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Phytotoxic essential oil from black turmeric (*Curcuma caesia* Roxb.) rhizome: Screening, efficacy, chemical basis, uptake and mode of transport

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ABSTRACT

Plant-based phytotoxins play an important role in plant-plant interaction and are of great promise for the development of bioherbicide. In this study, screening of essential oils from six different *Curcuma* rhizomes identified black turmeric (*Curcuma caesia* Roxb.) oil as an efficient growth inhibitor in wheatgrass coleoptile bioassay (IC_{50} 57.1 μg/mL). The phytotoxic efficacy of this oil was further confirmed through the dose dependent (10.0–300.0 μg/mL) inhibition of germination, coleoptile and radicle growth of wheatgrass seeds in water medium (IC_{50} 176.7, 90.6 and 93.0 μg/mL respectively) and the pre-germinated seeds in agar medium. Activity-guided fractionation and purification of the crude oil identified curzerenone, a major furanosesquiterpene in this oil as the most active phytotoxin (IC_{50} 13.0 μg/mL in coleoptile bioassay; 188.3, 34.7 and 36.7 μg/mL respectively in water medium). Structure-activity relationship study indicated the importance of all the functional groups for its phytotoxicity. A significant contribution by the oxygenated monoterpenes towards oil phytotoxicity was also evidenced. Though inhibitory efficacy of the oil was comparatively weak in soil, a profound activity through aerial diffusion was observed against germination and/or growth of fresh and pre-germinated wheatgrass seeds (IC_{50} 1.4–6.2 mg/L air). The study also confirmed the uptake of active phytotoxins with no detectable transformation by the seeds in water and their persistence in the soil for at least one and half months. Further, its applicability was demonstrated through an efficient post-emergence growth inhibition (IC_{50} 37.0–81.4 μg/mL) of bermudagrass weed in water and agar medium.

1. Introduction

Plant produces an arsenal of secondary metabolites often as a part of its survival strategy to counter the natural enemies and competitors such as pathogenic microbes, insect pests, weeds *etc*. Some of these metabolites exert phytotoxicity through the inhibition of germination and/or growth of the same or other plant species (Duke, 2010; Li et al., 2010; Macías et al., 2019, 2007; Wang et al., 2021). The metabolites which are involved in plant-plant interaction, exhibiting a direct or indirect influence on germination and/or growth within an ecological niche are called as allelochemicals (Duke, 2010; Li et al., 2010). These phytotoxins can be the source of natural herbicides for the weed management as an eco-friendly alternative to largely-used synthetic candidates (Bajwa et al., 2015; Jabran et al., 2015; Macías et al., 2019, 2007). Weeds are one of the greatest competitors of the crop and may lead to decreased crop productivity by up to 34% (Jabran et al., 2015; Oerke, 2006). These natural phytotoxins bear the potential to be an integral part of the sustainable agriculture and are worth to be explored.

Phytotoxic molecules have been reported from several parts of the plants including leaves, stem, bark, fruits, flowers, roots and rhizome. The secondary metabolites, both volatiles and non-volatiles produced by the underground tissues play a pivotal role in plant-plant interaction (Adedeji and Babalola, 2020; Rahman et al., 2019). Although the root allelochemicals have been explored widely there is only handful of systematic studies on rhizome phytotoxins especially VOCs (volatile organic compounds) therein. The VOCs in the form of essential oil from *Hedychium coronarium*, *Acorus calamus*, *Curcuma longa*, *C. zedoaria*, *Zingiber officinale*, *Z. montanum* and *Cyperus giganteus* rhizome have been

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demonstrated to be phytotoxic (Bora et al., 2021; Costa et al., 2019; Rolli et al., 2014; Satyal et al., 2013; Vilhena et al., 2009). Besides, rhizome oils have been known to be effective against plant enemies such as insects and microbes, indirectly indicating their role in plant defense (Ali et al., 2015; Damalas, 2011; Sharma et al., 2008; Sivasothy et al., 2011; Zhou et al., 2021). Therefore, rhizome VOCs can be a potential target in pursuit of phytotoxic metabolites.

The genus *Curcuma* comprises of rhizomatous aromatic herbs belonging to the family Zingiberaceae. They are of immense traditional as well as commercial values. Their uses as the phytomedicines, spices, nutraceuticals and as a part of regional cultures are well-known (Basak et al., 2010; Dosoky and Setzer, 2018; Rajkumari and Sanatombi, 2017; Schaffer et al., 2015; Sun et al., 2017). Southeast Asian countries and northeast India are known for the genetic diversity in Zingiberaceae species (Basak et al., 2010; Sun et al., 2017). A thorough literature survey revealed six commonly known ethnomedicinally important *Curcuma* species from the northeast region of India including *C. longa* (turmeric), *C. caesia* (black turmeric), *C. zedoaria* (zedoary), *C. amada* (mango ginger), *C. angustifolia* (east Indian arrowroot) and *C. aromatica* (wild turmeric) (Basak et al., 2010). Among these, phytotoxicity of turmeric and zedoary oil has been reported previously (de Melo et al., 2017; Ibáñez and Blázquez, 2019; Rolli et al., 2014). However, no detailed investigation has been made till date on these *Curcuma* rhizomes to identify the active phytotoxins, their mode of action and role in plant-plant interaction (Pandey et al., 2021). Considering the wide-spectrum bioactivities especially phytotoxicity of *Curcuma* essential oils, the authors hypothesize that rhizome VOCs from these species may be the source of potent phytotoxins which deserve a thorough investigation (Dosoky and Setzer, 2018; Rolli et al., 2014). Thus, the aim of the current study includes (i) screening of commonly known *Curcuma* rhizome oils from northeast India for phytotoxic activity, (ii) to identify the active phytotoxins from the most potent candidate and (iii) to test their efficacy, uptake, selectivity and mode of transport.

2. Material and methods

2.1. Chemicals and reagents

Deionized water for the experiments was obtained from Milli-Q system (Merck Millipore, MO, USA). Deuterated chloroform (99.8 atom %D), allyl alcohol and HPLC grade solvents were purchased from Sigma-Aldrich (MO, USA). Silica gel, sodium hypochlorite, Tween 80, sucrose, buffer components, anisaldehyde and other chemicals for preparing the staining agent were procured from Avra Synthesis (Hyderabad, India). Phytojars and other plasticwares were supplied by Tarsons (Kolkata, India). Thin layer chromatography (TLC) plates and Whatman No. 1 filter paper were obtained from Merck (Darmstadt, Germany). For the column chromatography and extraction purpose technical grade solvents were distilled before use.

2.2. Collection and extraction of the plant materials

Organic seeds (wheatgrass, rice, fenugreek, bermudagrass, green gram, chickpea and mustard seeds) were collected or purchased locally. Mature rhizome of *Curcuma* species (*C. caesia*, *C. longa*, *C. zedoaria*, *C. amada*, *C. angustifolia* and *C. aromatica*) were harvested from the experimental farm of CSIR-NEIST, Jorhat, India (26°44 15.6948 N and 94°9 25.4622 E, altitude 94 m). Their identity was confirmed by the plant breeder Dr. M. Lal, one of the authors of this article. The essential oil was extracted from these rhizomes through hydrodistillation in a Clevenger-type apparatus as described in the previous reports (Mahanta et al., 2020, 2021). The % oil yield was calculated with respect to the fresh weight of the rhizome sample (Table S1). The chemotaxonomic assessment of the collected *Curcuma* rhizomes through metabolite-profiling of the extracted oils in gas chromatography-mass spectrometry (GC-MS) further supported their identity.

2.3. Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was performed on silica gel G precoated (0.25 mm) aluminium plates (height 3.5 cm) using 1:1 *v/v* dichloromethane and *n*-hexane mixture as the mobile phase. The plate was developed half of its height, followed by drying and finally developed fully (solvent front position 85%). The spots were visualized through anisaldehyde staining.

2.4. High performance thin layer chromatography (HPTLC)

For the HPTLC analysis, Camag (CAMAG, Switzerland) automatic band applicator (ATS4), humidity controlled development chamber (ADC2), derivatizer and photodocumentation system (TLC Visualizer 2) were used. Silica gel G coated aluminium plates (10 cm × 10 cm × 0.25 mm, Merck) were employed for the analysis. The samples were dissolved in ethyl acetate to apply as the bands (8 mm) on the plate. A mixture of dichloromethane and *n*-hexane (3:1 *v/v*) was the mobile phase and anisaldehyde was the staining agent. VisionCATS software was used for organizing the work-flow, data documentation and analysis.

