

Inhibitory activities of essential oils from *Syzygium aromaticum* on emergence of *Echinochloa crus-galli*

xianzhi Ni

Hunan University of Humanities, Science and Technology

haodong bai

Hunan University of Humanities, Science and Technology

jincai Han

Hunan Academy of Agricultural Sciences

yong Zhou

Hunan Academy of Agricultural Sciences

zhendong Bai

Hunan University of Humanities, Science and Technology

siquan Luo

Hunan University of Humanities, Science and Technology

jingjing Xu

Hunan Academy of Agricultural Sciences

chenzhong Jin

Hunan University of Humanities, Science and Technology

Zuren Li (✉ lizuren88214@hunaas.com)

Hunan Academy of Agricultural Sciences

Article

Keywords: vacuum distillation, essential oil, *echinochloa crus-galli*, defense enzyme activity

Posted Date: May 30th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2947818/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Echinochloa crus-galli is a serious weed species in rice paddies. To obtain a new potential bioherbicide, we evaluated the inhibitory activities of 13 essential oils and their active substances against *E. crus-galli*. Essential oil from *Syzygium aromaticum* (L.) Merr. & L. M. Perry (SAEO) exhibited the highest herbicidal activity ($EC_{50} = 3.87 \text{ mg mL}^{-1}$) among the 13 essential oils evaluated. The SAEO was isolated at six different temperatures by vacuum fractional distillation, including 164 °C, 165 °C (SAEO – 165), 169 °C, 170 °C, 175 °C and 180 °C. The SAEO – 165 had the highest inhibitory rate against *E. crus-galli*. Gas chromatography-mass spectrometry and high phase liquid chromatography identified Eugenol ($EC_{50} = 4.07 \text{ mg mL}^{-1}$), α -caryophyllene ($EC_{50} = 17.34 \text{ mg mL}^{-1}$) and β -caryophyllene ($EC_{50} = 96.66 \text{ mg mL}^{-1}$) as the three compounds in SAEO. Results from a safety bioassay showed that the tolerance of rice seedling (~ 20% inhibition) was higher than that of *E. crus-galli* (~ 70% inhibition) under SAEO stress. Additionally, defense enzymes in *E. crus-galli* and rice seedling showed decreased activities, but only CAT levels were affected under SAEO treatment. Our results indicate that SAEO has a potential for development into a new selective bio-herbicide. They also provide an example of a sustainable management strategy for *E. crus-galli* in rice paddies.

Introduction

Weeds pose a growing menace to global food production^[1]. Barnyard grass (*Echinochloa crus-galli*) is currently among the most economically damaging weeds in global agriculture^[2, 3]. *E. crus-galli* releases a number of allelochemicals into paddy soils to suppress rice growth, but at a low rate^[4]. However, the extensive and repeated use of chemical herbicides, has greatly damaged the environment, but has also resulted in resistant barnyard grass accessions to herbicides, with different mechanisms of action^[5, 6]. More recently, *E. crus-galli* has acquired or evolved target-site and non-target-site herbicide resistance^[7]. In addition, *E. crus-galli* was also reported to have developed multiple resistance to cyhalofop-butyl and several ALS inhibitors, in Okayama in Japan^[8]. Consequently, the search for environmental-friendly botanical herbicides is a hot research domain.

Essential oils serve as sources of bioactive natural products which have attracted the attention of researchers. They are considered as an important class of herbicidal agents as they have high diversity, are biodegradable and show high sensitivity to organisms^[9, 10]. They play specific roles in plant signaling due to their dispersal and aromatic characteristics^[11]. Plant secondary metabolites are sources of bioherbicides which they release into the circumambient edatope to protect themselves against other plants or pathogenic bacteria^[12, 13]. At present, most of the studies conducted on plant extracts, have focused on their use as bacteriostatics, antioxidants and insecticides^[14–16]. However, the allelopathic effects of essential oils can be used for weed control in agricultural production. For example, essential oil from *Ambrosia artemisiifolia* was shown to have had significant inhibitory activity against *Setaria viridis* and *Amaranthus retroflexus*^[5]. And the plant extracts of *Bidens pilosa* exerted strong suppression against

the growth of *E. crus-galli*^[17]. Additionally, essential oils from *Nepeta glocephalata* Rech.f. were reported to have had phytotoxic effects on barnyard grass, but the herbicidal activity was not high^[18]. Further, the essential oil, trans-pinocarveol, from *Eucalyptus carnei* species was also shown to have had a significant inhibitory effect on annual ryegrass^[19]. Thus, it is essential to screen more essential oils, to identify promising bioactive compounds, which can be applied for the efficient control of barnyard grass in rice fields.

To screen a high variety of essential oils with bioactivities, we chose thirteen crude essential oils from plants species which grow abundantly in China. This included *Mentha × piperita* Linnaeus, *S. aromaticum*, *Cinnamomum cassia* Thunb, *Zingiber boehm*, *Cymbopogon citratus* Presl, *Cinnamomum camphora* (L.) Presl, *Capsicum annuum* linn. var. *grosso* (L.) Sendt., *Camellia sinensis* (L.) O. Ktze., *Artemisia caruifolia* var. *schochii*, *Citrus sinensis* Osb. var. *brasiliensis* Tanaka, *Citrus limon* (Linnaeus) Osbeck, and *Citrus reticulata* Blanco. These essential oils were applied to treat *E. crus-galli* to assay their herbicidal effects. The high-effective oils were isolated by vacuum fractional distillation and their activities evaluated in bioassay experiments. The specific chemical constituents of these oils were identified by gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) and their herbicidal activities were evaluated in bioassay experiments. We also evaluated the effect of one promising essential oil, SAEO on antioxidant enzymes such as catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) in *E. crus-galli*. The study serves as a baseline for the development of new selective biological herbicides for the control of *E. crus-galli* in rice paddies.

