees and collembola at all doses tested. In contrast, severe cardiac abnormalities were observed in zebrafish embryos at concentrations as low as 25 μg/mL. In addition, MAE showed toxicity at 1.6 μg/mL and 6.25 μg/mL in FB-21 and SH-SY5Y cells, respectively. Finally, MAE inhibited seed germination with inhibitory concentrations starting from 5.50 μg/mL in *B. rapa*, 20 μg/mL in *S. vulgare*, and 31 μg/mL in *C. sativus*.

Although *M. azedarach* extracts are considered valuable natural insecticides, their ecological impact cannot be underestimated. Even the use of an environmentally friendly solvent (an aqueous solution), for the first time, is not without side effects. Therefore, the data collected in this study show the importance of evaluating the dosages, modes of administration and production methods of *M. azedarach* phytoextracts in agricultural settings.

1. Introduction

Current agricultural practices, such as planting monocultures and the wide use of fertilizers and pesticides, have negative environmental impacts as they pollute air, soil, water resources, and reduce the biodiversity of ecosystems; also, they are no longer efficient in response to climate change(Tilman et al., 2017). The rising cost of fertilizers and, more importantly, the demand for food free from synthetic pesticides has led to the search for an alternative approach to solve these problems. In many parts of the world, natural products are used as alternatives to synthetic pesticides to counteract the advance of harmful insects to humans, animals, or crops. For instance, plant extracts from *Fabaceae, Apocynaceae*, *Meliaceae*, and *Asclepiadaceae* have long been used to control *Locusta migratoria* (Abdellaoui et al., 2019), *Aedes albopictus* (Pham et al., 2023), *Anopheles stephensi* (Kusman et al., 2023), *Agrilus planipennis* (McKenzie et al., 2010), *Drosophila melanogaster* (Bezzar-Bendjazia et al., 2016), *Atta sexdens* (Amaral et al., 2018), and *Spodoptera frugiperda* (Zhao et al., 2022). However, not all natural substances are devoid of environmental side effects. Plant-based insecticides can upset the balance in aquatic (where many insects breed) or soil ecosystems by interfering with natural biological controls or beneficial insects (such as bees) (Stockstad, 2021).

Plants belonging to the *Meliaceae* family are known for their insect-repelling qualities, which make them virtually immune to attacks by phytophagous insects (Dadé et al., 2018). This feature has been used by farmers in many parts of the world for the protection of grain as well as horticultural crops (Isman, 2005). The most widely cultivated and studied *Meliaceae* worldwide are *Azadirachta indica* A. Juss. (Neem) and *Melia azedarach* L. In particular, *Melia azedarach* (MA) (Fig. 1 A), also known as the “chinaberry tree”, is a deciduous tree, native to Asia and northern Australia, but also present in Africa, Europe, North and South America (Orwa et al., 2009). It typically reaches a height of 7–15 m and can be recognized by its greyish bark on the trunk and mature branches, reddish bark on young branches, lanceolate green leaves, light purple flower clusters, and spherical drupes (Orwa et al., 2009). The efficacy of extracts from fruits and leaves of *M. azedarach* has been confirmed against larvae and adults of many insects, such as: *Atta sexdens* (Amaral et al., 2018), *Liriomyza huidobrensis* (Banchio et al., 2003), *Drosophila melanogaster* (Bezzar-Bendjazia et al., 2016), *Spodoptera frugiperda* (Bullangpoti et al., 2012), *Sesamia nonagrioides* (Juan et al., 2000), *Agrilus planipennis* (McKenzie et al., 2010) and *Cnaphalocrocis medinalis* (Nathan, 2006). The insect-repelling effect of aqueous and organic MA phytoextracts is predominantly linked to the presence of limonoids, which have insect antifeedant activity (Bohnenstengel et al., 1999; Huang et al., 1994; Itokawa et al., 1995). Among these, the triterpenoid azadirachtin (AZA) ranks among the most biologically active compounds of this plant (Morgan, 2009) and has been extensively studied. This compound serves as an insect growth regulator, disrupting the typical development of insects, such as *Aedes, Anopheles,* and *Culex* mosquitoes (Chaudhary et al., 2017). AZA and other limonoids derived from *Meliaceae* have demonstrated effectiveness against a range of insects, including *Agrilus planipennis* (Emerald ash borer) (McKenzie et al., 2010), *Drosophila melanogaster* (Fruit fly) (Bezzar-Bendjazia et al., 2016), *Atta sexdens* (Leaf cutter ant) (Amaral et al., 2018), and *Spodoptera frugiperda* (Fall armyworm) (Zhao et al., 2022). However, the chemical composition of these plants and their products has been elucidated only partially, hence it remains possible that other secondary metabolites such as flavonoids, quinones, alkaloids, steroids, and conjugates (Muhammad et al., 2015; Peng et al., 2021) could contribute to the insecticidal properties. On the other hand, as observed for other natural pesticides (Catania et al., 2023), *Meliaceae* extracts can be toxic also for humans (Phua et al., 2008) and other species such as plants, pollinators and other ecologically valuable insects, despite their wide use. However, there is currently a lack of data concerning this aspect. Some authors have investigated the anti-tumoral properties of MA ethanolic phytoextracts (Ervina et al., 2020; Song et al., 2022), while others have examined their antibacterial (Della Bona and Nedel, 2015) and antiviral (Hemdan et al., 2023) properties, and still others have explored potential medical applications (Khan et al., 2014; Lee et al., 2022). However, their ecotoxicological properties have been limitedly explored.

The purpose of this manuscript was to determine whether an eco-friendly extract (an aqueous solution) of *M. azedarach* can be toxic for some organisms (plants and animals) that can be present in agricultural ecosystems. Additionally, to evaluate toxicity through the major routes of exposureto pollutants in humans, the cutaneous and neuronal toxicity of MAE phytoextracts will also be tested in various human cellular models. To date, few authors have produced *M. azedarach* extracts in aqueous solution and limited studies have evaluated its ecotoxicological impact, including the effects on beneficial insects or other agronomic crops.

2. Materials and methods

2.1. Plant material and extraction method

Aerial parts of *Melia azedarach* L. were collected in May 2023 in Leno, Brescia, Italy (45°21′52.3″ N, 10°11′53.9″ E.). One part (two kg) was allocated for aqueous extraction while the other part was used for the micromorphological investigation. The plant material was crushed, placed in a sealed flask, and macerated in 3.5 L of water for 48 h at room temperature. A sample of the extract was stored at the Department of