2.5. Gas chromatography-mass spectrometry (GC-MS)

Gas chromatography-mass spectrometry (GC-MS) analysis was conducted on an Agilent 8890 gas chromatograph coupled with Agilent 7010B triple quadrupole mass spectrometer. For the separation of the analytes, HP-5MS UI capillary column (30 m × 0.25 mm × 0.25 μm) was used. Helium was employed as the carrier gas with a flow rate of 1.0 mL/min. The injector temperature was maintained at 250 °C and the initial column temperature was kept at 50ºC for 1.0 min. A temperature gradient of 8ºC/min was set till 200 °C and a hold for 2.0 min followed by a ramp of 15 °C/min up to 260ºC and a hold for 3 min (total runtime 28.75 min). The MS data were recorded in the full scan mode and mass range *m/z* 40–400. GC-MS samples were prepared in HPLC grade ethyl acetate (~150 ppm) and 0.3 μL of the sample solution was injected with a split ratio of 10:1. Area under the peaks represented relative percent of the individual analytes. Agilent MassHunter Qualitative Analysis 10.0 software was used for the data processing and integrated NIST 2017 Mass Spectral Library for the structural prediction. The linear retention index (RI) value of the peaks was calculated in relation to the series of C_8-C_{20} *n*-alkanes.

2.6. ^{1}H nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy experiments were carried out in a 400YH FT NMR spectrometer (400 MHz), Jeol, Japan. Samples were prepared in deuterated chloroform (600 μL) for ^{1}H NMR spectroscopy. MestReNova software was used for the data analysis. Chemical shift values were reported in ppm and the residual solvent peak at 7.26 ppm was used as the reference in the proton NMR spectrum.

2.7. Wheatgrass coleoptile bioassay

The coleoptile bioassay was carried out following earlier reports by Macías and co-workers with few modifications (Macías et al., 2010). The wheatgrass seeds (without sterilization) were germinated in sterile water taken in a Petri dish at 22 ± 2 °C for 2 days. A stock solution (3.0 mg/mL) of the inhibitor (oil or oil fractions or purified metabolite) was prepared in HPLC grade methanol. The required stock solution to attain the test concentration in 2.0 mL final volume was transferred to a 5 mL glass vial. Then, methanol was evaporated under a stream of nitrogen and the inhibitor was added with 2.0 mL of phosphate-citrate buffer of pH 5.0 containing 2% sucrose (*w/v*) and 0.1% Tween 80. The mixture was vortexed vigorously for the homogeneous dispersion of the test sample. The roots and caryopses of the germinated seeds were detached from the coleoptiles, the apical portion (~2.0 mm) of the individual

coleoptile was cut off manually and the next 4.0 mm was used for the bioassay. Eight coleoptiles were placed in each vial and they were allowed to grow at 22 ± 2 °C for 36 h with a photoperiod of 12/12 h light/dark and occasional shaking. Each individual experiment was set up in duplicate. The buffer solution containing coleoptiles, sucrose and Tween 80 without any inhibitor was considered as the control. The length of the individual coleoptile after the incubation was measured digitally. The percent growth inhibition was determined as [(average increase in length of the coleoptiles in control – average increase in length of the coleoptiles in treated) / average increase in length of the coleoptiles in control] × 100%.

2.8. Phytotoxicity study of various Curcuma oils

Wheatgrass coleoptile bioassay was performed to screen the phytotoxic potential of the rhizome oil from six *Curcuma* species (*C. caesia*, *C. longa*, *C. zedoaria*, *C. amada*, *C. angustifolia* and *C. aromatica*) in the concentration range 50.0, 100.0, 200.0 and 300.0 μg/mL. The commercial herbicide allyl alcohol was assayed as the positive control in the concentration range 10.0–100.0 μg/mL.

2.9. General procedure for the surface sterilization of the seeds

The seeds (wheatgrass, rice, fenugreek, bermudagrass, green gram, chickpea and mustard) for phytotoxicity assay were surface sterilized by dipping into 1.0% sodium hypochlorite solution for *ca.* 1.0 min. After sterilization they were thoroughly washed with sterile deionized water for at least five times. The residual water on the seed surface was soaked over a blotting paper and further used directly for the assay.

2.10. Phytotoxicity bioassay (wheatgrass) in water medium

The phytotoxic activity was evaluated on wheatgrass seeds through germination and growth inhibition bioassay in water medium. The experiment was carried out following the previously reported protocol with few modifications (Liu et al., 2016). The phytotoxicity was tested for the black turmeric oil over a range of concentrations *i.e.* 10.0, 50.0, 100.0, 200.0 and 300.0 μg/mL. The test solutions were prepared in sterile deionized water (10.0 mL) containing 0.5% methanol (HPLC grade) and they were individually taken into sterile Petri dishes of 90 mm diameter. The solution without an inhibitor (oil) was treated as the control. Twenty surface sterilized seeds were evenly placed on each Petri dish and incubated in a controlled environment at 22 ± 2ºC with a photoperiod of 12/12 h light/dark. Each experiment was performed in triplicate. The incubation was continued for 3 days when more than 80% seeds were found to be germinated in control. Then, the number of germinated seeds and the length of coleoptile and radicle were recorded individually. The germination was assessed by a noticeable protrusion (>1 mm) of the radicle through the seed-coat. The radicle and coleoptile were detached carefully from the germinated seeds before measuring their length. Allyl alcohol was used as the positive control. The percent inhibition of seed germination was calculated as [(average no. of germinated seeds in control – average no. of germinated seeds in test sample) / average no. of germinated seeds in control] × 100%. Similarly, percent growth inhibition was determined as [(average length of the coleoptile or radicle in control – average length of the coleoptile or radicle in treated) / average length of the coleoptile or radicle in control] × 100%. The inhibitory potential was expressed through the half maximal inhibitory concentration (IC_{50}) value.

2.11. Phytotoxicity bioassay on different monocots and dicots

The phytotoxicity of black turmeric oil was also tested on different monocot and dicot seeds (3 each). The monocot seeds included rice (*Oryza sativa*), fenugreek (*Trigonella foenum-graecum*) and bermudagrass (*Cynodon dactylon*). Green gram (*Vigna radiata*), chickpea (*Cicer arietinum*) and mustard seeds (*Sinapis alba*) were selected in dicots. The phytotoxic potency of the oil on these seeds was studied through phytotoxicity bioassay in water medium in the concentration range 50.0, 100.0, 200.0 and 300.0 μg/mL.

2.12. Agar medium bioassay

The seedling growth inhibitory potential of black turmeric oil was tested through agar medium bioassay carried out in phytojars at 10.0, 50.0, 100.0, 200.0 and 300.0 μg/mL. The protocol was adopted with modifications from the article published by Shajib and coworkers (Shajib et al., 2012). Surface sterilized seeds of wheatgrass were pre-germinated for the assay at 22 ± 2ºC for 15 h. Sterile agar solution (0.7% *w/v*, 50 mL) was poured into each phytojar and the calculated amount of the inhibitor, taken in 40 μL Tween 80 was added into the agar solution. The mixture was agitated on a vortex shaker for the homogenous dispersion of the inhibitor in the medium before it gelatinized. Twenty pre-germinated seeds were sown in each phytojar. Then, the jars were sealed with parafilm tape and kept in a controlled environment at 22 ± 2ºC with a photoperiod of 12/12 h light/dark. Agar solution containing 40 μL Tween 80 without an inhibitor was kept as the control. Two replications were performed for each test concentration and the control. The seedlings were harvested after 3 days, thoroughly washed with water and the length of radicle and coleoptile were measured. The percent growth inhibition was calculated and represented as described in 'phytotoxicity bioassay in water medium'.

For the agar medium bioassay with bermudagrass, the seeds were pre-germinated at 26 ± 2ºC for 3 days and the assay was carried out at 50.0, 100.0, 200.0 and 300.0 μg/mL of oil concentration. The percent growth inhibition was determined as [(average increase in length of the coleoptile or radicle in control – average increase in length of the coleoptile or radicle treated) / average increase in length of the coleoptile or radicle control] × 100%.

2.13. Activity guided purification of active phytotoxin(s)

The black turmeric essential oil from the same batch was subjected to the fractionation using silica gel column chromatographic technique. The crude oil (400 mg) was loaded on a silica gel column (60–120 mesh, 27 cm × 1.4 cm H×ID) and eluted with an increasing percentage of ethyl acetate in *n*-hexane. Eluted fractions were concentrated individually under reduced pressure (200 mbar) at 40 °C to produce three fractions (**A**-**C**) as follows: (**A**) eluent: 100% *n*-hexane, 61 mg; (**B**) eluent: 1.0% *v/v* ethyl acetate in *n*-hexane, 230 mg; (**C**) eluent: 50.0% *v/v* ethyl acetate in *n*-hexane, 55 mg. Fraction **B** contained the major metabolites mainly curzerenone (**1**), germacrone (**2**) and camphor (**3**), all appearing at a very close R_f on TLC plate. Further purification of the fraction **B** was carried out similarly through silica gel column (60–120 mesh, 30 cm × 1.4 cm H×ID), eluting isocratically with 0.3% *v/v* ethyl acetate in *n*-hexane. It yielded pure germacrone (**2**, 27 mg), curzerenone (**1**, 45 mg) and camphor (**3**, 17 mg). The remaining mass (~100 mg) eluted impure as a mixture of **1** and **3** which was not used further. The fractions (**A**-**C**) and purified metabolites (**1**-**3**) were analyzed through GC-MS and ^{1}H NMR techniques. Also, an HPTLC analysis was performed in reference to the crude oil. The samples were stored in dark at 4 °C until further experiment.