Materials and Methods

Material and culture conditions. The seeds of *E. crus-galli* and rice used in the experiment were collected from the base of our laboratory (Gaoqiao Base in Changsha city in Hunan Province, China (113°14' 57" E 28°16' 49"N)) and can be used for the experiment described in this article. Plants were grown in the greenhouse (12 h photoperiod (light / dark cycle), 110–140 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 27 / 22 °C (day / night temperature regime)). Spraying was done with a 3WP-2000 walking spray tower (sprayer delivered 225 L ha^{-1} , walking speed 0.12–1.2 m/s, spraying pressure 15 pa) purchased from Nanjing Institute of Agricultural Mechanization, Ministry of Agriculture and Rural Affairs).

Test plant essential oils. Thirteen plant essential oils were obtained by steam distillation. They included (*M. piperita* essential oil (MPEO) # 1, *S. aromaticum* essential oil (SAEO) # 2, *E. caryophyllata* essential oil (ECEO) # 3, *C. cassia* essential oil (CCEO) # 4, *Zingiber* essential oil (ZEO) # 5, *C. citratus* essential oil (CIEO) # 6, *C. camphora* essential oil (CAEO) # 7, *C. annuum* linn essential oil (CNEO) # 8, *C. sinensis* essential oil (CSEO) # 9, *A. caruifolia* essential oil (ACEO) # 10, *C. sinensis* essential oil (CEEO) # 11, *C. limon* essential oil # (CLEO) 12, and *C. reticulata* essential oil (CREO) # 13).

Seed germination bioassay. Fifteen healthy rice and *E. crus-galli* seeds were germinated in Petri dishes (diameter = 9 cm) lined with a single layer of Whatman # 2 filter paper and grown in the greenhouse. Different concentrations of the essential oil, SAEO (pH = 5.0, essentially nonvolatile), were prepared (1, 2,

2.5, 4, 5 mg essential oil into 0.5 mL of Dimethylformamide (DMF), and into 10 mL of 6 / 1000 Tween 80 aqueous solution). Then 5 mL of each was added separately to the petri dishes, each which had fifteen rice seeds planted in them. Treatment solution without essential oil was used as control. Twenty *E. crus-galli* and rice seeds were also planted in Petri dishes lined with filter paper. All the Petri dishes were kept in a growth chamber set at 12 h photoperiod (light / dark cycle), 110–140 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 27 / 22 °C (day / night temperature regime). The number of seeds that germinated were recorded after seven days and the germination ratio of each group was calculated based on the data. The experiment was repeated three times. Parameters were calculated as follows:

$$\text{Germination percentage} = (\text{number of germinated seeds} / \text{total number of seeds}) \times 100 \text{ (1)}^{[20]}$$

Seedling growth bioassay. Fifteen healthy rice and *E. crus-galli* seedlings were grown in pots (volume = 9 × 9 cm) with 100 g of garden soil (content of organic matter ≤ 3%, pH = 5.42) of perlite / manure (200 g L⁻¹) mixture. Different concentrations of SAEO (*E. crus-galli*: 0.01, 0.05, 0.1, 0.2, 0.3 g, rice: 0.1, 0.3, 0.5, 0.8, 1 g essential oil into 0.5 mL of DMF, and into 10 mL of 6 / 1000 Tween 80 aqueous solution, pH = 5.0, essentially nonvolatile) were sprayed (10 mL) evenly on the barnyard grass (2 - leaf stage) and rice seedlings (2 - leaf stage) and kept in the greenhouse. The plants were used in experiments when they were at the 2 - leaf stage. In all bioassay experiments spraying was applied to the foliage. Plant samples were harvested after seven days and their fresh weights recorded. Inhibition ratios of the essential oil were calculated based on the fresh weight data. The experiment was repeated three times. The seedlings inhibition ratio was calculated using the formula:

$$(\text{Control-test sample}) / \text{Control} \times 100\% \text{ (2)}^{[21]}.$$

Collection and isolation of essential oil by vacuum fractional distillation. The active substance in SAEO was obtained by isolation using vacuum fractional distillation^[22] (fraction temperature: 165 °C, 170 °C, 180 °C; fraction temperature: 164 °C, 169 °C and 175 °C; pressure: – 95 kPa).

Bioassays were then conducted with different concentrations (100, 300, 500 mg essential oil into 0.5 mL of DMF, and into 10 mL of 6 / 1000 Tween 80 aqueous solution) of the collected condensed SAEO (at 165 °C, 170 °C and 180 °C). They were sprayed evenly on barnyard grass seedlings (2 - leaf stage). Grass samples were harvested after seven days and their fresh weights recorded. Inhibition ratios of the SAEO were calculated based on the fresh weight data. The experiment was repeated three times. The experimental group with the highest inhibition ratio was selected for a further secondary distillation at 164 °C, 169 °C and 175 °C. Bioassay experiments were then conducted as described before and the distillate collected. The experimental group which exhibited the highest inhibition ratio, was selected for gas chromatography-mass spectrometry (GC-MS) analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis. The chemical constituents of SAEO were identified using GC-MS. The data were acquired on a Shimadzu GC-MS-QP2010 gas chromatograph

(Shimazu, Japan). The chromatographic column conditions were as follows: the chromatographic column was RTX-5MS (30 m × 0.25 mmID × 0.25 µm df) and the carrier gas was highly pure helium, which had a flow rate of 0.8 mL min⁻¹, a diversion ratio of 3.0 and vaporization temperature of 300 °C. The temperature program was a starting temperature of 100 °C, maintained at 0.0 min, a heating rate of 20.0 °C min⁻¹ to 180 °C, maintained at 1.0 min, a heating rate of 5.0 °C min⁻¹ to 200 °C, maintained at 1.0 min, and a heating rate of 20.0 °C min⁻¹ to 280 °C, maintained at 4.0 min. The injection rate volume was 1 µL and the delay time of solvent was 3.15 min. Ion source: EI source, electron energy: 70 eV, ion source temperature: 240 °C, interface temperature: 280 °C, and scanning mass range: m / z 33–600. The nist05, nist05s and wiley7 libraries were used for identification of compounds. The relative amount of extract was determined using the peak area of the total ion chromatograms (TIC).