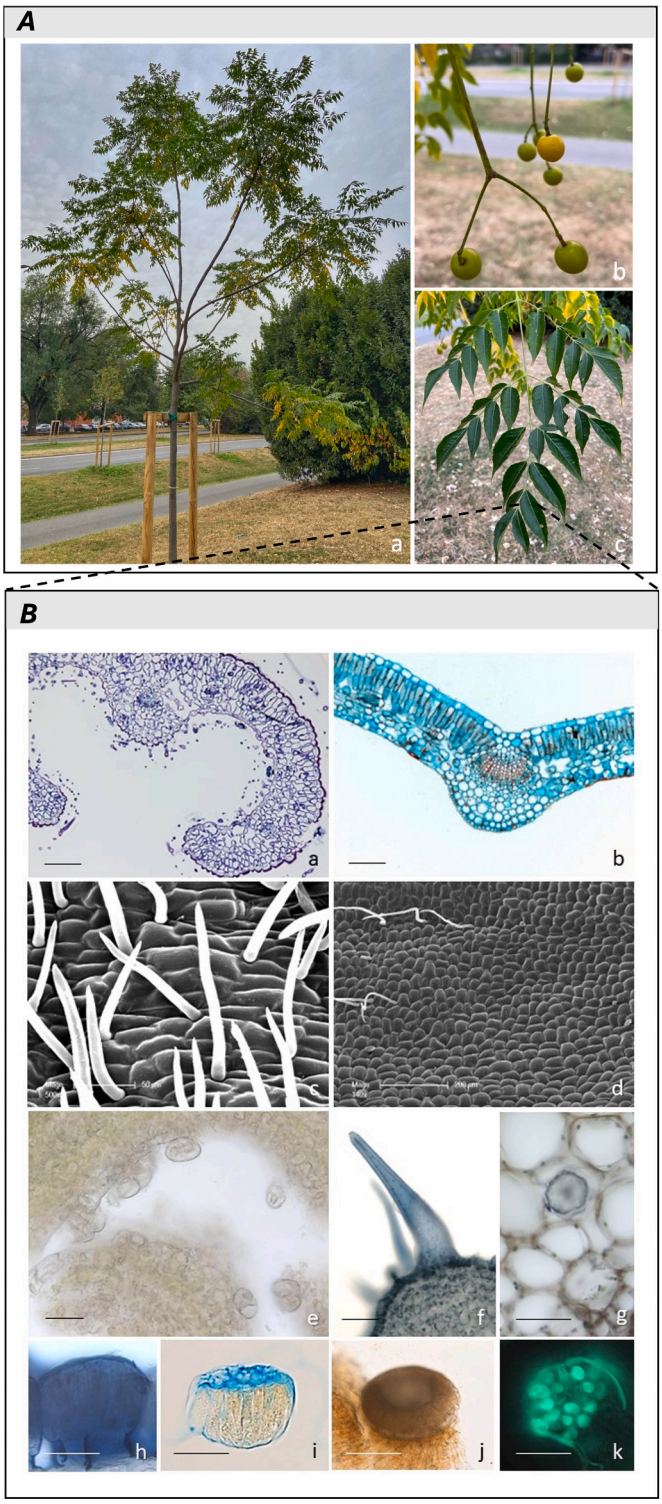

Fig. 1. A (a-c): *Melia azedarach* L.: whole plant (a), fruits (b) and leaves (c). B (a-k): a-g. Micromorphology of *Melia azedarach* leaflet. a, b LM micrographs showing cross sections of a young leaflet stained with Toluidine Blue (a) and of a full-expanded leaflet stained with Safranin O-Fast Green (b); c, d. SEM micrographs showing the epidermis of the abaxial lamina (c) and of the adaxial lamina (d). e-g LM micrographs showing cross sections of a young leaflet with glandular trichomes (e, colorless), of a non-glandular trichome (f, stained with Toluidine Blue) and a detail of a druse in the mesophyll (g, colorless). h-k LM micrographs showing the histochemistry of the glandular trichomes on young leaflets: Nadi reagent (h), Alcian Blue (i), Wagner Reagent (j), $AlCl_3$ (k). *Scale bars: 200 mm (d); 100 mm (a, b); 50 mm (c, e, g); 25 mm (f, h-k).*

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2.2. Micromorphological investigation

The micromorphology of *M. azedarach* leaves was studied using scanning electron microscopy (SEM), light microscopy (LM), and fluorescence microscopy (FM), alongside a histochemical survey. Both fresh material and fixed samples embedded in HistoResin (Technovit® 7100) were used for this purpose.

2.2.1. Scanning Electron Microscopy (SEM)

Leaflets were hand-prepared, fixed in FAA solution (formaldehyde: acetic acid: ethanol 70 % = 5: 5: 90) for 72 h, dehydrated in an ascending ethanol series up to absolute, and critical-point dried. The samples were mounted on aluminum stubs and gold coated. At least ten trials/replicates were analyzed to evaluate the variability level of the micromorphological features. Observations were performed under a Zeiss® EVO MA15 SEM operating at 10 kV at the Interdepartmental Center for Electron Microscopy and Microanalysis Services (M.E.M.A.) of the University of Florence (Florence, Italy).

2.2.2. Light Microscopy (LM) and Fluorescence Microscopy (FM)

Free-hand sections of fresh leaflets and fixed samples were subjected to histochemical assays to detect the sites responsible for the production of the main secondary metabolites.

For the fresh material, free-hand sections obtained with a razor blade were used. Samples were also fixed in F.A.A. solution (Formaldehyde: Acetic Acid: Ethanol 70 % = 5:5:90) for 48 h at 4 °C. Subsequently, the samples were washed in 70 % ethanol for 24 h; they were then progressively dehydrated with 80 % ethanol for 2 h, 95 % ethanol for 2 h, and then twice in absolute ethanol for 2 h/each. Pre-embedding was performed first with ethanol and HistoResin in a 1:1 ratio for one night, then with 1:2 ratio for 2 h and in pure HistoResin for 3 h. Finally, the embedding was done in a polypropylene capsule with the addition of hardener in a 1:15 ratio of basic resin. The HistoResin samples were sectioned using an ultramicrotome.

The following dyes were employed: Toluidine Blue and Safranine O-Fast Green as general stains (Mazzi and Beccari, 1966); Fluoral Yellow for total lipids (Brundrett et al., 1991), Nile Red for neutral lipids (Greenspan et al., 1985), Nadi reagent for terpenes (Carde and David, 1964), Ruthenium Red (Jensen, 1962) and Alcian Blue for acid polysaccharides (Mazzi 1966), Ferric Trichloride for polyphenols (Gahan, 1984), Aluminum Trichloride for flavonoids (Delaveau et al., 1971), and Wagner reagent for alkaloids (Mazzi and Beccari, 1966). Ten trials/replicates were performed for each reagent. Fresh, unfixed, and unstained sections of leaves and leaflets were used as a negative control. Observations were made under the Leitz DM-RB Fluo Optic microscope equipped with a Nikon digital camera.

2.3. Phytochemical characterization of extracts

2.3.1. HPLC-DAD analysis

A Prominence-i HPLC instrument (Shimadzu, Kyoto, Japan) equipped with a Kinetex Evo C18 column (Phenomenex, Torrance, CA, USA) and coupled to a diode-array detector (DAD) was used for the separation and quantification of AZA. The mobile phases were (A) HPLC grade water and (B) acetonitrile, with a gradient of 0 to 2 min (0 % solvent B), 2 to 12 min (0 % to 95 % solvent B), 12 to 14 min (95 % solvent B), 14 to 15 min (95 % to 0 % solvent B), and 15 to 17 min (0 % solvent B). The flow rate was 1.0 mL/min, the column temperature was set at 30 °C, and the injection volume for all samples was 10 μL; the wavelength to detect AZA was 217 nm. For this analysis, AZA standard was purchased from Sigma-Aldrich (Darmstadt, Germany), dissolved in ethanol (5 mg/mL), and further diluted in ethanol. The presence of AZA in the samples was confirmed by ESI-MS analysis as reported in the following paragraph.

Finally, a calibration curve (25–2000 μg/mL) was performed to quantify AZA in the samples.

2.3.2. ESI-MS analysis of AZA

Mass spectra were recorded by direct infusion electrospray ionization (ESI) on an LCQ Fleet ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Qual Browser Thermo Xcalibur 4.0.27.13 software was used for data processing (Thermo Fisher Scientific, Waltham, MA, USA). The ESI parameters for sample analysis performed in positive ionization mode (ESI+) were as follows: spray voltage at 5.5 kV, capillary temperature at 180 °C, capillary voltage at 45 V, and tube lens at 100 V. Gas flow rates (arb) were set at sheath 10, aux 0, and sweep 0. The infusion flow rate was set at 5 μL/min. ESI-MS spectra were obtained after injection of a 100 μg/mL solution prepared by dissolving in methanol the lyophilized samples (aqueous extract and AZA standard) obtained from the manual collection of fractions from HPLC-DAD. Collision-induced dissociation (CID) experiments were carried out to confirm the identity of the detected species, and fragmentation patterns were compared with literature data (Sarais et al., 2008).

2.3.3. UHPLC-HRMS untargeted analyses

MAE was diluted 1:10 with MQ water, centrifuged at 13,300 rpm for 10 min prior to analysis, and 1 mL of the supernatant was transferred to a polypropylene vial. Chemical characterization was performed by liquid chromatography (UHPLC) coupled to high-resolution mass spectrometry (HRMS), using an untargeted approach. Data were acquired in both negative ESI- and positive ESI+ ion modes using different instrumentations and methods. ESI- analysis was performed using a Waters Acquity UHPLC coupled to a Waters Xevo G2 QToF-MS. An Agilent Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm) was used as stationary phase, and was maintained at 40 °C. The mobile phase was a mixture of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B). The elution gradient was the following: 0–1 min, 98 % A; 11 min, 15 % A; 16 min, 0 % A; 20 min, 0 % A; 21 min, 98 % A; 24 min, 98 % A. The flow rate was set at 0.3 mL/min and the injection volume was 2 μL. MS data were acquired in both ESI- and ESI+ ionization modes, covering the 50–2000 Da mass range. The MS parameters were set as follows: sampling cone voltage, 40 V; source offset, 80 V; capillary voltage, 3.5 kV; nebulizer gas: N_2 at a flow rate of 800 L/h; desolvation temperature, 450 °C. Mass accuracy and reproducibility were maintained by lock mass (leucine-enkephalin) infusion via lockspray at a flow rate of 20 μL/min. MS^e experiment was performed with a fixed collision energy of 30 V to obtain structural information on the eluted compounds.

Chromatographic (retention time, R.T.; area under the curve, AUC, sum-normalized to total AUC) and MS (*m/z* values of parent ions) data of eluted metabolites were extracted from raw chromatograms and MS spectra using the MarkerLynx XS platform (Waters). The data matrix, exported as a .csv file, was used to select the most intense ions for further identification. Preliminary identification of variables (metabolites) was performed by comparing experimental m/z values, calculated molecular formulas, and fragmentation spectra with open-source databases such as Metlin (https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage), KNApSAcK (http://www.knapsackfamily.com/Knapsack_core/top.php), and PubChem (https://pubchem.ncbi.nlm.nih.gov/). In addition, the same data were compared with the available literature.

Regarding HRMS data in ESI+, they were collected analyzing MA on a Q-Exactive™ Focus Hybrid Quadrupole-Orbitrap MS (Thermo Scientific, Waltham, MA, USA) coupled to a Vanquish UHPLC pump and equipped with heated electrospray ionization (HESI)-II probe (Thermo Scientific, USA). The instrumental conditions are reported in the supplementary materials. Before analysis, samples were centrifuged at 8000 ×*g* × 10 min, filtered with 0.22 μm cellulose syringe filters, and then injected (6 μL) in the system.