A single point inhibition of seed germination and growth was studied on wheatgrass seeds for the fractions (**A**-**C**) and purified molecules (**1**-**3**) in reference to the crude oil at 200.0 μg/mL through phytotoxicity bioassay in water. Besides, a wheatgrass coleoptile bioassay was set up to comparatively evaluate the inhibitory efficacy of the crude oil, chromatographic fractions **A**-**C** and purified metabolites (**1**-**3**) at single point concentration of 50.0 μg/mL.

2.14. Isolation of curzerene (4) and zederone (6)

Curzerene (**4**) was isolated from the black turmeric essential oil of a different batch through column chromatography technique. Briefly, 1.5 gm of the crude essential oil was loaded on to a silica gel column (60–120 mesh, 25 ×2 cm H×ID) and eluted with *n*-hexane (500 mL). Further purification of this concentrated fraction (236 mg) through silica gel column (60–120 mesh, 27 cm × 0.9 cm H×ID, mobile phase: *n*-pentane 150 mL) yielded pure curzerene (**4**, 29.0 mg).

Zederone (**6**) was isolated from the acetone extract of the black turmeric rhizome. Ground and 3 days air-dried (25.0 g) rhizome was extracted with acetone (100 mL) to obtain 1.1 g brown oleoresin. It was fractionated through silica gel column chromatography using 3.0% *v/v* ethyl acetate in *n*-hexane to obtain three fractions (0.25, 0.11 and 0.08 g) and finally eluted with ethyl acetate (0.58 g). Further purification of the third fraction (0.08 g) through silica gel column chromatography (60–120 mesh, 31 cm × 1.4 cm H×ID) using increasing percentage of ethyl acetate (1.0–5.0% *v/v*) in *n*-hexane yielded pure zederone (**6**, 18.0 mg). The identity and purity of **4** and **6** was investigated through GC-MS, ^{1}H and ^{13}C NMR techniques which matched well with the previous reports (Hamdi et al., 2015; Yang et al., 2011).

2.15. Semi-synthesis of tetrahydrocurzerenone (5)

Curzerenone (**1**, 30.0 mg, 0.130 mmol) in 4.0 mL methanol (HPLC grade) was added with about 50 mg 10% Pd on activated carbon and allowed to shake continuously under 50 psi pressure of hydrogen. After 4 h, the reaction mixture was filtered through a celite bed (dichloromethane as the eluent) and concentrated to obtain 29.5 mg gummy liquid. It was further chromatographed over silica gel column (60–120 mesh, 22 cm × 1.4 cm H×ID) using a gradient of ethyl acetate (0.0–0.2% *v/v*) in *n*-hexane to furnish 15.0 mg (0.064 mmol, yield 49.2%) gummy liquid. The GC-MS, ^{1}H and ^{13}C NMR analyses data revealed the reduction of terminal unconjugated double bonds in the structure, identifying it as tetrahydrocurzerenone (**5**).

2.16. Structure-activity relationship (SAR) study

Four molecules, structurally related to curzerenone *viz.* germacrone (**2**), curzerene (**4**), tetrahydrocurzerenone (**5**), zederone (**6**) were selected for the structure-activity relationship (SAR) study. The phytotoxicity of these selected compounds was investigated through wheatgrass coleoptile bioassay in the concentrations 10.0 50.0, 100.0, 200.0 and 300.0 μg/mL and the efficacy was expressed through their IC_{50} values.

2.17. Air diffusion bioassay

The assay protocol to test the phytotoxic potential of black turmeric rhizome volatiles through aerial diffusion was inspired and partially adopted by the experimental set up and method described previously for *Dittrichia viscosa* and *Calamintha nepeta* (Araniti et al., 2013, 2017). Polypropylene jars *i.e.* wide-mouth cylindrical containers of capacity 4 L (16 cm × 20 cm) were taken and a hole (0.5 cm) was made at the center of the lid which was fitted with a cotton plug (Fig. 3**E**). The required amount of crude oil for different test concentrations (1.0, 2.0, 4.0 and 8.0 mg/L of air) was dissolved in 0.3 mL acetone, loaded on a Whatman No. 1 filter paper (90 mm) and acetone was allowed to evaporate. A Petri dish (90 mm), lined with the oil-loaded filter paper was kept at the base of every jar. In the control jar, acetone without oil was loaded. In each of the five jars (four tests and one control), three uncovered Petri dishes (75 mm) each containing 7.0 mL sterile deionized water and twenty surface-sterilized wheatgrass seeds at three different stages of germination *i.e.* (i) fresh, (ii) pre-germinated for 24 h and (iii) pre-germinated for 48 h were laid on a wire structure at the mid-depth. Prior to this set up, average radicle and coleoptile length were measured for the pre-germinated (48 h) seeds. Finally, the lids were closed, sealed with parafilm tape and the jars were kept at 22 ± 2 °C with a photo period of 12/12 h light/dark. After 3 days of incubation, the length of radicle and coleoptile were measured. The percent inhibition and IC_{50} values were calculated for the fresh and pre-germinated seeds (24 and 48 h) individually at different test concentrations as described in 'phytotoxicity bioassay in water medium'. To note, in the case of pre-germinated seeds (48 h) an average increase in the length during incubation was considered for this calculation.

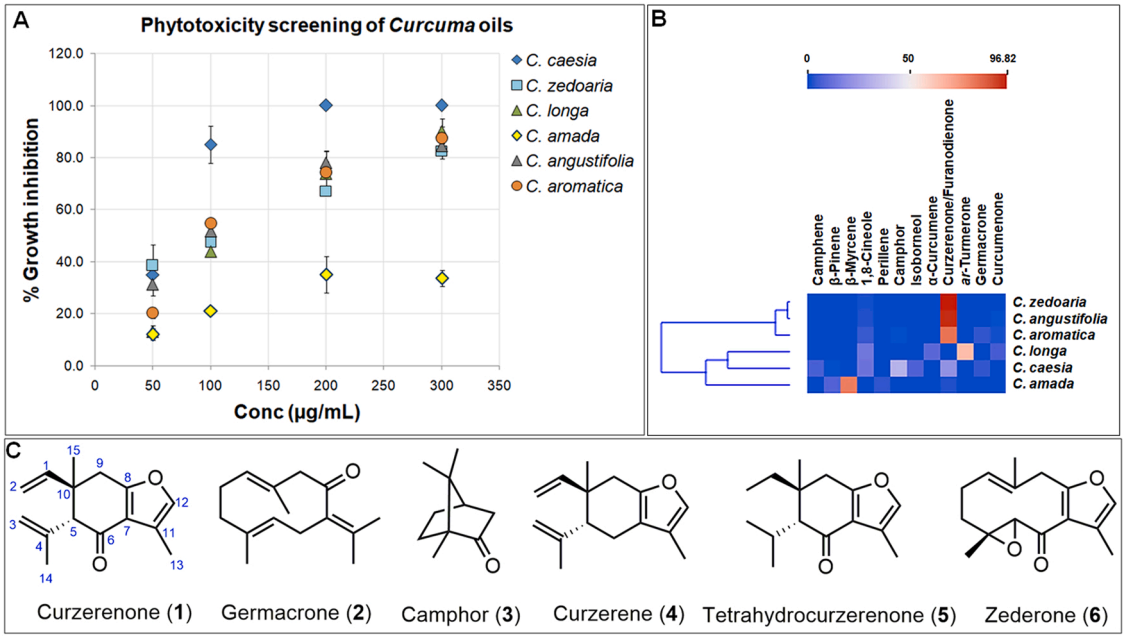

Fig. 1. (A) Phytotoxicity evaluation of six *Curcuma* oils (50.0–300.0 μg/mL) through wheatgrass coleoptile bioassay; (B) hierarchically clustered heatmap for the tested *Curcuma* species generated on the basis of their essential oil composition; (C) chemical structures of curzerenone, germacrone, camphor and related molecules.