Bioassay with major compounds. Toxicity tests were carried out using three major compounds identified in SAEO from the GC-MS (eugenol, β-caryophyllene and α-caryophyllene). Eugenol was purchased from Macklin Biochemical Co., Ltd (Shanghai, China). β-caryophyllene and α-caryophyllene were purchased from J&K Scientific Ltd. (Beijing, China). Different concentrations of the three compounds were prepared (10, 50, 100, 300, 500 mg added to the mixture of 0.5 mL of DMF, and into 10 ml of 6 / 1000 Tween 80 aqueous solution) and each sprayed on *E. crusgalli* seedlings at the 2 - leaf stage. Grass samples were harvested after seven days and their fresh weight recorded. Inhibition ratios for each concentration were calculated based on the fresh weight data. The experiment was repeated three times.

High performance liquid chromatography (HPLC). The eugenol content in SAEO collected at 165 °C (SAEO – 165) was determined using HPLC, which was performed on a Shimazu LC-10AD pump and an SPD-10A UV detector, with a C₁₈ column (4.6 mm × 200 mm, packing: Hypersil, particle size: 5 µm) from Dalian Elite Analytical Instruments. The acetonitrile and methanol used for the HPLC were chromatographic pure and the water was also aseptic. The chromatographic condition was (mobile phase: methanol and water (65 : 35), flow rate: 1 mL min⁻¹, detection wavelength: 280 nm, column temperature: 25, injection volume: 20 µL). The eugenol standard used was purchased from Shanghai Macklin Biochemical Co., Ltd (99% purity).

Preparation of standard: 20 mg of eugenol was weighed and added to the mobile phase, which was shaken and diluted to a concentration of 100 mg L⁻¹. Preparation of test solution: SAEO – 165 was dissolved in sterile water and diluted to a ratio of 1: 100, 000. A volume of 20 µl each of the reference and test solutions was injected into the liquid chromatograph and ran according to the above chromatographic conditions. The chromatogram and peak area were recorded and the content was calculated based on the peak area.

Defense enzyme activities. Standard SAEO (0.5 g) was mixed with 0.5 mL of DMF and 10 ml of 6 / 1000 Tween 80 aqueous solution and sprayed on *E. crusgalli* and rice seedlings at the 2 - leaf stage. Grass sample were collected at 1, 4, 8, 24, 48 and 72 h after spraying SAEO, and stored at – 70 °C until the measurement of defense enzymes activities and plant total proteins. The activities of the antioxidant enzymes, superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) were determined with kits

following the manufacturer's instructions. Total protein (TP) was determined using the Coomassie bright blue method. All kits were purchased from Nanjing Jiancheng Bioengineering Institute. The data were expressed as relative activity (%).

Statistical analyses. The data were analysed by one-way analysis of variance (ANOVA), and treatment values were compared at $P \leq 0.05$. Data variance was determined using the data processing system (DPS) software and error analysis was carried out in MS Excel 2019. Graphs were produced using Origin 2017. The statistical analyses were performed using SPSS software (SPSS Inc., Chicago, Illinois, USA).

Results

SAEO inhibits the growth of *E. crus-galli*. The inhibitory effects of 13 essential oils on barnyard grass was studied in bioassay experiments. SAEO and CCEO had inhibitory effects on *E. crus-galli*, seven days after spraying seedlings with the 13 essential oils at 10 mg mL^{-1} (Fig. 1A). The other 11 essential oils had no significant inhibitory effect (Figure S1). Of the two, SAEO had more pronounced inhibitory effect. It showed an inhibition ratio of 86.46% on *E. crus-galli* seedlings at a concentration of 10 mg mL^{-1} (Fig. 1A). The inhibition ratio on seed germination was 33.33% when the concentration of SAEO was 0.1 mg mL^{-1} , and 99.17% when the concentration was 0.4 mg mL^{-1} (Fig. 1B). The EC_{50} of SAEO at the seed germination stage of *E. crus-galli* was $0.1549 \text{ mg mL}^{-1}$, and at the seedling stage was 3.87 mg mL^{-1} (Table S1). At the seedling stage (2 - leaf stage), the inhibition ratio was 15.27% when the concentration of SAEO was 1 mg mL^{-1} , and 94.56% when the concentration was 30 mg mL^{-1} (Fig. 1C). The inhibitory effect significantly increased with increase in SAEO concentration (Fig. 1D). The inhibitory effects of the 2 fraction distilled SAEO on *E. crus-galli* are shown in Figure S2.