2.4. Biological activity

2.4.1. Cell culture toxicity

Human neuroblastoma cells (SH-SY5Y) and primary human fibroblasts (FB-21) were cultured according to the previously published protocol (Abate et al., 2021). Briefly, SH-SY5Y cells were incubated at 37 °C in 5 % CO_2 in Dulbecco's modified Eagle's medium (DMEM) until 80 % confluence was reached. Cells were then seeded in 96-well plates and treated with medium alone (control) or with different concentrations of MAE (from 100 μg/mL to 0.2 μg/mL) for 48 h. Cell viability was assessed using a colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to measure the activity of mitochondrial enzymes that reduce MTT to formazan.

2.4.2. Zebrafish embryo acute toxicity test

All experiments took place at the Zebrafish Facility, Department of Molecular and Translational Medicine, University of Brescia, Italy. All experiments were conducted in accordance with the Italian and European rules on animal care and the standard rules defined by the Local Committee for Animal Health (Organismo per il Benessere Animale) and authorized by the Italian Ministry of Health (Aut N° 585/2018, date of approval 31 July 2018 and DM n° 92/2009-A, date of approval 19/05/2009).

To study MAE toxicity in zebrafish, Fish embryo toxicity (FET) test was performed to evaluate mortality and embryotoxicity as described in OECD protocols (OECD, 2013). Adult zebrafish (AB wild-type line) were kept at 28.5 °C and 7.0–7.4 pH in a recirculating water tank on a 14 h light/10 h dark cycle (Li et al., 2019) and, after breeding by natural crosses, the embryos obtained were used for the FET test. Zebrafish embryos were exposed to the phyto-extract and evaluated for mortality and morphology at 48 and 72 hpf (hours post fertilization). A dose-response survival curve was plotted for each dose and time endpoint. Briefly, embryos were cultured in fish water (0.1 g/L "Instant Ocean" sea salt, 0.1 g/L sodium bicarbonate, 0.19 g/L calcium sulfate) with incubation at 28.5 °C until experiments. Embryos were exposed to MAE from the gastrula stage (4 hpf) until 48 and 72 hpf according to the previously published protocol (Zizioli et al., 2023). To improve uptake of MAE, embryos were dechorionated and then transferred to Petri dishes containing 5 mL of MAE at different dosages (from 1.6 to 50 μg/mL) for both time-points. Embryos were also incubated with fish water only (negative control) or 3,4-dichloroaniline (3.7 mg/L) used as a positive control. Each treatment was tested on 30 zebrafish larvae and the entire experiment was repeated three times. Morphological assessments were performed at 72 hpf by daily visual inspection of anaesthetized embryos (0.4 % tricaine, Sigma-Aldrich) under a Zeiss Axiozoom V13 microscope. Morphological endpoints included head and tail morphology, antero-posterior (AP) axis, presence or absence of pericardial edema, and yolk sac size and morphology. Images were acquired in the lateral position at 20× magnification using a Zeiss Axiozoom V13 microscope equipped with a PlanNeoFluar Z 1 × 0.25 FWD 56 mm objective and Zen Pro software.

2.4.3. Honey bee chronic exposure test

The ecotoxicological experiment with *Apis mellifera ligustica* was performed in July 2023 at the Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore (Piacenza, Italy). Through oral administration, worker honey bees were exposed to two solutions (sugar and water) with MAE at concentrations of 25 μg/mL and 3.12 μg/mL. A brood frame with capped cells from one bee colony was selected and kept in a climatic chamber at 36 ± 1 °C and 60 % relative humidity (RH) (Papa et al., 2021). First, we tested the palatability of the two MAE concentrations. Then, ten newly enclosed bees, for each concentration (25 μg/mL and 3.12 μg/mL) and a control group (sugar syrup), were randomly collected from the combs and actively fed with 2 μL of solution (Fig. 5 A). The bees were then moved into cages (18 × 15 × 9.5 cm) and kept in the climatic chamber at 35 ± 1 °C and 60 % RH, where they had

ad libitum access to the same food solution (Fig. 5 A). Three replicates have been performed for each concentration. After 24, 48, 72, and 96 h we recorded the mortality and the behavioral abnormalities according to the categories described in the OECD chronic test (OECD, 2017). To evaluate the effect of MAE concentrations (25 μg/mL and 3.12 μg/mL) on the mortality of the honey bees, an ANOVA test accounting for repeated measures was applied as implemented in the R program (Team, 2021).

2.4.4. Collembola chronic exposure test

The ecotoxicological experiment performed on the collembola species *Folsomia candida* was carried out from October to November 2023 at the Department of Sustainable Crop Production, Università Cattolica del Sacro Cuore (Piacenza, Italy). *F. candida* was exposed to MAE (25 μg/mL and 3.12 μg/mL), whereas a control group was kept in standard natural soil Lufa 2.2 (Speyer, Germany). The day preceding the treatment, 15 g of soil for each concentration were mixed with 4 mL MA solution (MAE and water) in a glass beaker and dried overnight at room temperature. Then, ten individuals 9–12 days old of *F. candida* were placed into Petri dishes prepared with a base of plaster of Paris with graphite, 5 g of treated soil, and ~2.5 mg baker yeast. The experiment was conducted in triplicate (10 collembola × 3 replicates x each concentration) in a climatic chamber at 21 ± 1 °C and 80 % RH and lasted 28 days. On day 28, the collembolas were extracted from the soil via floatation, and adult and juvenile stages were counted. To evaluate the effects of the two concentrations of the phytoextract on collembola a generalized linear model (GLM) with a negative binomial error family, as implemented in the 'MASS:glm.nb' R function, was used (Team, 2021; Venables and Ripley, 2002).

2.4.5. Seed germination and root elongation assay

To evaluate the phytotoxicity, the effects of aqueous extract of MAE on seed germination and root elongation were measured. In particular, the assay was performed according to the Italian Environmental Agency guidelines. Briefly, seeds of two dicotyledons (*Cucumis sativus* L. and *Brassica rapa* L.) and one monocotyledon (*Sorghum vulgare* L.) not treated with fungicides were preliminary checked for vitality in distilled water in the dark at 25 ± 2 °C for 72 h (germination rates >90 %). We used cucumber (*Cucumis sativus* L.), tatsoi (*Brassica rapa* L.), and sorghum (*Sorghum vulgare* L.) seeds purchased from a commercial retailer. Seeds were exposed to different MAE concentrations ranging from 50 μg/mL to 1.6 μg/mL and incubated in the dark at 25 ± 2 °C for 72 h. At the end of the exposure, the germinated seeds were counted and the length (to the nearest millimeter) of the root system emerging from the seeds was measured using a ruler. The effect on germination and root elongation was expressed as the percent germination index (GI). The final calculation expresses the germination index (GI):

$$\boldsymbol{GI} = (\boldsymbol{Gs} \times \boldsymbol{Ls})/(\boldsymbol{Gc} \times \boldsymbol{Lc}) \times \mathbf{100\%}$$

where: Gs is the number of germinated seeds in the sample, Gc is the number of germinated seeds in the control, Ls is the root length of the sample, and Lc is the root length of the control.

2.5. Chemicals

All chemicals used were of analytical grade. Ethanol (98 % pure), acetonitrile, MQ water (all LC-MS grade) were purchased from Meck (Darmstadt, Germany). The standards used for the UHPLC-QTOF semi-quantitative analyses were purchased from Sigma-Aldrich (Merck KGAA, Darmstadt, Germany).

2.6. Statistical analysis

2.6.1. MTT assay

All experiments, including analytical and cell viability assays, were performed in triplicate, and data are presented as mean ± standard error of the mean (SEM). Cell viability data were analyzed using a two-way ANOVA test. All statistical analyses were conducted using Microsoft Excel 16.52 and GraphPad V8 (GraphPad Software, La Jolla, CA, USA).

2.6.2. Zebrafish toxicity test

All the experiments were repeated at least three times, and data are reported as mean ± SEM. The comparison between control and treated embryos was done using GraphPad V8 (La Jolla, CA USA) with *p-value < 0.05 considered statistically significant. The significance test of all the data was analyzed by one-way analysis of variance (one-way ANOVA).

2.6.3. Seed germination test

For the seed germination test, the experiments were repeated at least three times, and data are reported as mean ± SEM. The comparison between samples treated with MAE and control was done using GraphPad V8 (La Jolla, CA USA) with *p-value < 0.05 considered statistically significant. The significance test of all the data was analyzed by one-way analysis of variance (one-way ANOVA).

2.6.4. Correlation analyses

Pearson's correlation analysis was performed using the "corrplot" and "ggplot2" packages through R studio software (v. 4.2.3.) among bioactive metabolites identified using the LC-MS approach and biological assays including cytotoxicity assessment, FET test examination and seed germination test.