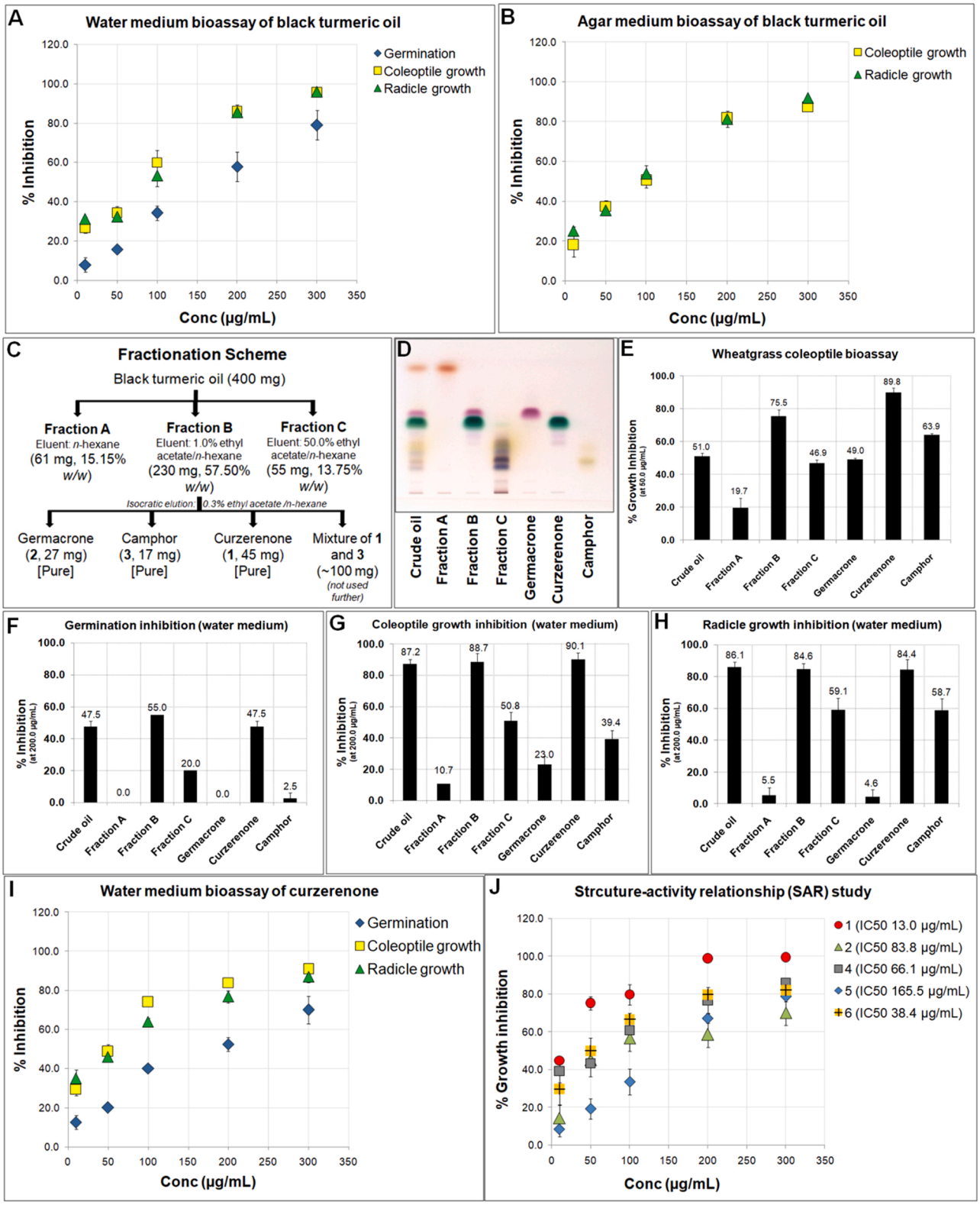

Fig. 2. Phytotoxicity bioassay (wheatgrass) of black turmeric oil (10.0–300.0 µg/mL) in (A) water medium with fresh seeds and (B) agar medium with pre-germinated seeds; (C) silica gel column chromatographic fractionation and purification scheme for the black turmeric oil; (D) image of the HPTLC plate for the crude oil, fractions **A-C**, **2**, **1** and **3** (to note, the spot for camphor is not visible possibly due to its sublimation in HPTLC condition); (E) wheatgrass coleoptile bioassay for the crude oil, fractions **A-C**, **2**, **1** and **3** at 50.0 µg/mL; (F-H) phytotoxicity bioassay (wheatgrass) in water medium for the crude oil, fractions **A-C**, **2**, **1** and **3** at 200.0 µg/mL showing the inhibition of (F) germination, (G) coleoptile and (H) radicle growth; (I) phytotoxicity bioassay (wheatgrass) of curzerenone (**1**, 10.0–300.0 µg/mL) in water medium; (J) structure-activity relationship (SAR) study: wheatgrass coleoptile bioassay for **1** and related molecules (**2**, **4**, **5** and **6**) at 10.0–300.0 µg/mL.

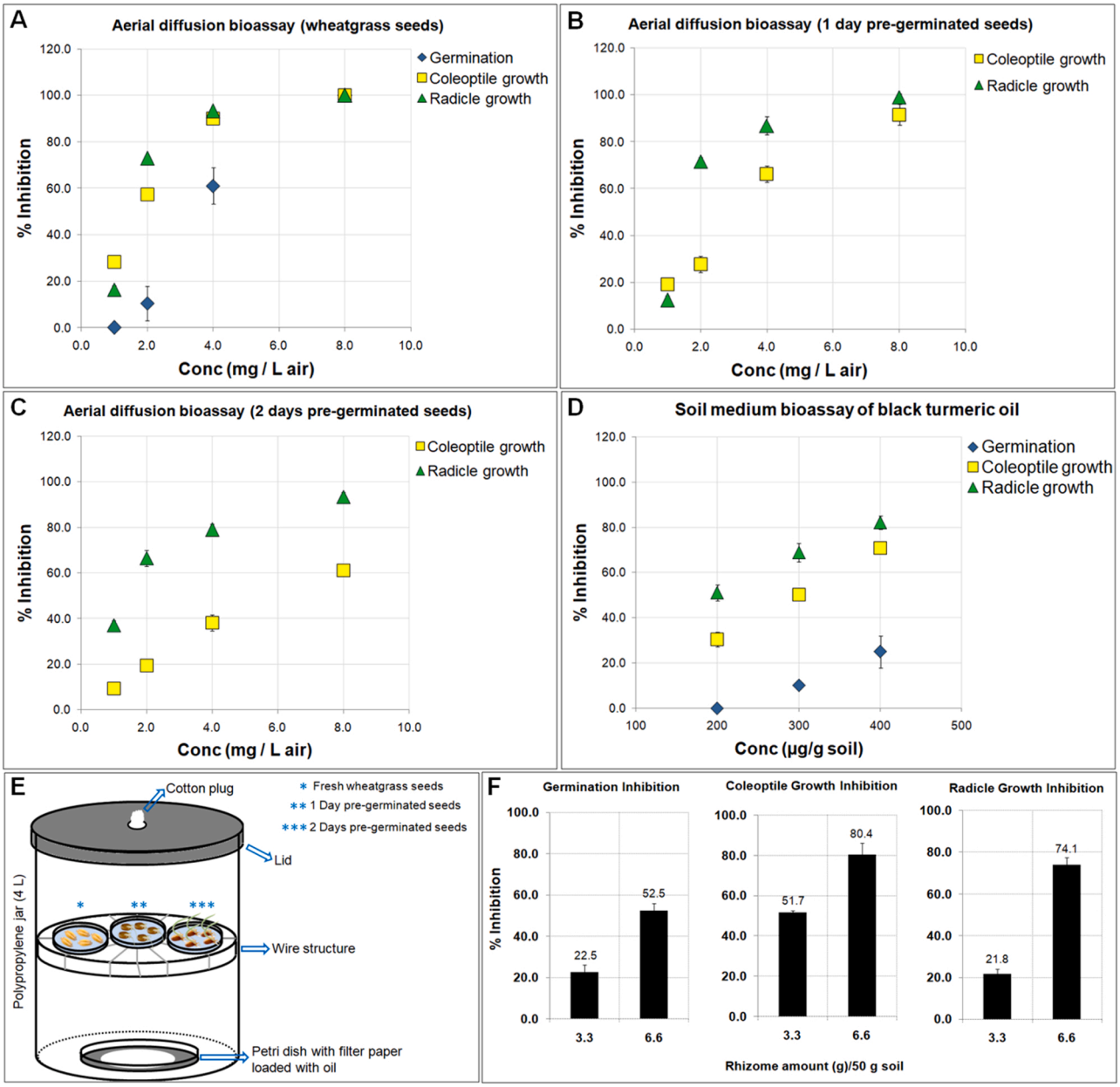

Fig. 3. (A-C) Phytotoxicity bioassay (wheatgrass) of black turmeric oil through aerial diffusion (1.0–8.0 mg/L air) on (A) fresh seeds, (B) one day pre-germinated seeds and (C) two days pre-germinated seeds; (D) Phytotoxicity bioassay (wheatgrass) of black turmeric oil (200.0–400.0 µg/mL) in soil medium; (E) cartoon representation of the aerial diffusion bioassay set-up; (D) germination, coleoptile and radicle growth inhibition of the wheatgrass seeds by *n*-hexane extract of the soil-rhizome (ground) mixture incubated at an ambient condition for 45 days (3.3 and 6.6 g / 50 g soil).

2.18. Soil medium bioassay

The protocol for soil medium bioassay was adopted with significant modifications from 'sandwich agar method' reported by Young et al. (Young and Bush, 2009). Dry clay type garden soil was collected from the institute campus, crumbled and taken in a Petri dish. The soil was watered and twenty wheatgrass seeds were sown to ensure that the soil had no significant inhibitory effect on the seed germination and their growth. Further, the soil sample (30 g) was taken in phytojars and the black turmeric oil in 5.0 mL sterile deionized water containing 0.1% Tween 80 was added at three different concentrations (200.0, 300.0 and 400.0 µg/g of the soil). They were thoroughly mixed and packed at the bottom of the jar. Sterile agar solution (0.7% *w/v*, 15 mL) was dispensed to create an even thin layer on the top of the soil and waited until gelatinized. The soil sample without any added oil was considered as the control. Twenty wheatgrass seeds were sown on the agar in the individual jar. Each test concentration including the control was set up in duplicate. The sealing and incubation of the phytojars and finally the analysis were carried out similarly as described in 'agar medium bioassay'.