SAEO components analysis by GC-MS and its bioassay. Three major chemical constituents were identified by GC - MS (Fig. 2A) in SAEO - 165 and these were eugenol (73.35%), β -caryophyllene (23%), and α -caryophyllene (3.65%) (Table S2). The EC_{50} of eugenol, α -caryophyllene and β -caryophyllene recorded at the seedling stage of *E. crus-galli* were 4.07 mg mL^{-1} , 17.34 mg mL^{-1} and 96.66 mg mL^{-1} , respectively (Table S3). Eugenol, α -caryophyllene and β -caryophyllene showed significant inhibitory effect and their inhibition ratios recorded at a concentration of 5 mg mL^{-1} were 74.11%, 28.06% and 12.3%, respectively (Fig. 2B, C, D). The inhibition ratios of the three compounds at 30 mg mL^{-1} were 88.69%, 69.65% and 40.95% respectively. Eugenol showed better herbicidal efficacy than β -caryophyllene and α -caryophyllene.

High performance liquid chromatography (HPLC) analysis. To identify the active fraction which can serve as a bioherbicide, we obtained purified SAEO by distillation (fraction temperature: 165°C , 170°C , 180°C ; fraction temperature: 164°C , 169°C and 175°C , pressure: -95 kPa). The order of inhibitory effect for fraction I temperatures was $165^\circ\text{C} > 170^\circ\text{C} > 180^\circ\text{C}$ and that for fraction II was $164^\circ\text{C} > 175^\circ\text{C} > 169^\circ\text{C}$ (Figure S2).

The HPLC results indicated that the peak area of eugenol purified from SAEO – 164 was 50488 and its content was 50.488%, SAEO – 165 was 73770 and its content was 74.77%, SAEO – 169 was 68906 and its content was 68.906%, SAEO – 170 was 83364 and its content was 83.364%, SAEO – 175 was 67807 and its content was 67.807%, SAEO – 180 was 82306 and its content was 82.306% (Fig. 3, Table S4).

SAEO is relatively safe for rice seedlings. To verify whether SAEO was safe for crops, we evaluated the safety of rice under SAEO stress (Fig. 4). The inhibitory ratios of SAEO at concentrations of 0.1 and 0.5 mg mL⁻¹ on rice at the germination stage were 34.44% and 95.56% respectively (Fig. 4A). At the 2 - leaf stage, the inhibition ratio was 19.71% when the concentration of SAEO was 10 mg mL⁻¹, and 93.92% when the concentration was 100 mg mL⁻¹ (Fig. 4B). The inhibitory effect of SAEO on rice are shown in Fig. 4C. The EC₅₀ of SAEO to rice seed germination and seedling stage were 0.1831 mg mL⁻¹ and 24.9431 mg mL⁻¹, respectively (Table S5). Results showed that the tolerance of rice to SAEO at seedling stage, was higher than that of *E. crus-galli* at the same concentration.

SAEO inhibits the activities of defense enzymes in *E. crus-galli* and rice. The CAT activity in *E. crus-galli* and rice treated with SAEO first showed an increasing trend, then a decreasing trend (Fig. 5). CAT activity in *E. crus-galli* was 31.753 U gprot⁻¹ at 0 h, reached a peak value of 72.744 U gprot⁻¹ at 8 h and then decreased to 9.143 U gprot⁻¹ at 72 h, which was lower than that of the control group (Fig. 5A). The CAT activity in *E. crus-galli* increased by 56.35% (0–8 h, compared to control), and decreased (0–72 h, compared to the control) by 71.21%. CAT activity in rice was 26.368 U gprot⁻¹ at 0 h, reached a peak value of 36.828 U gprot⁻¹ at 8h and then decreased to 27.009 U gprot⁻¹ at 72 h, which was higher than that of the control group (Fig. 5B). The CAT activity in rice increased by 28.4% (0–8 h, compared to control), and decreased (0–72 h, compared to the control) by -2.43% (Fig. 5B).

E. crus-galli SOD activity was 41.103 U gprot⁻¹ at 1 h, reached a peak value of 141.891 U gprot⁻¹ at 8 h and then decreased to 39.199 U gprot⁻¹ at 72 h (Fig. 5C). SOD activity of rice was 145.13 U gprot⁻¹ at 0 h, reached a peak value of 152.461 U gprot⁻¹ at 1 h and then decreased to 73.831 U gprot⁻¹ at 72 h (Fig. 5D).

POD activity in *E. crus-galli* was 34.924 U gprot⁻¹ at 1 h, reached a peak value of 66.075 U gprot⁻¹ at 8 h and then decreased to 23.482 U gprot⁻¹ at 72 h, which was nearly the same as that of the control group (Fig. 5E). POD activity in rice was 52.32 U gprot⁻¹ at 0 h, reached a peak value of 131.026 U gprot⁻¹ at 72 h (Fig. 5F).

The TP content in *E. crus-galli* was 1.356 gprot L⁻¹ at 0 h, decreased to the lowest value of 0.484 gprot L⁻¹ at 8 h, and gradually increased to 1.647 gprot L⁻¹ between 8 h to 72 h (Fig. 5G). The rice TP content was 1.357 gprot L⁻¹ at 0 h, which decreased to the lowest value of 0.601 gprot L⁻¹ at 72 h (Fig. 5H). The relative activities of CAT, POD and SOD were calculated based on the TP.

Discussion

In this study, we evaluated the phytotoxicities of 13 essential oils. Bioassay results showed that SAEO had significant phytotoxicity and inhibition against the growth of *E. crus-galli* in a dose-dependent manner. Similar to our results, rice leaf essential oil was shown to have had phytotoxicity to *E. crus-galli* ($IC_{50} = 964.3 \text{ ug mL}^{-1}$), with the inhibitory effect proportional to the applied doses of oil^[23]. Also, the essential oil from *Cymbopogon citratus* was reported to have significantly reduced the seed germination, root length and shoot lengths of *E. crus-galli* at 8 μL concentration^[24]. The germination of *E. crus-galli* was reported to have been reduced by 92% in response to treatment with 0.5 mg mL^{-1} *Satureja montana* essential oil^[25]. *Thymbra capitata* essential oil (2 $\mu\text{L mL}^{-1}$) was also shown to have inhibited the germination of *E. crus-galli* seeds^[26].