3. Results

3.1. Micromorphological investigation

The leaves of *M. azedarach* are bipinnately compound, alternate and grouped towards the apical portion of the branches. The number of leaflets ranges from 3 up to 11. The cross-section of young leaflets and full-expanded leaflets showed a dorsal-ventral arrangement with adaxial and abaxial epidermises covered by an evident cuticle that appeared thicker on the adaxial one (Fig. 1 Ba, Bb). The palisade parenchyma was arranged in 1–2 cell layers and the spongy parenchyma in 2–3 cell layers. In the young leaflet, the abaxial lamina was characterized by the presence of abundant epidermal appendages, i.e., non-glandular and glandular trichomes (Fig. 1Ba), that were sporadically or totally absent in the full-expanded leaflet (Fig. 1Bb).

The adaxial and abaxial laminas consisted of polygonal cells with straight anticlinal walls; they were isodiametric on the abaxial epidermis and elongated on the adaxial one (Fig. 1 Bc, Bd). Unicellular simple non-glandular trichomes were observed, especially on adaxial lamina. They were pointed at the apex, erect, curved, or bent at an obtuse angle near the middle (Fig. 1 Bc, Bd, Bf).

Glandular trichomes of peltate type were observed on the very young leaflets, mostly on the adaxial surface: they exhibited a short stalk and a round multicellular head, generally surmounted by a wide subcuticular chamber (Fig. 1 Be). They appeared occasionally on the full expanded leaflets. Several cluster crystals of calcium oxalate in the form of druses were scattered in the palisade and spongy mesophylls and resulted especially abundant in the parenchyma of the midrib region (Fig. 1 Bg).

The histochemical results on the glandular trichomes revealed a high level of consistency for all the analyzed trials/replicates (Fig. 1 Bh—Bk), both on young and full-expanded leaflets. The secreted material exhibited a complex chemical composition. All the dyes specific for lipophilic compounds gave positive reactions, especially to the Nadi reagent indicating the presence of terpenes (Figure 1 Bh). Additionally, the production of mucopolysaccharides, alkaloids and flavonoids was highlighted, following the applications of the Alcian Blue, the Wagner reagent and $AlCl_3$ tests, respectively (Figure 1 Bi, Bj, Bk).

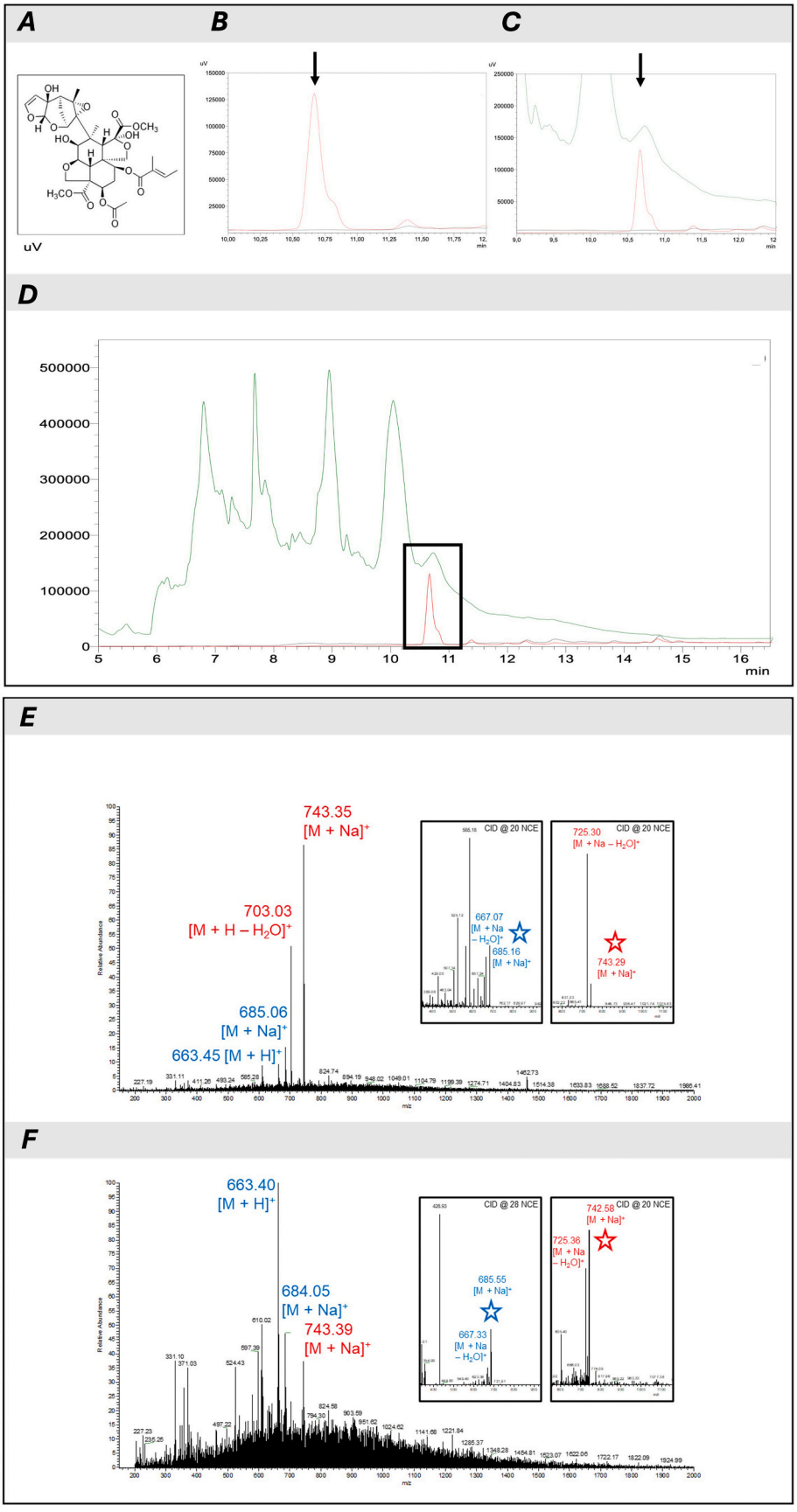

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Fig. 2. A-D: Chromatograms of MAE and of the standard azadirachtin. (A) Chemical structure of azadirachtin; (B) details of elution of STD AZA (red) and (C) the AZA in MAE sample; and (D) superimposed chromatograms of the AZA standard and MAE.
E-F: (E) ESI-MS spectra obtained from the fraction manually collected from the HPLC-DAD analysis of the AZA standard. The main panel includes the full ESI-MS spectrum. In the right inset, the spectrum resulting from the CID experiment (normalized collision energy, NCE = 20) is reported: the fragmentation pattern is in agreement with literature data for AZA-A (Sarais et al., 2008). In the left inset, the spectrum resulting from the CID experiment (NCE = 20) is reported: the fragmentation pattern suggests the presence of AZA-B (Sarais et al., 2008). (F) ESI-MS spectra obtained from the fraction manually collected from the HPLC-DAD analysis of the aqueous phytoextract of MA. The main panel includes the full ESI-MS spectrum. In the right inset, the spectrum resulting from the CID experiment (NCE = 20) is reported: the fragmentation pattern is in agreement with literature data for AZA-A (Sarais et al., 2008) and is comparable to the one obtained for the AZA standard. In the left inset, the spectrum resulting from the CID experiment (NCE = 28) is reported: the fragmentation pattern suggests the presence of AZA-B and is again similar to that of the standard (Sarais et al., 2008).

3.2. Quantification of azadirachtin (AZA) in the aqueous phytoextract

Chromatograms show that AZA STD elutes around 10 min and MAE shows a peak corresponding to AZA STD (Fig. 2 C, D). The concentration of AZA in the aqueous extract of MA was determined to be ~108.34 μg/mL. To confirm the presence of AZA in the MA aqueous extract, eluate was collected for about 10 min and direct infusion ESI-MS analysis was performed on this fraction.

The analysis revealed the presence of two azadirachtins (AZA-A and AZA-B) both in the AZA standard and in the collected sample. Although these compounds were eluted under the same peak in the HPLC analysis, the mass spectrometer accurately identified them as separate entities.

The signals detected in positive ionization mode and the corresponding adducts or fragments are reported below and have been labeled in red for azadirachtin-A (AZA-A) and in blue for azadirachtin-B (AZA-B) in the graphical representation (Fig. 2 E, F). The signals that were isolated and fragmented in CID studies have been highlighted with a star.

First, the AZA standard was analyzed (Fig. 2 E), and the following signals were identified in the full spectrum: $m/z = 743.35$ $[M + Na]^+$ and $m/z = 703.03$ $[M + H - H_2O]^+$, representing AZA-A, together with $m/z = 685.06$ $[M + Na]^+$ and $m/z = 663.45$ $[M + H]^+$, related to AZA-B. As depicted in the inset of the figure, the fragmentation study allowed to confirm the identity of the species: the signals at $m/z = 725.30$ $[M + Na - H_2O]^+$ and $m/z = 667.07$ $[M + Na - H_2O]^+$, peculiar of AZA-A and AZA-B, were detected.