To test the possibility of transport of these phytotoxins from rhizome to soil and to assess their soil stability, a simulated experiment was designed. The soil (50 g) sample used in the 'soil medium bioassay' was thoroughly mixed with fresh and ground rhizome (3.3 and 6.6 g in two separate experiments) and taken in glass beakers (500 mL). The control experiment contained no added rhizome in the soil. The beakers were covered with aluminium foil and kept in shade at ambient condition (25 ± 3 °C and relative humidity 65 ± 10%). After 45 days, the beakers were added with 250 mL *n*-hexane, stirred for 3 h and kept undisturbed overnight. Further, the organic layers were filtered individually through

Whatman No. 1 filter paper and concentrated at 40 °C under reduced pressure (200 mbar) to obtain 17.0 mg (3.3 g rhizome), 21.1 mg (6.6 g rhizome) and 2.0 mg (control) brownish sticky extracts. They were analyzed through GC-MS and subjected to phytotoxicity bioassay in water medium to check their phytotoxic efficacy through single point inhibition at 200.0 μg/mL.

2.19. Uptake and transformation study

The phytotoxicity bioassay was set up with wheatgrass seeds and black turmeric oil at 200.0 μg/mL in water medium. The control experiment did not contain any oil. Both the experiments were performed in duplicate. After 3 days of incubation, the seeds were separated individually with forceps from the aqueous medium, surface was washed with ethyl acetate (3–5 drops) and taken into a mortar and pestle. The ethyl acetate that was used for washing the seed-surface was taken with water medium. Then, the seeds were added with 200.0 μg eugenol as an internal standard, crushed and extracted with ethyl acetate (3 ×1.0 mL). The combined organic layer was filtered, passed through anhydrous sodium sulfate, concentrated up to 2.0 mL under a stream of nitrogen and analyzed through GC-MS. Similarly, 200.0 μg eugenol was added to the water medium as an internal standard and extracted with ethyl acetate (3 ×1.0 mL). The combined organic layer was treated, concentrated and analyzed similarly.

2.20. Statistical analysis

'Orange3' (version 3.30.1), an open source visual programming software package (https://orangedatamining.com) was used for generating the heatmap from GC-MS based quantitative oil profile of the studied *Curcuma* species. In the heatmap output, columns represented individual chemical constituents whereas the rows were represented by different *Curcuma* species. A dendrogram, associated with the heatmap showed the clustering of *Curcuma* species on the basis of their similarity in essential oil profile. The percent inhibition values in different phytotoxicity bioassays were expressed as mean ± standard deviation. MS Office Excel 2007 (Microsoft Corporation, Redmond, USA) was used for all these statistical analyses.

3. Results and discussion

3.1. Screening of Curcuma oils for phytotoxic activity

The essential oils from six different *Curcuma* (*C. caesia*, *C. longa*, *C. zedoaria*, *C. amada*, *C. angustifolia* and *C. aromatica*) rhizomes were screened for their phytotoxic potential through wheatgrass coleoptile bioassay. A concentration dependent coleoptile growth inhibitory activity was observed in all the oil samples in the range 50.0–300.0 μg/mL (Fig. 1A). The essential oil from black turmeric (*C. caesia*) showed the highest potency with a half maximal inhibitory concentration (IC_{50}) 57.1 μg/mL. It exerted very high growth inhibition (85.0%) even at 100.0 μg/mL. *C. angustifolia*, *C. zedoaria*, *C. aromatica* and *C. longa* also showed high inhibition with comparable efficacy among themselves (IC_{50} 91.4, 91.6, 102.3 and 116.5 μg/mL respectively). The observed phytotoxicity was low in the case of *C. amada* oil with $IC_{50} > 300.0$ μg/mL. In reference, a synthetic herbicide *viz.* allyl alcohol (positive control) showed IC_{50} 52.9 μg/mL in this bioassay. To the best of our knowledge, a comparative phytotoxicity was investigated among *Curcuma* oils for the first time in this study. Considering the high phytotoxic potency shown by *C. caesia*, it was selected for further studies.

GC-MS analysis of the oil samples revealed camphor (31.53%), 1,8-cineole (13.33%), curzerenone/furanodienone (20.85%) and germacrone (4.04%) as the major constituents of tested black turmeric oil (Table 1, Fig. 1C). To note, furanodienone is a thermolabile furanosesquiterpene which rearranges to curzerenone at an elevated temperature. When injected in GC-MS, both the molecules appeared as a

Table 1
GC-MS analysis of the essential oil composition in six different *Curcuma* oils that were tested for phytotoxicity [blank cell indicates 'not detected'].

R_t (min)	Chemical identity	RI $_{Calc}$	RI $_{Lit}$	Relative %					
				C. caesia	*C. zedoaria*	*C. angustifolia*	*C. aromatica*	*C. longa*	*C. amada*
5.77	α-Pinene	937	937	0.76					
6.05	Camphene	952	954	6.78					
6.57	β-Pinene	980	979	1.65					7.96
6.78	β-Myrcene	991	988	0.66					79.78
7.44	*p*-Cymene	1027	1025					1.40	
7.52	Limonene	1031	1030	1.80					
7.59	1,8-Cineole	1035	1035	13.33	2.04	2.45	5.05	13.40	
7.86	β-Ocimene	1049	1037						1.79
8.82	Linalool	1101	1101	1.21					
8.87	Perillene	1104	1102						3.70
9.75	Camphor	1151	1150	31.53			1.69		
9.82	Norborneol	1155	1167	1.57					
9.97	Isoborneol	1163	1160	7.57					
10.13	Borneol	1171	1167	1.30					
10.56	α-Terpineol	1194	1199	0.89					
12.30	Isobornyl acetate	1296	1285	1.00					
14.06	β-Elemene	1399	1403	1.24		1.07	1.55		
15.49	α-Curcumene	1492	1486					9.77	
15.74	Curzerene	1508	1500	2.90	1.14				
17.12	Caryophyllene oxide	1598	1587					2.95	1.19
17.36	Curzerenone/furanodienone	1615	1606	20.85	96.82	95.24	82.21		2.40
18.19	*ar*-Turmerone	1675	1678					64.03	
18.71	Germacrone	1713	1707	4.04			4.22		
19.46	Unidentified furanosesquiterpene (M^+ 230)	1767	–				0.85		
20.18	Ambrial	1817	1815						2.05
20.48	Unidentified sesquiterpene (M^+ 234)	1837	–				1.23	2.23	
20.79	Curcumenone	1857	1860			1.24	2.31	6.22	
23.11	Zederone	2012	2008				0.88		
	Monoterpene hydrocarbons (MH)			11.65	0.00	0.00	0.00	1.40	89.53
	Oxygenated monoterpenes (OM)			58.40	2.04	2.45	6.74	13.40	3.70
	Sesquiterpene hydrocarbons (SH)			1.24	0.00	1.07	1.55	9.77	0.00
	Oxygenated sesquiterpenes (OS)			27.79	97.96	96.48	91.70	75.44	5.64

single peak (R_t 17.36 min) which was also observed in our previous studies (Fig. S1) (Mahanta et al., 2020, 2021). However, ^{1}H NMR spectroscopic data of the black turmeric oil confirmed exclusive presence of curzerenone (**1**) in the tested sample and no trace of furanodienone was detected (Fig. S2). *C. angustifolia*, *C. zedoaria* and *C. aromatica* oils were similar in composition and mainly consisted of curzerenone/furanodienone mixture (82–97%) and 1,8-cineole (2–5%), hence showing comparable efficacies. The presence of furanodienone was observed along with **1** in these oils through ^{1}H NMR analysis. Turmeric (*C. longa*) oil was majorly composed of 1,8-cineole (13.40%), curcumenone (6.22%), α-curcumene (9.77%) and *ar*-turmerone (64.04%). The major portion of mango-scented *C. amada* oil was shared by monoterpene hydrocarbons (MH, 89.53%) *viz.* β-myrcene (79.78%), β-pinene (7.96%) and β-ocimene (1.79%). The profiling results matched well with the previous reports and further supported their individual botanical identity (Angel et al., 2014; Jena et al., 2017; Mahanta et al., 2020; Xiang et al., 2017; Zhang et al., 2017).

The oil composition in these six different *Curcuma* oils was further visualized through an easy to perceive hierarchically clustered heatmap (Fig. 1**B**). It was generated with the selected constituents having relative abundance > 3.0% in any of the tested oil samples. The dendrogram clearly showed that *C. angustifolia*, *C. zedoaria* and *C. aromatica* formed a single clade among which first two formed a sub-clade. These correlations corroborated close IC_{50} values for these three species. *C. caesia* and *C. longa* formed a sub-clade probably due to the common abundance of 1,8-cineole (13.33% and 13.40% respectively) in both the samples. *C. amada* oil, dominated by different class of metabolites (MH) showed a weak correlation with the rest. Thus, *Curcuma* oils rich in oxygenated mono and sesquiterpenes (OM and OS) exhibited a higher phytotoxicity in comparison to hydrocarbon-dominating one.