In our study, results showed that the main component of the essential oils extracted from *S. aromaticum* was eugenol. This result is in accordance with the work of Ulanowska M and Olas B^[27] and Hu Q^[28]. Eugenol is a phenylpropanoid with a wide range of documented biological activities^[29]. Eugenol has been reported to be used as antimicrobial^[30], allelochemical^[25], antioxidant^[31], insecticidal agents^[32] and other uses^[33]. Results from the plant bioassay, showed that eugenol, α -caryophyllene and β -caryophyllene had inhibitory effects on *E. crus-galli*, but eugenol had the strongest inhibitory effect than the others. Similar to our results, eugenol was reported to have shown greater phytotoxicity to the germination of *Lolium rigidum*. It was completely inhibited by the application of eugenol at a concentration of 640 nL mL^{-1} ^[34]. It also inhibited germination (60%) of *Avena fatua* at a concentration of 1000 μM ^[35]. These show that SAEO has the potential to be developed as a herbicide against *E. crus-galli* in fields.

The safety of herbicides should not be ignored in the development process. For example, essential oil from lavender was shown to be effective against *Lavandula angustifolia* but safe for cucumbers^[36]. Also, apple pectin cross-linked by citric acid which contained savory essential oil was shown to have significantly inhibited the seed germination of *A. retroflexus*, but was relatively safe on tomato at a concentration of 3 $\mu\text{L mL}^{-1}$ ^[37]. Interestingly, in our research, SAEO had no obvious inhibitory effect on rice (2 - leaf stage) (Fig. 4D). Similar to our results, *Hyptis suaveolens* essential oil (2 mg mL^{-1}) was reported to have inhibited germination and seedling growth of *E. crus-galli*. But the inhibitory effect on rice germination was lower (~ 40% inhibition)^[38]. *Mentha longifolia* essential oil (250 $\mu\text{g mL}^{-1}$) was also reported to have inhibited the germination of *E. crus-galli*. However, the impact was comparatively lesser on rice germination (~ 40% inhibition)^[39]. Furthermore, rice leaf essential oil contains many substances with antioxidant properties (such as momilactones A and B), which are phytotoxic to different weeds (*E. crus-galli* and *Bidens pilosa* L.), but the same applied doses of EO slightly stimulated (5–13%) the growth of the rice^[23]. These show that the SAEO could be developed into a novel safe bioherbicide for the control of *E. crus-galli* in rice paddies.

The antioxidant enzyme system in plants influence their physiological functions such as growth, development, stress defense and response and metabolic processes^[40]. The effect of essential oils on the antioxidant enzyme system in plants have been evaluated in past studies^[41, 42]. We therefore evaluated

the inhibition effect of SAEO on the activities of antioxidant enzymes (CAT, SOD and POD) in *E. crus-galli* and rice. Interestingly, only *E. crus-galli* CAT decreased significantly after 8 h compared to the control. However, the CAT activity in rice did not decrease significantly compared with the control group. The SOD and POD activities in *E. crus-galli* did not also change significantly after 72h compared with the control. Similar to our results, *A. retroflexus* treated with 2.5% *Eupatorium adenophorum* leachate solution for 24–72h showed increased SOD, POD, and CAT activities, but only CAT levels were affected by the treatment^[43]. Furthermore, the CAT activity in *E. crus-galli* first increased, then decreased after treatment with *Veronica persica* ethanolic extracts^[21]. Our results indicate that the application of SAEO may lead to a rapid accumulation of ROS in barnyard grass than in rice, indicating that SAEO may be relatively safe for rice. SAEO effectively inhibited the growth and development of barnyard grass seedlings, which might be related to its effect on the CAT activity in barnyard grass. This however needs further research.

Conclusions

In conclusion, this study showed that among 13 plants essential oils studied, LIA had a high inhibitory activity against *E. crus-galli* in a dose-dependent manner. The LIA was collected and purified by vacuum distillation under different conditions, and that which was collected at 165°C (LIA-165) had the strongest inhibitory activity. GC-MS and HPLC analyses showed that LIA-165 contained three main compounds (eugenol, β -caryophyllene and α -caryophyllene) and also had an effect on the activities of antioxidant enzymes in *E. crus-galli*. Further, LIA was relatively safe to rice plants at the 2-leaves-stage and may serve as an effective and safe post-emergence herbicide against *E. crus-galli* in rice production. Further work should focus on stable and safety formulations of LIA and field experiments to evaluate its efficacy.

Declarations

Author Contributions: Conceptualization, L.Z.R. and J.C.Z.; Methodology, L.Z.R.; investigation, B.H.D, N.X.Z. and H.J.C.; Software, Z.Y. and N.X.Z.; data curation, B.H.D., B.Z.D. and N.X.Z.; Resources, N.X.Z., L.S.Q. and X.J.J.; writing—original draft preparation, B.H.D.; writing—review and editing, L.Z.R.; supervision, J.C.Z. and L.Z.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the China Agriculture Research System of MOF and MARA (grant number CARS - 16 - E19), Natural Science Foundation of Hunan Province (grant number 2021JJ20034), Innovation Platform Open Funds for Universities in Hunan Province (grant number No.19K049), Hunan Agriculture Research System (grant number 2022-31), Hunan Key Research and Development Programme (grant number 2020NK2046), Scientific-Innovative of Hunan Agricultural Sciences and Technology (grant number 2019LS05, 2019LS06, 2019TD03, 2020CX58 and 2020CX60, 2022CX134), Scientific-Innovative of Hunan Postgraduate Sciences (grant number CX20211224), China.