The same pattern was observed in MAE (Fig. 2 F), as in the full spectrum we detected the following signals: $m/z = 743.39$ $[M + Na]^+$, corresponding to AZA-A, and $m/z = 663.40$ $[M + H]^+$, representing AZA-B. A peak at $m/z = 685.06$ (putative $[M + Na]^+$ for AZA-B) was also observed. Again, CID experiments confirmed the identity of the species, as diagnostic fragments were detected for AZA-A ($m/z = 725.36$ $[M + Na - H_2O]^+$) and AZA-B (667.33 $[M + Na - H_2O]^+$) in the spectra. In both cases, CID patterns were found to be in agreement with literature data (Sarais et al., 2008).

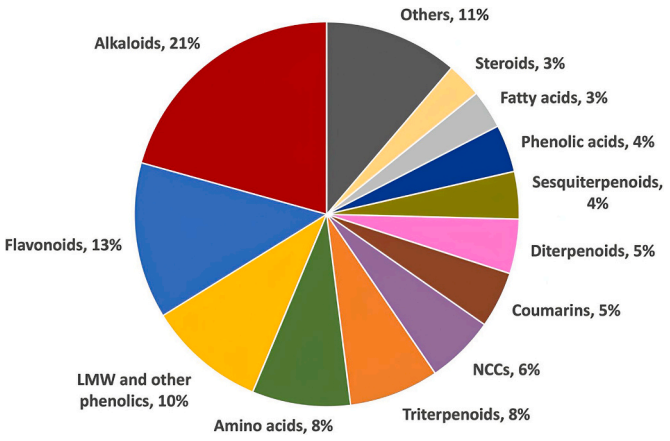

Fig. 3. Pie chart of 13 main classes of bioactive compounds identified in MAE. The percentage value represents the number of compounds belonging to that class. Abbreviations: LMW – low molecular weight; NCCs – nitrogen-containing compounds.

3.3. Phytochemical screening of extracts

The profiling of phytochemical constituents of MAE was conducted using an untargeted metabolomics approach through the UHPLC-HRMS instrumentation. This method allowed to comprehensively annotate 677 compounds, mostly represented by plant secondary metabolites. The comprehensive list of annotated compounds is reported in the supplementary tables (Supplementary tables "dataset"), inclusive of their specific annotation information such as average retention time (min), ontology, general compound classification, MS1 isotopic spectrum, MS/MS spectrum, and samples abundances among others.

Metabolic profiling of MAE revealed 13 primary classes of bioactive compounds (Fig. 3, Table 1), with alkaloids, flavonoids, low molecular weight phenols (LMW), and other phenolics emerging as the most represented families. Additionally, amino acids, terpenoids (mono-, sesqui-, di-, and tri-terpenes), and nitrogen-containing compounds (NCCs) garnered significant attention in the metabolite profiling of MAE (Fig. 3, Table 1).

Considering MAE alkaloid content, the most abundant were 3-alkyl indoles (4-(1H-indol-3-yl) butane-2-one), aconitine-type diterpenoid alkaloids (e.g., bulleyaconitine A, eriodictyol, and benzoylhypaconine), and harmala alkaloids (e.g., harmine hydrochloride). Among flavonoids, flavanones (e.g., pinocembrin, purpurin, and glabrol), chalcones (e.g., 3-(4-hydroxyphenyl)-1-phenyl-2-propen-1-one and loureirin B), and isoflavones (e.g., irisflorentin, 6,8-diprenylorobol, and genistein) were highly represented. These were then enriched with a high amount of LMW and other phenolic compounds, such as 6-hydroxybenzofuran-3(2H)-one, fraxetin, lindenenol, (*S*)-gingerol, and others. The MAE was also abundant in terpenoid compounds, with significant representation of tri-, di-, and sesquiterpenes. Triterpenoids were rich in bruceantin, and lucidenic acid B, followed by diterpenoids like guggulsterone, isoabienol, and 20-*O*-acetylingenol-3-angelate, and sesquiterpenoids including furanodienon, nerolidol, bergamottin, and atractylon.

Moreover, MAE exhibited an abundance of amino acids, steroids, NCCs, fatty acids, tannins, and coumarins, collectively contributing to the general bioactivity that characterizes these extracts (Supplementary tables).

3.4. Cytotoxicity assay on human FB-21 and SH-SY5Y cells

To assess the potential toxic effects of MAE through the major routes of pollutant exposure in humans, cutaneous and neuronal toxicity were tested in vitro. For this purpose, human neuroblastoma cells (SH-SY5Y) and human fibroblasts (FB-21) were selected as they are well-recognized alternative animal models used in toxicological studies.

Specifically, the cytotoxicity of MAE was evaluated by MTT assay determining the percentage of cell survival after 48 h of MAE exposure. For this purpose, SH-SY5Y and FB-21 human cells were treated with MAE at different concentrations ranging from 50 μg/mL to 1.6 μg/mL for 48 h. The concentrations of the phytoextract were calculated according to the AZA calibration curve obtained from HPLC analysis. The results from the MTT assay show a concentration-dependent cytotoxic effect on both FB-21 and SH-SY5Y cells (p-value $>$ 0.05) (4 A, B). The

Table 1
Extract of the mostly relevant bioactive compounds identified inclusive of their specific annotation information such as average retention time (min), ontology, general compound classification, MS1 isotopic spectrum, MS/MS spectrum, and samples abundances among others. The comprehensive list of annotated compounds is reported in the supplementary tables "dataset".

Metabolite name	Adduct type	Ontology	General classification	Abundances
4-(1H-indol-3-yl) butan-2-one	[M + H]+	3-alkylindoles	Alkaloids	1.867.718.272,00
Bulleyaconitine A	[M + H]+	Aconitane-type diterpenoid alkaloids	Alkaloids	14.025.496,33
Benzoylhypaconine	[M + H]+	Aconitane-type diterpenoid alkaloids	Alkaloids	9.543.092,00
Harmine hydrochloride	[M + H]+	Harmala alkaloids	Alkaloids	5.950.381,00
Eriodictyol	[M + H]+	Flavanones	Flavonoids	4.791.368,33
Pinocembrin	[M + H]+	Flavanones	Flavonoids	43.916.364,00
purpurin	[M + H]+	8-prenylated flavanones	Flavonoids	20.004.909,33
Glabrol	[M + Na]+	8-prenylated flavanones	Flavonoids	8.457.607,33
Glabrol	[M + H]+	8-prenylated flavanones	Flavonoids	7.193.914,33
3-(4-hydroxyphenyl)-1-phenyl-2-propen-1-one	[M + H]+	Retrochalcones	Flavonoids	21.326.199,33
Loureirin B	[M + H]+	Retro-dihydrochalcones	Flavonoids	12.667.819,67
Irisflorentin	[M + H]+	4'-*O*-methylisoflavones	Flavonoids	11.453.484,00
6,8-Diprenylorobol	[M + Na]+	6-prenylated isoflavanones	Flavonoids	10.287.153,33
6,8-Diprenylorobol	[M + H]+	6-prenylated isoflavanones	Flavonoids	3.309.757,67
Genistein	[M + H]+	Isoflavones	Flavonoids	3.410.129,00
6-hydroxybenzofuran-3(2H)-one	[M + ACN + H]+	Benzofurans	LMW and other phenolics	480.015.338,67
Fraxetin	[M + H]+	7,8-dihydroxycoumarins	LMW and other phenolics	186.181.381,33
lindenenol	[M + H]+	Naphthofurans	LMW and other phenolics	91.998.970,67
(*S*)-[6]gingerol	[M + Na]+	Gingerols	LMW and other phenolics	25.682.686,67
Dictamnine	[M + Na]+	Furanoquinolines	LMW and other phenolics	4.480.145,00
[6]-Gingerol	[M + H]+	Gingerols	LMW and other phenolics	3.941.078,00
Bruceantin	[M + Na]+	Quassinoids	Triterpenoids	9.519.972,67
Lucidenic acid B	[M + H]+	Triterpenoids	Triterpenoids	8.197.638,33
Guggulsterone E&Z	[M + H]+	Androgens and derivatives	Diterpenoids	39.101.400,00
isoabienol	[M + H]+	Diterpenoids	Diterpenoids	9.217.798,33
20-O-Acetylingenol-3-angelate	[M + H]+	Tigliane and ingenane diterpenoids	Diterpenoids	7.013.269,67
Furanodienon	[M + Na]+	Germacrane Sesquiterpenoids	Sesquiterpenoids	67.810.889,33
Nerolidol	[M + H]+	Sesquiterpenoids	Sesquiterpenoids	25.883.981,33
Bergamottin	[M + H-H_2O]+	Terpene lactones	Sesquiterpenoids	16.453.635,00
Atractylon	[M + H]+	Eremophilane, 8,9-secoeremophilane and furoeremophilane sesquiterpenoids	Sesquiterpenoids	5.998.441,67

phytoextract EC_{50} values were 10.70 μg/mL and 19.31 μg/mL for FB-21 and SH-SY5Y cells respectively. In all the assays, treatments that reduced viability by >80 % were considered cytotoxic.