3.2. Seed germination and growth inhibition by black turmeric oil

The phytotoxic potential of black turmeric oil was further established through the germination and growth (coleoptile and radicle) inhibition assays on wheatgrass seeds in water and agar medium separately. When the efficacy was tested for this oil in water medium (taken in Petri dish), a concentration dependent inhibition of germination, coleoptile and radicle growth was observed in the concentration range 10.0–300.0 μg/mL with IC_{50} 176.7, 90.6 and 93.0 μg/mL respectively (Fig. 2A). The growth inhibition was found to be very high at 200.0 μg/mL after 3 days (86.2% and 85.4% for coleoptile and radicle respectively). In comparison, IC_{50} for the positive control (allyl alcohol) was calculated as 22.8, 16.5 and 15.4 μg/mL respectively in the same assay. Similarly, a concentration dependent inhibition of coleoptile and radicle growth was observed for the pre-germinated (15 h) wheatgrass seeds when assayed in 0.7% agar medium taken in phytojars (Fig. 2B). In this assay, the IC_{50} was determined as 111.3 and 100.4 μg/mL respectively, indicating its ability to inhibit the post-germination growth.

The phytotoxic ability of black turmeric oil was demonstrated for the first time in the current study. However, rhizome essential oil from several Zingiberaceae species mainly belonging to the genera *Curcuma*, *Zingiber* and *Hedychium* has been investigated previously in this regard (Bora et al., 2021; Costa et al., 2019; de Melo et al., 2017; Ibáñez and Blázquez, 2019; Mirmostafaee et al., 2020; Rolli et al., 2014). For example, turmeric oil exhibited pre-emergence inhibition of the seed germination (16.3%), root (68.6%) and hypocotyl (49.2%) growth of tomato at 1.0 μL/mL (Rolli et al., 2014). In case of ginger (*Z. officinalis*) oil, the observed inhibition was 4.2%, 73.8% and 58.2% respectively. In another study, turmeric oil extremely affected the germination and seedling growth of a weed namely *Cortaderia selloana* at 1.0 μL/mL (Ibáñez and Blázquez, 2019). *C. zedoaria* oil also has shown its ability to inhibit the germination (~60–70%) and growth (65–90%) of lettuce and tomato at 1.0% dose (de Melo et al., 2017). In our recent study, essential oil of cassumunar ginger (*Z. montanum*) efficiently exerted the phytotoxicity on wheatgrass seeds with IC_{50} 89.4 (germination), 23.1 (shoot) and 52.1 (root) μg/mL (Bora et al., 2021). The oil extracted from the rhizome of *H. coronarium*, an invasive species in Brazilian riparian habitat inhibited the germination and radicle growth of four native tree species *in vitro* (Costa et al., 2019). The efficacy of black turmeric oil was higher or comparable to these previous reports on rhizome oil phytotoxicity within its family, indicating its potential as an herbal phytotoxin. This scientific knowledge may be a step forward towards the value addition and utilization of this endangered crop.

3.3. Activity-guided identification of phytotoxic principle(s)

The identification of active constituent(s) in a crude extract provides the chemical basis of bioactivity and it is an important step towards the development of natural product based technologies. To identify the phytotoxins in black turmeric oil, activity-guided fractionation and purification strategy was adopted (Fig. 2C). Silica gel column chromatographic fractionation of the crude oil (Fractions A-C) followed by phytotoxicity evaluation of the individual fractions in water medium at 200.0 μg/mL (*i.e.* close to the IC_{50} value for germination) led to the identification of active fractions (Fig. 2F-H). The composition of individual fractions was analyzed through extensive chromatographic, spectroscopic and spectrometric (GC-MS, ^{1}H NMR and HPTLC) techniques. During GC-MS analysis the constituents were identified on the basis of their mass spectral library hits and retention index (RI) values (Table 2, Fig. S1). The identity of major constituents was further confirmed through ^{1}H NMR spectral data (Fig. S2). These results were supported by HPTLC analysis of the fractions in reference to the crude oil (Fig. 2D). It was observed that non-polar fraction **A**, highly rich in sesquiterpene hydrocarbons showed very weak/no inhibition against germination (0.0%), coleoptile (10.7%) and radicle growth (5.5%) of the wheatgrass seeds. Fraction **B**, major mass of the oil (57.5% *w/w* of the oil) containing curzerenone (**1**, 67.1%), germacrone (**2**, 12.3%) and camphor (**3**, 20.6%) exhibited strongest inhibition of germination and growth (55.0%, 88.7% and 84.6% respectively) that was comparable to the crude oil. Relatively polar fraction **C**, comprising of monoterpene alcohols (majorly isoborneol 62.9%, borneol 11.9% and α-terpineol

Table 2
GC-MS analysis of the column chromatographic fractions (**A-C**) of the black turmeric essential oil.

R_t (min)	Chemical identity	RI $_{Calc}$	RI $_{Lit}$	Relative %	Class
Fraction A					
13.16	δ-Elemene	1346	1338	1.88	SH
14.08	β-Elemene	1400	1391	41.86	SH
14.58	Caryophyllene	1433	1420	0.82	SH
14.74	γ-Elemene	1443	1434	0.75	SH
15.03	β-Gurjunene	1462	1432	0.74	SH
15.44	γ-Gurjunene	1489	1473	5.22	SH
15.50	α-Amorphene	1493	1482	0.81	SH
15.65	β-Eudesmene	1502	1486	15.52	SH
15.77	β-Guaiene	1510	1490	23.87	SH
16.04	γ-Cadinene	1528	1513	1.38	SH
16.15	δ-Cadinene	1535	1524	4.11	SH
16.38	Sesquiterpene hydrocarbon (M^+ 204)	1550	–	0.94	SH
16.48	Sesquiterpene hydrocarbon (M^+ 204)	1556	–	0.97	SH
17.13	Caryophyllene oxide	1599	1581	1.12	OS
Fraction B					
9.76	Camphor	1151	1142	20.64	OM
17.38	Curzerenone	1617	1623	67.06	OS
18.73	Germacrone	1714	1693	12.30	OS
Fraction C					
8.83	Linalool	1101	1099	6.08	OM
9.83	2-Norbornanol	1155	1148	6.48	OM
9.97	Isoborneol	1163	1157	62.9	OM
10.13	Borneol	1172	1167	11.92	OM
10.33	4-Terpineol	1182	1177	2.49	OM
10.57	α-Terpineol	1195	1189	9.17	OM

9.2%) also showed significant inhibition (20.0%, 50.8% and 59.1% respectively), yet weaker than the crude. The structural identity of isoborneol and borneol in the fraction **C** was also confirmed through its ^{1}H NMR analysis.

Further purification of the most active fraction **B** yielded pure **1**, **2** and **3**. The identity of these purified molecules was confirmed through ^{1}H NMR spectra and GC-MS analysis which matched well with our previous report (Mahanta et al., 2020). When these three purified molecules were tested for their phytotoxic ability individually, highest inhibition was exhibited by curzerenone (**1**). It showed 47.5% inhibition of germination, 90.1% inhibition of coleoptile growth and 84.4% inhibition of radicle growth at 200.0 μg/mL. Camphor (**3**) exerted much weaker inhibition (germination 2.5%, coleoptile growth 39.4% and radicle growth 58.7%) than **1** whereas germacrone (**2**) showed a weak inhibition. These results indicated curzerenone (**1**) as the major phytotoxic principle in black turmeric oil. A similar activity trend was observed through the coleoptile bioassay at 50.0 μg/mL (*i.e.* close to the IC_{50} value for crude oil in coleoptile bioassay) with the fractions and purified molecules (Fig. 2**E**). Fraction **B** showed highest activity (75.5% inhibition) among all the fractions (**A-C**) which was even higher than the crude (51.0% inhibition). Curzerenone exhibited highest inhibition (**1**, 89.8%) followed by camphor (**3**, 63.9%) and germacrone (**2**, 49.0%). This bioassay data further substantiated our findings in the water medium assay.

In a separate experiment, curzerenone (**1**) showed concentration dependent (10.0–300.0 μg/mL) inhibition of germination and growth of wheatgrass seeds in water medium with significantly higher potency in comparison to the crude oil. The obtained IC_{50} values were 188.3 μg/mL (819 μM) for germination, 34.7 μg/mL (151 μM) for coleoptile and 36.7 μg/mL (160 μM) for radicle growth (Fig. 2I). In the coleoptile bioassay, curzerenone exhibited very potent activity with an IC_{50} 13.0 μg/mL (56 μM) (Fig. 2J), much stronger than the activity observed with the crude oil (IC_{50} 57.1 μg/mL). It further confirmed curzerenone as the majorly abundant most active phytotoxin in this essential oil. A number of phytotoxic sesquiterpene lactones (*e.g.* lappalone, costunolide, parthenin, guaianolides, inuloxins) and alcohols with IC_{50} in the range 20–180 μM have been reported previously from the plant and microbial sources through coleoptile bioassay (Macías et al., 2019). However, identification of a furanosesquiterpene as the major phytotoxic principle in an essential oil through activity-guided fractionation was demonstrated for the first time in this study. Besides, the role of major and minor oxygenated monoterpenes (camphor, isoborneol, borneol, α-terpineol, 4-terpineol) for the phytotoxicity of black turmeric oil was also significant according to the observed results. The phytotoxicity of these monoterpenes has also been clearly established in the previous reports which support our current observation (Bora et al., 2021; Chaimovitsh et al., 2017; Martino et al., 2010; Rolli et al., 2014; Vokou et al., 2003).