Acknowledgments: The authors thank editors and reviewers for their hard work and kind comments for this paper. We would like to thank Dr. Margaret Baker for professional english language editing services.

Conflicts of Interest: The authors declare no conflict of interest.

Statement on the collection of plant material: The studies comply with local and national guidelines and regulations.

Data availability: All data generated or analyzed during this study are included in this published article [and its supplementary information files].

References

1. Storkey, J., Mead, A., Addy, J. & MacDonald, A.J. Agricultural intensification and climate change have increased the threat from weeds. *Glob chang biol.* **27**, 2416-2425 (2021).
2. Bagavathiannan, M.V. & Norsworthy, J.K. Pollen-mediated transfer of herbicide resistance in *Echinochloa crus-galli*. *Pest Manag Sci.* **70**, 1425-1431 (2014).
3. Nguyen, T.H., Malone, J.M., Boutsalis, P., Shirley, N. & Preston, C. Temperature influences the level of glyphosate resistance in barnyardgrass (*Echinochloa colona*). *Pest Manag Scie.* **72**, 1031-1039 (2016).
4. Khanh T.D., Xuan T.D., Chung I.M. & Tawata S. Allelochemicals of barnyardgrass-infested soil and their activities on crops and weeds. *Weed Biol. Manage.* **8**, 267-275 (2008).
5. The International herbicide-resistant weed database. <http://www.weedscience.org>. Accessed: April 2 (2021).
6. Kaloumenos, N.S., Chatzilazaridou, S.L., Mylona, P.V., Polidoros, A.N. & Eleftherohorinos, I.G. Target-site mutation associated with cross-resistance to ALS-inhibiting herbicides in late watergrass (*Echinochloa oryzicola* Vasing.). *Pest Manag Sci.* **69**, 865-873 (2013).
7. Guo, L. *et al.* *Echinochloa crus-galli* genome analysis provides insight into its adaptation and invasiveness as a weed. *Nat Commun.* **8**, 1031 (2017).
8. Iwakami, S., Hashimoto, M., Matsushima, K.I., Watanabe, H., Hamamura, K. & Uchino, A. Multiple-herbicide resistance in *Echinochloa crus-galli* var. *formosensis*, an allohexaploid weed species, in dry-seeded rice. *Pestic. Biochem. Physiol.* **119**, 1-8 (2015).
9. Abd-ElGawad, A.M., Elshamy, A.I., Al-Rowaily, S.L. & El-Amier, Y.A. Habitat affects the chemical profile, allelopathy, and antioxidant properties of essential oils and phenolic enriched extracts of the invasive plant *Heliotropium Curassavicum*. *Plants (Basel)*. **8**, 482 (2019).
10. El-Gawad, A.A., Elshamy, A., El Gendy, A.E., Gaara, A. & Assaeed, A. Volatiles profiling, allelopathic activity, and antioxidant potentiality of *Xanthium Strumarium* Leaves essential oil from egypt: evidence from chemometrics analysis. *Molecules.* **24**, 584 (2019).
11. Abd-ElGawad, A. *et al.* Essential oil of *Bassia muricata*: chemical characterization, antioxidant activity, and allelopathic effect on the weed *Chenopodium murale*. *Saudi J Biol Sci.* **27**, 1900-1906 (2020).

12. Bais, H.P., Vepachedu, R., Gilroy, S., Callaway, R.M. & Vivanco, J.M. Allelopathy and exotic plant invasion: From molecules and genes to species interactions. *Science*. **301**, 1377-1380 (2003).
13. Schandry, N. & Becker, C. Allelopathic plants: models for studying plant–interkingdom interactions. *Trends Plant Sci*. **25**, 176-185 (2019).
14. Cazella, L.N. *et al.* Antimicrobial activity of essential oil of *Baccharis dracunculifolia* DC (Asteraceae) aerial parts at flowering period. *Front. Plant Sci*. **10**, 27 (2019).
15. Limam, H., Jemaa, M.B., Tammar, S., Ksibi, N. & Msaada, K. Variation in chemical profile of leaves essential oils from thirteen *Tunisian Eucalyptus* species and evaluation of their antioxidant and antibacterial properties. *Ind. Crops Prod*. **158**, 112964 (2020).
16. Sheng, Z., Jian, R., Xie, F., Chen, B. & Hong, W.D. Screening of larvicidal activity of 53 essential oils and their synergistic effect for the improvement of deltamethrin efficacy against *Aedes albopictus*. *Ind. Crops Prod*. **145**, 112131 (2020).
17. Xuan, T.D. *et al.* Weed allelochemicals and possibility for pest management. *International Letters of Natural Sciences*. **56**, 25-39 (2016).
18. Dyanat, M. & Asgari, F. Phytotoxic effects of essential oils from *Nepeta glocephalata* Rech.f. and *N. ispanica* Boiss. on selected weed species. *Acta agriculturae Slovenica*. **4**, 117 (2021).
19. Li, A. *et al.* A strategy of rapidly screening out herbicidal chemicals from *Eucalyptus* essential oils. *Pest Manag Sci*. **76**, 917-927 (2020).
20. Kamran, M. *et al.* Pre-sowing seed treatment with kinetin and calcium mitigates salt induced inhibition of seed germination and seedling growth of choysum (*Brassica rapa* var. parachinensis). *Ecotox Environ Safe*. **227**, 112921 (2021).
21. Li, Z.R., Liu, Y.B., Zhou, X.M., Li, X.G. & Bai, L.Y. Allelopathic herbicidal effects of crude ethanolic extracts of *Veronica persica* (Lour.) Merr. on weeds. *Allelopathy J*. **46**, 85-96 (2019).
22. Do, D.N. *et al.* Fractionating of lemongrass (*Cymbopogon citratus*) essential oil by vacuum fractional distillation. *Processes*. **9**, 593 (2021).
23. Minh, T.N. *et al.* Phytochemical analysis and potential biological activities of essential oil from rice leaf. *Molecules*. **24**, 546 (2019).
24. Poonpaiboonpipat, T., Pangnakorn, U., Suvunnamek, U., Teerarak, M., Charoenying, P. & Laosinwattana, C. Phytotoxic effects of essential oil from *Cymbopogon citratus* and its physiological mechanisms on barnyardgrass (*Echinochloa crus-galli*). *Ind. Crops Prod*. **41**, 403-407 (2013).
25. Angelini, L.G., Carpanese, G., Cioni, P.L., Morelli, I., Macchia, M. & Flamini, G. Essential oils from *Mediterranean Lamiaceae* as weed germination inhibitors. *J. Agri. Food. Chem*. **51**, 6158-6164 (2003).
26. Verdeguer, M. *et al.* Herbicidal activity of *Thymbra capitata* (L.) Cav. essential oil. *Molecules*. **25**, 2832 (2020).
27. Ulanowska, M. & Olas, B. Biological properties and prospects for the application of eugenol-a review. *Int J Mol Sci*. **22**, 3671 (2021).