3.5. Zebrafish acute toxicity test

Zebrafish embryos were exposed to MAE and their mortality was measured at 48 hpf. The phenotype of the embryos was evaluated at 72 hpf. Data in Fig. 4 C demonstrated that zebrafish embryos treated with MAE showed the highest mortality rate at 50 μg/mL (p-value <0.05). In addition, at lower dosages, a dose-dependent mortality rate was still significant and evident up to 6.25 μg/mL (p-value < 0.05).

From 3.12 μg/mL MAE appeared to be completely harmless to the fish (Fig. 4 C). Similar findings have been observed also for the embryos at 72 hpf (Fig. 4 D), while embryos treated with the 50 μg/mL concentration exhibited severe necrosis (Fig. 4 D). Embryos treated with concentrations ranging from 25 μg/mL to 3.12 μg/mL showed variable levels of pericardial edema (Fig. 4 D). Overall, this data confirmed that MAE induces alteration in zebrafish embryos phenotypes up to a concentration of 3.12 μg/mL.

3.6. Seed germination test

According to Barral and Paradelo (Barral and Paradelo, 2011), GI values below 50 % indicate high phytotoxicity; values between 50 % and 80 % indicate moderate phytotoxicity; and values above 80 % indicate the absence of phytotoxicity. As shown in Fig. 4 E, MAE has a significant impact on seeds germination and root elongation of all the three plant species studied. As the concentration of the extract increases, there is a noticeable increase in phytotoxicity (GI < 50 %). Specifically, *B. rapa* GI was lower than 50 % (Fig. 4 E, blue line) at MAE concentration ranging from 50 μg/mL to 6.25 μg/mL; MAE treatment cause high phytotoxicity at 50 μg/mL and 25 μg/mL in *S. vulgare* (Fig. 4 E, red line), while this was true for *C. sativus* (Fig. 4 E, green line) only at 50 μg/mL. Notably, cucumber appears to be the most resilient species to the MAE, as its germination index reaches 50 % at a concentration of 30.99 μg/mL. On the contrary, *B. rapa* is undoubtedly the most vulnerable species to this treatment, displaying noticeable inhibition even at lower doses; indeed, its germination index reaches 50 % at a concentration of 5.50 μg/mL. Interestingly, *S. vulgare* exhibits a peculiar behavior where it is more resistant than cucumber at lower doses but becomes increasingly sensitive when the dose exceeds 25 μg/mL; it reaches a germination index value of 50 % at a concentration of 19.91 μg/mL, showing higher sensitivity than cucumber but greater resistance than tatsoi to MAE. To sum up, this germination test allowed to determine that MAE concentrations at or below 3.12 μg/mL are not harmful to these particular plant species. However, concentrations above 25 μg/mL appear to significantly inhibit the germination of both monocots and dicots plants. MAE didn't show phytotoxicity (GI>80 %) in *S. vulgare* and *C. sativum* at lower MAE concentration (from 5.25 μg/mL to 1.6 μg/mL). Conversely, *B. rapa* GI remained above the threshold of phytotoxicity.

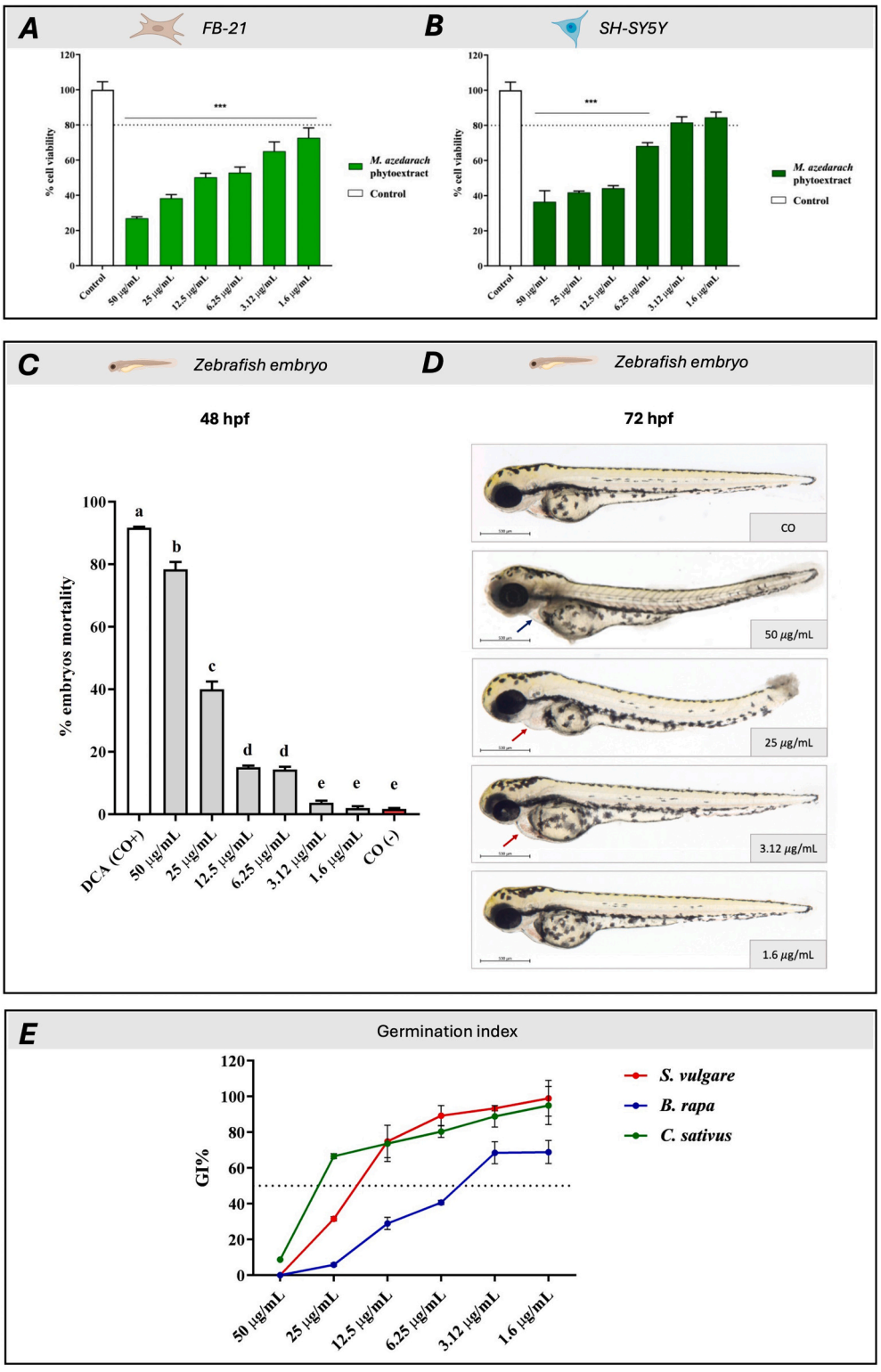

Fig. 4. A-B: Effects of MAE on FB-21 and SH-SY5Y cells viability. The bars indicate the percentage of cell viability of FB-21 (A) and SH-SY5Y (B) cells treated with the indicated concentrations (ranging from 50 µg/mL to 1.6 µg/mL) of MA extract for 48 h and subjected to an MTT assay. The concentrations are referred to the AZA content within the extract, quantified by HPLC. Data are representative of three replicates and shown as mean ± standard deviation: *** $p < 0.05$.
C-D: C) Mortality of zebrafish embryos at 48 hpf after exposure to MAE by immersion method. Zebrafish embryos were exposed at the gastrula stage (4hpf) to several MAE concentrations. Control embryos were treated with fish water (CO-) and with 3,4-dichloroaniline (CO+). Results are expressed as mean ± SEM (N embryos = 30). Statistical analysis is reported using letters (a,b,c,d,e,), and the difference in *p*-values between columns is $p < 0.05$. D). Representative pictures of the effects of the phytoextract exposure on zebrafish embryos. All figures are lateral views with dorsal to the top and anterior to the left. Malformations are indicated with arrows: (blue) indicates severe necrosis and (red) pericardial edema. CO, control embryos; hpf, hours post fertilization.
E: Percentage germination index (GI%) of *S. vulgare*, *B. rapa*, and *C. sativus* exposed to treatments with different concentration (ranging from 50 µg/mL to 1.6 µg/mL) of MAE for 72 h. The concentrations are referred to as the AZA content within the extract, quantified by HPLC. Data are representative of three replicates and shown as mean ± SEM.