3.4. Structure-activity relationship (SAR) study of curzerenone (1)

To understand the role of different functional groups/moieties of curzerenone (**1**) in phytotoxicity, four structurally related molecules (**2**, **4-6**) were isolated or semi-synthesized (Supplementary Material Fig. S3-S8). The phytotoxicity of these selected compounds was tested through wheatgrass coleoptile bioassay in the concentration range 10.0–300.0 μg/mL. These compounds varied in their functional groups and/or overall structural architecture in comparison to **1** (Fig. 1**C**). The keto group at C-6 was absent in curzerene (**4**) whereas two terminal unsaturations were hydrogenated in tetrahydrocurzerenone (**5**). Zederone (**6**) was a 4,5-epoxidized analogue of furanodienone (retro-Cope rearranged analogue of **1**) containing a ten-membered ring. Germacrone (**2**) also contained a ten-membered ring and lacked the furan moiety in its structure. Though all these tested molecules showed a dose dependent response, they were found be less potent phytotoxins in comparison to **1**. The obtained IC_{50} values were 66.1 μg/mL (306 μM), 165.5 μg/mL (707 μM), 38.4 μg/mL (156 μM) and 83.8 μg/mL (384 μM) for curzerene (**4**), tetrahydrocurzerenone (**5**), zederone (**6**) and germacrone (**2**) respectively (Fig. 2**J**). It indicated that all the functional groups in **1** were essentially important for its high phytotoxic potency.

3.5. Mode of transportation

A plant-based phytotoxin may transport through different routes such as air (diffusion), water (precipitation) or soil (biodegradation/root exudates) before it exerts toxicity to the neighboring plants (Liu et al., 2016; Nakano et al., 2006; Song et al., 2019). To assess the efficacy and stability in different media is important for the fundamental understanding as well as future application of the phytotoxin. Black turmeric oil has shown its efficacy against wheatgrass seed germination and growth when assayed in water and agar medium. Besides, there is a possibility of transport through air (aerial diffusion of volatiles) or soil (degradation of rhizome) which has been studied and described as under.

3.5.1. Phytotoxic efficacy of black turmeric oil through aerial diffusion

The aerial phytotoxicity of black turmeric oil was tested in a closed vessel of 4 L capacity in the concentration range 1.0–8.0 mg/L of air (Fig. 3**E**). Diffusion of the volatiles from the oil (loaded on a filter paper) at an ambient condition, concentration dependently inhibited the growth of fresh and pre-germinated wheatgrass seeds in the tested range (Fig. 3A-C). The calculated IC_{50} for the inhibition of coleoptile growth were 6.2 (pre-germinated for 2 days), 3.6 (pre-germinated for 1 day) and 1.7 (fresh) mg/L. It indicated that inhibitory efficacy was higher during the initial phase of germination in the aerial mode. On the other hand, radicle growth inhibition was similar for all three stages, exhibiting IC_{50} as 1.4, 1.8 and 1.7 mg/L respectively. In the case of fresh seed, it also inhibited the germination with an IC_{50} of 4.2 mg/L. In a previous study, Dudai and co-workers studied the allelopathic potential of 32 essential oils through aerial diffusion (Dudai et al., 1999). Several monoterpene-rich essential oils were shown to inhibit the germination of wheatgrass with IC_{50} as low as 28 ppm. In another investigation, Vokou et al. identified several monoterpene ketones and alcohols *viz.* terpinen-4-ol, carvone and dihydrocarvone (among 47 monoterpenoids studied) that extremely inhibited the germination and growth of *Lactuca sativa* seeds at 25 ppm (Vokou et al., 2003). The efficacy exhibited by black turmeric oil in the current study was significantly higher than the previously reported oils/molecules, demonstrating it as an efficient phytotoxin against the germination and post-emergence growth of wheatgrass through aerial mode.

3.5.2. Phytotoxic efficacy and stability of black turmeric oil in soil

Black turmeric oil also exhibited dose dependent inhibition of germination and growth (wheatgrass) in the soil, though it was weaker than the observed activity in water or agar medium (Fig. 3D). At 300.0 μg/mL the inhibition of germination, coleoptile and radicle growth were 10.0%, 50.1% and 68.9% respectively (in reference to 79.0%, 95.8% and 96.0% respectively in the water medium). The observed activity was relatively low probably due to the (a) restricted mobility (of phytotoxins) through soil medium resulting in a lower exposure of the phytotoxins to the seeds and (b) trapping of volatile phytotoxins in the medium, thus limiting its evaporation and exposure through the aerial mode. Despite a weaker activity, this study showed the ability of black turmeric oil to exert its phytotoxicity in soil medium as well. A detailed and systematic comparison of efficacy in different media for a phytotoxic essential oil was performed for the first time in the current study.

Further, a simulated experiment was designed to assess the possibility of transport of these phytotoxins into the soil through rhizome degradation and their stability (or persistence) in this medium. Fresh and ground rhizome was mixed with garden soil (3.3 and 6.6 g in 50 g soil) and kept for 45 days in an ambient condition. The presence of the

identified phytotoxins was further analyzed in the *n*-hexane extracts of these soil-rhizome mixtures through GC-MS technique. Curzerenone (**1**) was detected as one of the major constituents along with germacrone (**2**) and other solvent extracted metabolites [mainly (*E*)-labda-8(17),12-diene-15, 16-dial (a labdane dialdehyde) and oxygenated sesquiterpenes ($C_{15}H_{22}O_2$, M^+ 234)] (Fig. S9). Interestingly, major monoterpenes in the oil such as camphor, 1,8-cineole, isoborneol were not detected in these extracts. Prolong incubation of the ground rhizome in the soil plausibly led to the evaporation of these low-boiling monoterpenes from the soil-rhizome mixtures. A similar observation was made by Young and co-workers during their investigation on allelopathic potential of *Juniperus ashei* leaves. Headspace GC-MS analysis of the fresh leaf and litter leachates showed the major abundance of camphor followed by bornyl acetate and limonene whereas none of these phytotoxic monoterpenes was detected in the degraded litter (Young and Bush, 2009). When the extracts from both the experiments were tested for phytotoxicity in water medium (200.0 μg/mL), a high inhibition of germination (52.5%), coleoptile (80.4%) and radicle (74.1%) growth was observed for the sample with 6.6 g rhizome (Fig. 3F). The inhibition was significant for the other sample (3.3 g rhizome) as well. These results indicated that **1**, the major and most active phytotoxin in the black turmeric oil may transport into the soil through rhizome and persist for at least a period of one and half month therein. But, low-boiling monoterpenes escaped the soil during this period and may not be regarded as the key phytotoxins when a long-term effect in the soil is concerned.

3.6. Uptake and transformation study

The uptake of terpenic phytotoxins by the plant tissues interferes with different physiological processes causing disruption of microtubule assembly, membrane damage, increased oxidative stress, damaging photosynthetic machinery or altering the water status which finally lead the inhibition of germination and growth (Araniti et al., 2017, 2016; Chaimovitsh et al., 2017). Sometimes, these molecules are bio-transformed by the plant enzymes producing metabolite(s) with varied phytotoxicity (Chaimovitsh et al., 2017; Dudai et al., 2009). Therefore, the evidence of phytotoxin uptake provides chemical and physiological basis to the observed toxicity (Chaimovitsh et al., 2017; Dudai et al., 2009; Loi et al., 2008). The uptake and transformation of black turmeric oil constituents by wheatgrass seeds were studied at 200.0 μg/mL in water medium. After three days the composition of ethyl acetate extracts for the aqueous medium and seeds were analyzed separately through GC-MS (Table 3, Fig. S10). No monoterpene hydrocarbon was detected probably due to their high volatility. Oxygenated monoterpenes (1, 8-cineole, camphor, norborneol, isoborneol, borneol) and sesquiterpenes (curzerenone and germacrone) were identified in both the extracts (Table 3). Curzerenone (**1**), germacrone (**2**), camphor (**3**) and 1, 8-cineole were the major molecules found in the seed extract. The aqueous medium was dominated by camphor, curzerenone and borneol. All these molecules were originally present in the oil, thereby eliminating the possibility of detectable new transformation products.