28. Hu, Q., Zhou, M. & Wei, S. Progress on the antimicrobial activity research of clove oil and eugenol in the food antiseptics field. *J Food Sci.* **83**, 1476-1483 (2018).
29. Vitalini, S., Orlando, F. & Iriti, M. Selective phytotoxic activity of eugenol towards monocot and dicot target species. *Nat. Prod. Res.* **11**, 1-4 (2021).
30. Chaieb, K. *et al.* The chemical composition and biological activity of clove essential oil, *Eugenia caryophyllata* (*Syzygium aromaticum* L. Myrtaceae): a short review. *Phytother. Res.* **21**, 501-506 (2007).
31. Radünz, M. *et al.* Antimicrobial and antioxidant activity of unencapsulated and encapsulated clove (*Syzygium aromaticum*, L.) essential oil. *Food Chem.* **276**, 180-186 (2019).
32. Batiha, G.E., Beshbishy, A.M., Tayebwa, D.S., Shaheen, H.M., Yokoyama, N. & Igarashi, I. Inhibitory effects of *Syzygium aromaticum* and *Camellia sinensis* methanolic extracts on the growth of *Babesia* and *Theileria* parasites. *Ticks Tick-borne Dis.* **10**, 949-958 (2019).
33. González, J.N.H., Herrera, G.A.C, Velázquez, M.M. & Andrews, H.E. Clove essential oil (*Syzygium aromaticum* L. Myrtaceae): extraction, chemical composition, food applications, and essential bioactivity for human health. *Molecules.* **26**, 6387 (2021).
34. Vasilakoglou, I., Dhima, K., Paschalidis, K. & Ritzoulis, C. Herbicidal potential on *Lolium rigidum* of nineteen major essential oil components and their synergy. *J. Essent. Oil Res.* **25**, 1-10 (2013).
35. Ahuja, N., Singh, H.P., Batish, D.R. & Kohli, R.K. Eugenol-inhibited root growth in *Avena fatua* involves ROS-mediated oxidative damage. *Pestic. Biochem. Physiol.* **118**, 64-70 (2015).
36. Ibáñez, M.D. & Blázquez, M.A. Phytotoxic effects of commercial *Eucalyptus citriodora*, *Lavandula angustifolia*, and *Pinus sylvestris* essential oils on weeds, Crops, and invasive species. *Molecules.* **24**, 2847 (2019).
37. Taban, A., Saharkhiz, M.J. & Kavoosi, G. Development of pre-emergence herbicide based on Arabic gum-gelatin, apple pectin and savory essential oil nano-particles: A potential green alternative to metribuzin. *Int. J. Biol. Macromol.* **167**, 756-765 (2021).
38. Sharma, A., Singh, H.P., Batish, D.R. & Kohli, R.K. Chemical profiling, cytotoxicity and phytotoxicity of foliar volatiles of *Hyptis suaveolens*. *Ecotox Environ Safe.* **171**, 863-870 (2019).
39. Singh, N., Singh, H.P., Batish, D.R., Kohli, R.K. & Yadav, S.S. Chemical characterization, phytotoxic, and cytotoxic activities of essential oil of *Mentha longifolia*. *Environ. Sci. Pollut. Res.* **27**, 13512-13523 (2020).
40. Zhou, J., Zhang, C., Du, B., Cui, H., Fan, X., Zhou, D. & Zhou, J. Soil and foliar applications of silicon and selenium effects on cadmium accumulation and plant growth by modulation of antioxidant system and Cd translocation: comparison of soft vs. durum wheat varieties. *J. Hazard. Mater.* **402**, 123546 (2021).
41. Fatemi, H., Esmaili, P.B. & Rizwan, M. Foliar application of silicon nanoparticles affected the growth, vitamin C, flavonoid, and antioxidant enzyme activities of coriander (*Coriandrum sativum* L.) plants grown in lead (Pb)-spiked soil. *Environ. Sci. Pollut. Res.* **28**, 1417-1425 (2021).

42. Mohammad, P., Sabzi, N.M., Ali, M. & Filippo, M. Exploring the bio-control efficacy of *Artemisia fragrans* essential oil on the perennial weed *Convolvulus arvensis*: Inhibitory effects on the photosynthetic machinery and induction of oxidative stress. *Ind. Crops Prod.* **155**, 112785 (2020).