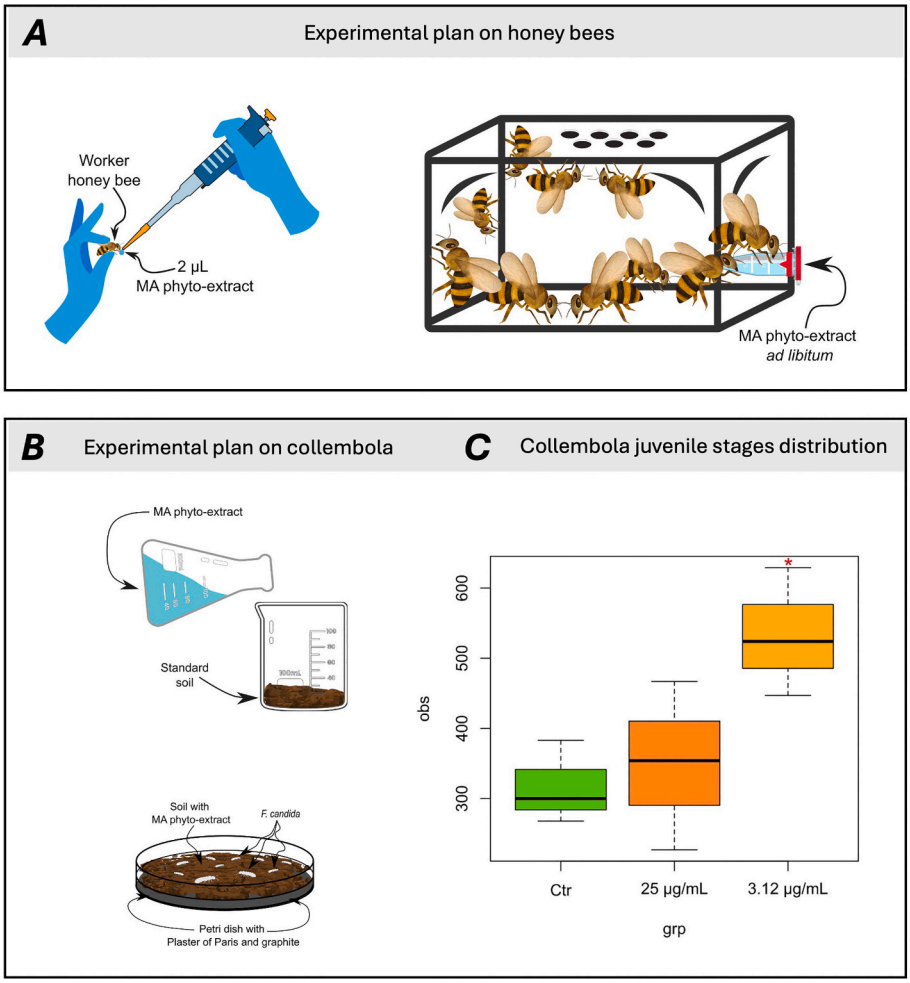

Fig. 5. A: Graphical representation of the experimental plan of honey bees chronic exposure test. Worker honey bees were fed with 25 µg/mL or 3.12 µg/mL of MA phyto-extract (left side) and an example of cage with 10 worker bees with MA phyto-extract ad libitum (right side).
B: Graphical representation of the experimental plan representation of the collembola chronic exposure test, including preparation of standard soil with MA phyto-extract (on top) and an example of Petri dish with soil and collembola (on bottom). C: Distribution of the collembola juvenile stages counts of the control, and collembola treated with 25 µg/mL and 3.12 µg/mL of MA phyto-extract.

3.7. Effects of chronic exposure on honey bees and collembola

For the entire duration of the experiment, the honey bees did not show any behavioral abnormality and the recorded mortality was low. The ANOVA results on mortality of honey bees showed a non-significant difference with the control group (p-value > 0.05).

For *F. candida*, the GLM analysis showed that the number of surviving adults in the treated and control experiments was not statistically different (p-value >0.05). The number of juvenile stages exposed to 25 µg/mL MAE was not significantly different from the control. Conversely, the number of juvenile stages exposed to 3.12 µg/mL MAE was significantly higher than the control (p-value <0.05) (Fig. 5 C, Table 2).

3.8. Pearson's correlation analysis

Pearson's correlation analysis was considered in this work to find out the correlation score (r) between compounds identified in MAE and the different toxicity assays. The correlation score and significance values (p-value < 0.05) are reported in the supplementary tables "Table Y". MAE exhibited a diverse composition of bioactive compounds, potentially contributing to various biological activities. Specifically, a clear negative correlation between the MTT vitality assay on FB-21 cells and the abundance of flavonoids like eriodictyol, phloretin, isoliquiritin, and hesperetin was observed, suggesting that they are mainly associated with MAE toxicity. Moreover, limonoids such as toosendanin; terpenoids including curdione, bilobalide, and alisol A, 24-acetate; and alkaloids like amygdalin, ranaconitine, and indaconitine were also negatively correlated ($r > 0.99$; p-value <0.05) (supplementary tables). Also, classes of alkaloids, terpenoids, and limonoids, such as sophocarpine, brusatol, furanodienon, vindoline, nomilinic acid, isotoosendanin, and azadirachtin B, were negatively associated with the MTT vitality assay on SH-SY5Y cells ($r > 0.99$; p-value <0.05). Furthermore, phenolic compounds like pinocembrin, imperatorin, isocurcumenol, and cepharanthine contributed to the toxicity of MAE on SH-SY5Y cells ($r > 0.99$; p-value <0.05).

Considering the germination index, alkaloids, di-, tri-, and sesquiterpenes, as well as tannins, including matrine, euphorbiasteroid, cucurbitacin E, vindoline, and furanodienon, exhibited negative correlations with the germination of *B. rapa*. Additionally, these classes exhibited inhibitory effects on the germination of *S. vulgare* and *C. sativus*. Notably, different metabolites within these classes displayed high negative correlations with the germination indexes of the respective plant species ($r > 0.99$; p-value < 0.05).

The percentage of zebrafish embryo mortality was positively correlated with cyclopamine, corynoxeine, dehydronuciferin, galanthamine, conessine, 3-acetylaconitine, and sinomenine hydrochloride (alkaloids class), 12alpha-hydroxyamoorstatin (limonoid class), 7-beta-hydroxylathyrol, telocinobufagin, and lucidenic acid B (terpenoids classes)

Table 2
Statistical results observed regarding the ecotoxicological experiments performed on honey bees and collembola. p-value is reported in the right column. p-value <0.05 is considered statistically significant.

Experiment	p-value
Honey bees	0.77
Collembola adults 25 μg/mL vs ctr	1.0
Collembola adults 3.12 μg/mL vs ctr	0.76
Collembola juveniles 25 μg/mL vs ctr	0.0301
Collembola juveniles 3.12 μg/mL vs ctr	<2e-16

(supplementary tables).

Among the MA phytoextract's bioactive compounds, limonoids (e.g., obacunone) and several polyphenols (e.g., gallic acid-O-dipentoside, dihydrocaffeic acid, and 3-hydroxy-5,7,4′-trimethoxyflavone) were identified as the only class contributing to zebrafish toxicity ($r > 0.99$; p-value <0.05).

4. Discussion

In order to perform an ecotoxicological evaluation of an aqueous phytoextract of *Melia azedarach* L., it was crucially important to conduct a micromorphological analysis of *Melia azedarach* leaflets. Thus, at first, we deepened our knowledge regarding the micromorphology and the secretory structures of *M. azedarach* leaves. As a whole, our observations are in accordance with those reported in the literature, with the exception of some morphological details. Previous authors (Abdel-Hameed, 2014; Jafari et al., 2013a, 2013b) conducted morphological studies with the aim of recognizing diagnostic microscopic features for the differentiation of samples belonging to *M. azedarach* and *Azadirachta indica* A. Juss, usually misidentified *taxa* in the pharmaceutical preparation of botanical drugs. Such authors reported the occurrence of polygonal cells with irregular anticlinal walls (which instead appeared straight in our samples), occasional druses, unicellular unbranched covering trichomes and glandular trichomes defined 'with multicellular foot and head' by Abdel-Hameed (Abdel-Hameed, 2014) and as 'peltate trichomes' by Jafari et al. (Jafari et al., 2013a, 2013b). More recently, Tinley et al. (Tilney et al., 2018) reported the occurrence of laminar and petiolar extrafloral nectars on the leaves of the target species. They also observed unicellular hairs and capitate glands (associated with very young leaflets) corresponding to the peltate glandular trichomes observed herein, although the terminology is not appropriate and consistent. These authors also mentioned the presence of stellate hairs occasionally visible abaxially, but not observed in our samples. An element of novelty of the present study is the definition of the histochemical profile of the peltate glandular trichomes, resulting in the secretion of material of complex chemical nature, including terpenes, mucopolysaccharides, flavonoids, and alkaloids.

One of the main constituents of the aqueous MA extract considered in this study was AZA (~108.34 μg/mL), a characteristic limonoid already described in extracts of *M. azedarach* (Khanavi et al., 2014; Mehdi et al., 2012), whose biological importance has been demonstrated by numerous studies (Amaral et al., 2018; Bezzar-Bendjazia et al., 2016; Fan et al., 2023; McKenzie et al., 2010). AZA is claimed as the main bioactive constituent of MAE regarding insecticidal activity; hence it was selected as a reference for expressing the dosages of phytoextract used in the toxicity assessments of MAE. Additionally, to further investigate the phytochemical composition of MAE, other secondary metabolites of the plant were analyzed by untargeted UHPLC-HRMS. This analysis revealed the presence of numerous alkaloids, flavonoids, and terpenoids. The extract contains a rich concentration of 3-alkyl indoles, making it the most abundant alkaloid. In addition, flavonoids and isoflavones are prominent among the secondary metabolites. As for terpenoid compounds, we found a noteworthy presence of triterpenes and sesquiterpenes, with bruceantin being particularly represented among the triterpenes. These findings align with previous researches that reported similar classes of molecules, even though using an ethanolic extract (Ahmed et al., 2012; Asadujjaman et al., 2013; Jeba Malar et al., 2020). It is worth noting that the composition of aqueous extracts of *M. azedarach* has not been as extensively studied as ethanolic extracts.