Interestingly, the distribution ratio of individual constituents in the medium and seed extracts varied widely (Table 3). It was observed that the abundance of oxygenated monoterpenes was high in the aqueous medium in comparison to the seeds. The distribution ratio of these compounds in the medium *versus* seed varied in the range 2.03–7.00. On the other hand, the uptake of sesquiterpenes such as **1** and **2** was higher, thus producing a lower distribution ratio (medium *versus* seed) 1.67 and 0.55 respectively. These results indicated that the uptake of phytotoxins (**1**, **3** and other oxygenated monoterpenes) by the seeds initiated and led the inhibition of germination and subsequent growth. In a previous study, volatiles released by the leaves of *Micromeria fruticosa* (mainly mono and sesquiterpenes) were detected and quantified in the germinating wheat seeds sown in the soil (Dudai et al., 2009). A quantitative variation in the uptake of volatile constituents by the seeds was observed when compared with the original composition in the fresh leaf volatile, supporting our observation.

3.7. Phytotoxicity of black turmeric oil in monocots and dicots

The phytotoxicity of black turmeric oil was further investigated on three monocot (bermudagrass, rice and fenugreek) and three dicot (chickpea, green gram and mustard) seeds in water medium. Though a concentration dependent inhibitory trend was observed in all the tested species, the potency was relatively weaker with IC_{50} (germination) > 300.0 μg/mL and IC_{50} (growth) > 150.0 μg/mL except the case of bermudagrass (Table 4, Fig. S11). The inhibitory activity was observed on bermudagrass with IC_{50} 156.7, 37.0 and 66.7 μg/mL respectively for the germination, coleoptile and radicle growth that was comparable to wheatgrass. At 200.0 μg/mL the growth inhibition was extensive (88.6% for coleoptile and 83.0% for radicle) (Fig. 4A). It indicated the ability of black turmeric oil to efficiently inhibit the bermudagrass seedling growth. To further evaluate its growth inhibition efficacy, an agar medium bioassay was performed with the pre-germinated seeds (3 days) of bermudagrass in the range 50.0–300.0 μg/mL. A dose dependent growth inhibition was visible (Fig. 4B-C). The observed IC_{50} were 81.4 and 70.8 μg/mL for the coleoptile and radicle growth respectively. A high growth inhibition (79.0% and 76.7% respectively) was observed even at 200.0 μg/mL. Bermudagrass is a noxious and aggressive weed and distributed widely all over the world. It affects many crops and only selected herbicides are effective for its control (Haq et al., 2010). Several essential oils have been reported previously as phytotoxic to bermudagrass. Catmint (*Nepeta meyeri*) oil, highly rich in nepetalactone analogues inhibited 46% and 80% germination at 0.01% and 0.02% (equivalent to 100 and 200 μg/mL) respectively in water medium in reference to 32% inhibition in the control containing 0.01% Tween 20 (Mutlu et al., 2011). In another study *Eucalyptus citriodora*, *Ocimum basilicum* and *Mentha arvensis* essential oils showed dose dependent visible injury, reduction in body weight, shoot weight and root weight when sprayed (30–60 μL per 10 plants in a pot) as the emulsion in water (Khare et al., 2019). Considering the efficacy exhibited by black turmeric oil in the current study, it can be a probable candidate especially for the post-emergence management of bermudagrass.

Table 3
GC-MS based quantitative analysis of uptake study (black turmeric oil constituents) by wheatgrass seeds at 200.0 μg/mL in water medium [IS: internal standard].

R_t (min)	Constituent	Water extract		Seed extract		(A)/ (B)
		Rel %	Constituent/IS (A)	Rel %	Constituent/IS (B)	
7.59	1,8-Cineole	3.34	0.065	2.42	0.032	2.03
9.76	Camphor	15.27	0.297	3.37	0.044	6.75
9.83	Norborneol	3.26	0.063	1.14	0.015	4.20
9.97	Isoborneol	4.86	0.095	1.88	0.025	3.80
10.13	Borneol	9.00	0.175	1.94	0.025	7.00
13.46	Eugenol (**IS**)	51.39	1.000	76.50	1.000	1.00
17.38	Curzerenone	10.91	0.212	9.71	0.127	1.67
18.73	Germacrone	1.12	0.022	3.06	0.040	0.55

Table 4
Phytotoxic potency of black turmeric oil on different monocot and dicot species, assayed in water medium.

	Common name	Scientific name	IC_{50} (µg/mL)		
			Germination	Coleoptile	Radicle
Monocotyledon	Wheatgrass	*Triticum aestivum*	176.7	90.6	93.0
	Bermudagrass	*Cynodon dactylon*	156.7	37.0	66.7
	Rice	*Oryza sativa*	> 300.0	151.3	> 300.0
	Fenugreek	*Trigonella foenum-graecum*	> 300.0	228.5	202.6
			Germination	**Hypocotyl**	**Radicle**
Dicotyledon	Green gram	*Vigna radiata*	> 300.0	235.0	215.5
	Chickpea	*Cicer arietinum*	> 300.0	170.5	248.7
	Mustard	*Sinapis alba*	> 300.0	246.4	64.7

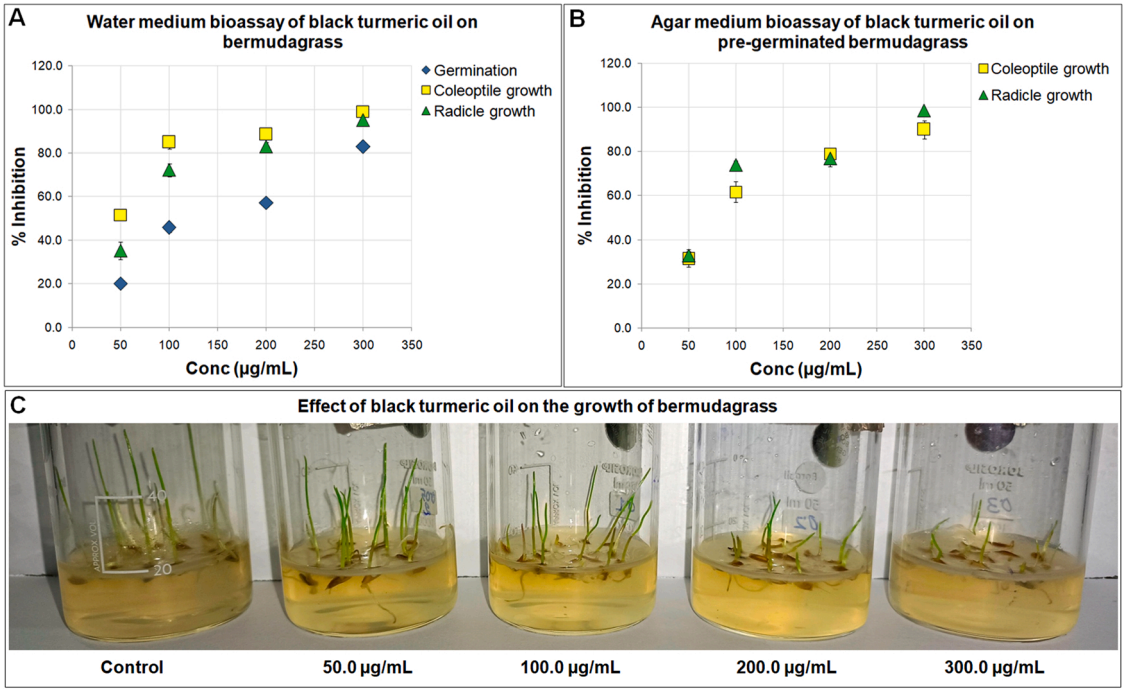

Fig. 4. Phytotoxicity bioassay (bermudagrass) of black turmeric oil (50.0–300.0 µg/mL) in (**A**) water medium and (**B**) agar medium with pre-germinated seeds; (**C**) photograph of the experimental set up clearly exhibiting a dose dependent inhibitory effect of black turmeric oil on the pre-germinated bermudagrass seedling growth.

4. Conclusion

In conclusion, the current study identified potent phytotoxic efficacy of black turmeric essential oil against the germination and growth of wheatgrass. The phytotoxic ability of this oil was demonstrated in water, agar and soil media and also through the aerial diffusion. The inhibition through water and aerial mode was comparatively more efficient. Curzerenone (**1**), a majorly abundant furanosesquiterpene in this oil was found to be the most active phytotoxic principle followed by oxygenated monoterpenes. Curzerenone in the ground rhizome may persist in the soil for at least one and half month. The uptake of phytotoxins without any detectable structural transformation by the wheatgrass seeds was also confirmed in water medium. This oil efficiently inhibited the post-emergence growth of bermudagrass weed while showing relatively weak toxicity towards several food crops. The scientific knowledge generated in this study indicates black turmeric oil as a potent candidate to be considered for the development of bioherbicide in future for the management of weeds.

CRediT authorship contribution statement

Bhaskar Protim Mahanta: Designing and execution of the experiments, Manuscript drafting. **Phirose Kemprai**: Performing experiments. **Pranjit Kumar Bora**: Reviewing the manuscript. **Mohan Lal**: Cultivation, authentication and supply of the plant materials. **Saikat Haldar**: Conceptualization and designing of the experiments, Manuscript drafting and reviewing.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2022.114788.

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