In general, the insecticidal properties of several alkaloids, flavonoids, and terpenes have been already reported in the literature. For instance, glycoalkaloids from *Solanaceae* plants exert a non-specific toxicity on several species of insects (Chowański et al., 2016); widely diffused flavonoids such as quercetin, kaempferol, apigenin, luteolin and their glycosides have toxic properties on insects such as *Spodoptera litura, Bemisia tabaci, Aphis gossypii*, and *Acyrthosiphon pisum*, (Pereira et al., 2024); lower molecular-weight terpenes, typical constituents of plant essential oils, as well as higher molecular weight terpenes can exert toxic effects on insects such as lice, cockroaches, and Triatominae bugs (Dambolena et al., 2016; Liu et al., 2021). Up to now, the contribution of flavonoids and alkaloids to the insecticidal properties of *M. azedarach* has not been investigated, although we can speculate that they may participate to the effects observed in this study, considering the published literature. On the other hand, the terpenes 1,3-dicinnamoyl-11-hydroxymeliacarpin, 1-cinnamoyl-3-methacryl-11-hydroxymeliacarpin, and 1-cinnamoyl-3-acetyl-11-hydroxymeliacarpin (not detected in our study) have been shown to exhibit a highly poisonous effect on *Spodoptera littoralis*(Lin et al., 2022). Overall, these data point out the need to carry out further investigations to better understand the role of flavonoids, terpenes, and alkaloids in the bioactivity of *M. azedarach* and its extracts.

In order to study the potential ecotoxicity of MAE, the exposure effects to the extract were monitored on multiple biological models. The models selected are representative of the agricultural ecosystem that comes into contact with MAE extracts. In fact, all products (synthetic or natural) used in agriculture to control phytophagous insects are not expected to have side effects on pollinating insects (bees) (Leska et al., 2021), beneficial soil insects (collembola) (Joimel et al., 2022), fish (contamination of culverts) (Rohani, 2023) or even seed germination processes (Shakir et al., 2016). At the same time, the choice of animal models was guided by the responsible use of animal experimentation. According to the 3Rs principle (Replace, Reduce, Refine), experiments with other animal models (e.g. rodents) were replaced whenever possible (Hubrecht and Carter, 2019). Therefore, two human cell lines were selected to evaluate potential cutaneous and neuronal toxicity and screen different dosages in vitro aiming to identify the not harmful doses of MAE. In-vitro, MAE showed toxicity on FB-21 cells starting from a concentration of 1.6 μg/mL, while on SH-SY5Y cells it exhibited toxicity starting from a concentration of 6.25 μg/mL.

In a study conducted by Jafari et al. (Jafari et al., 2013a, 2013b), methanolic extracts obtained from *M. azedarach* demonstrated a cytotoxic effect at similar doses on four cell lines, and this aligns with our findings. The toxicity of MAE was then assessed on zebrafish to understand its potential embryogenic toxic effect but also its influence on aquatic fauna and potential environmental effects if used as open-field insecticide that might reach groundwater sources. Regarding the toxicity test on zebrafish embryos, it was found that the extract caused significant mortality starting from a concentration of 25 μg/mL. Additionally, the surviving embryos treated with MAE at 50 μg/mL and 25 μg/mL exhibited severe necrosis and cardiac edema. These findings are consistent with those documented in another study examining the toxicity of a *Meliaceae* extract, indeed Plhalova et al. (Plhalova et al., 2018), explored a phytoextract derived from *A. indica*, which similarly induced mortality and morphological aberrations.

Lastly, we examined the effects of MAE on three plant species to investigate the impact of this product on the germination process, considering its use as natural insecticide. For the germination test, three different plant species were chosen to obtain more comprehensive and reliable data, including one monocot (sorghum) and two dicots (cucumber and tatsoi). The germination test revealed an inhibitory effect on germination. In particular, cucumber exhibited the highest resistance to

MAE, whereas tatsoi demonstrated the greatest susceptibility, with sorghum displaying intermediate susceptibility. Notably, at concentrations exceeding 25 μg/mL, substantial inhibition of germination was evident across all evaluated plant species. Our findings align with the results of other research groups, such as Carvalho et al. (Carvalho et al., 2019), who have observed an inhibitory effect on the germination of other plants from certain phytoextracts with a similar profile of secondary metabolites.

Regarding toxicity effects after chronic exposition in vivo, MAE exerted different effects in *A. mellifera* and *F. candida*. According to scientific literature, AZA is part of the insect growth disruptors (IGDs) for its multiple inhibitory effects, though it is also known for its antifeedant and repellent effects (Pener and Dhadialla, 2012). In worker honey bees, the oral administration of MAE at 25 and 3.12 μg/mL did not induce any lethal and sub-lethal effects (e.g., behavioral alterations). However, knowing that AZA can interfere with the endocrine system, a harmful effect on larvae, pupae, drone, queen bee, or other wild pollinators is still possible at different concentrations (Bernardes et al., 2018; Faria Barbosa et al., 2014; Thompson et al., 2005). Furthermore, AZA may also affect oogenesis and vitellogenesis (Sayah et al., 1996). Considering the collembola *F. candida*, MAE at 25 and 3.12 μg/mL did not cause a reduction in the reproduction rate. This effect was also observed by Zortéa and colleagues (Zortéa et al., 2018) who tested different concentrations, between 1.5 mg/kg and 140 mg/kg. In our work, we observed an increase of collembola juvenile stages at sublethal concentration (3.12 μg/mL), because it can be hypothesized that MAE that at low dose can positively impact traits such as growth, time to first molting and fecundity, similarly to what found when insects were exposed to sublethal concentrations (<32 mg/kg) of nonylphenol (Widarto et al., 2007). However, we cannot exclude a positive effect on the endosymbiont *Wolbachia* necessary for *F. candida* parthenogenetic reproduction (Negri et al., 2012).

A Pearson correlation analysis was also conducted to highlight the specific correlation between secondary metabolites of MAE and acute toxicity assays. The study revealed a negative correlation between the vitality of FB-21 cells and the presence of flavonoids and terpenes, which was similarly seen in SH-SY5Y cells with alkaloids, terpenes, and limonoids. These results align with previous studies conducted by Pertino et al. (Pertino et al., 2007) and Zhang et al. (Zhang et al., 2022), who reported cytotoxic effects on a variety of cell lines induced by terpenes and alkaloids derived from plants. Also, the germination index of the three plant species tested was negatively correlated to the presence of alkaloids, terpenes, and tannins in MAE. This observation was also confirmed in the study conducted by Ibrar Shinwari et al. (Ibrar Shinwari et al., 2017), who reported the inhibitory effect of MA aqueous extract in tomato seed germination. Lastly, in the zebrafish mortality test, a positive correlation was found between embryo mortality and the presence of alkaloids, terpenes, and limonoids. These findings shed light on the potential toxicological effects of these compounds on different biological systems.

5. Conclusions

Environmentalists push for the use of natural products in agriculture instead of synthetic alternatives since plant-based products are generally safer. However, the chemical composition of these products is often complex and not completely characterized and may present compounds that can be dangerous to plants, microorganisms, animals, or even humans, especially if they are improperly used. *M. azedarach* is widely used as a natural insecticide in the form of extract. The availability of this plant and the ease with which these extracts can be prepared demonstrate its wide applicability in agriculture and plant breeding. In this work we aimed at deepening the knowledge about the chemical composition of *M. azedarach* aqueous extract and preliminarily assessing its toxicity on different organisms, from plants (seeds) to animals (zebrafish and bees) and approaching human relevance with in vitro cellular models. The results of this study should give pause for thought because, despite being conceived as safe, this product can be harmful for plants, insects and, potentially, to humans when used at high doses. Hence, it will be crucial to evaluate the effectiveness of MAE within concentration ranges that do not have detrimental effects on the environment. Moreover, in a one-health oriented vision, agricultural operators should always be informed about the risks that these products may pose to humans and the environment and should be aware about standardized protocols for their application on plants.

Anyway, the data collected did not evaluate the mechanisms of action involved in the toxicological response, hence further studies in this direction are needed. In addition, all measurements were made in laboratory experimental models. Therefore, this analysis should be repeated in models closer to agricultural reality. In the future, more emphasis should be placed on the impact that natural products can have on soil, other plants (wild and cultivated) and animals. This approach aligns with the need to ensure sustainable agricultural practices and ecosystem health, in line with Sustainable Development Goals (SDGs) promoting environmental sustainability and biodiversity conservation.

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Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.175314.

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