43. Ma, J.H., Xing, G.F., Yang, W.X. & Lei, L. Inhibitory effects of leachate from *Eupatorium adenophorum* on germination and growth of *Amaranthus retroflexus* and *Chenopodium glaucum*. *Acta Ecologica Sinica.* **32**, 50-56 (2012).

Figures

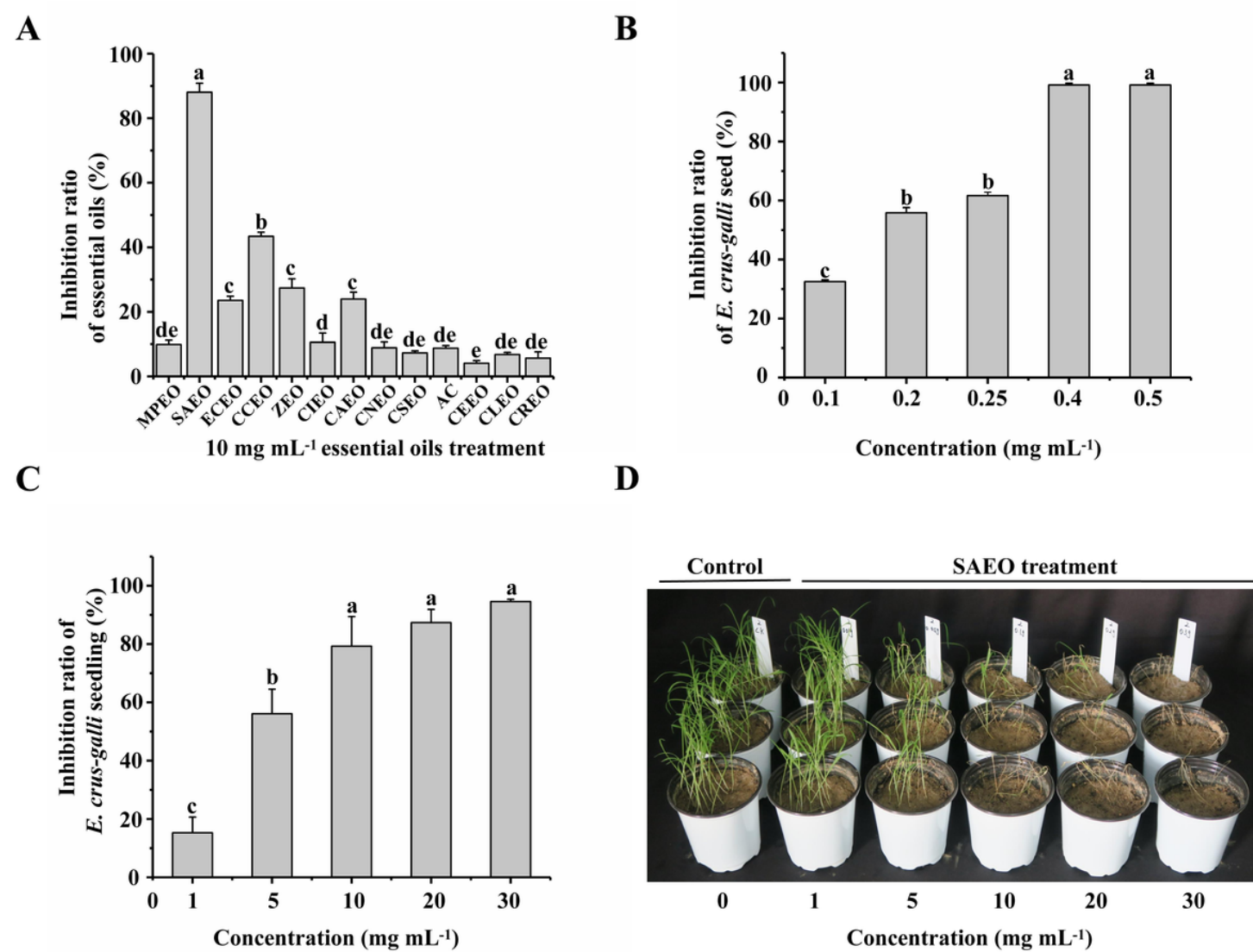


Figure 1

(A) Inhibition ratio of 13 essential oils (10 mg mL⁻¹) on seedling growth of *E. crus-galli* 7 days after treatment. (*M. piperita* essential oil (MPEO), *S. aromaticum* essential oil (SAEO), *E. caryophyllata* essential oil (ECEO), *C. cassia* essential oil (CCEO), *Zingiber* essential oil (ZEO), *C. citratus* essential oil (CIEO), *C. camphora* essential oil (CAEO), *C. annuum* essential oil (CNEO), *C. sinensis* essential oil

(CSEO), *A. caruifolia* essential oil (ACEO), *C. sinensis* essential oil (CEEEO), *C. limon* essential oil # (CLEO), and *C. reticulata* essential oil (CREO). The error bar is the standard error of the mean. The letter above the error bar indicates significant difference between means (ANOVA). The same letter indicates no significant differences between treatments. **(B)** Inhibitory effect of different SAEO concentrations (0.1, 0.2, 0.25, 0.4, 0.5 mg mL⁻¹) on *E. crus-galli* seed. **(C)** Inhibitory effect of different SAEO concentrations (1, 5, 10, 20, 30 mg mL⁻¹) on *E. crus-galli* seedling. **(D)** Inhibitory effect of different SAEO concentrations (0, 1, 5, 10, 20, 30 mg mL⁻¹) on *E. crus-galli* seedlings growth.

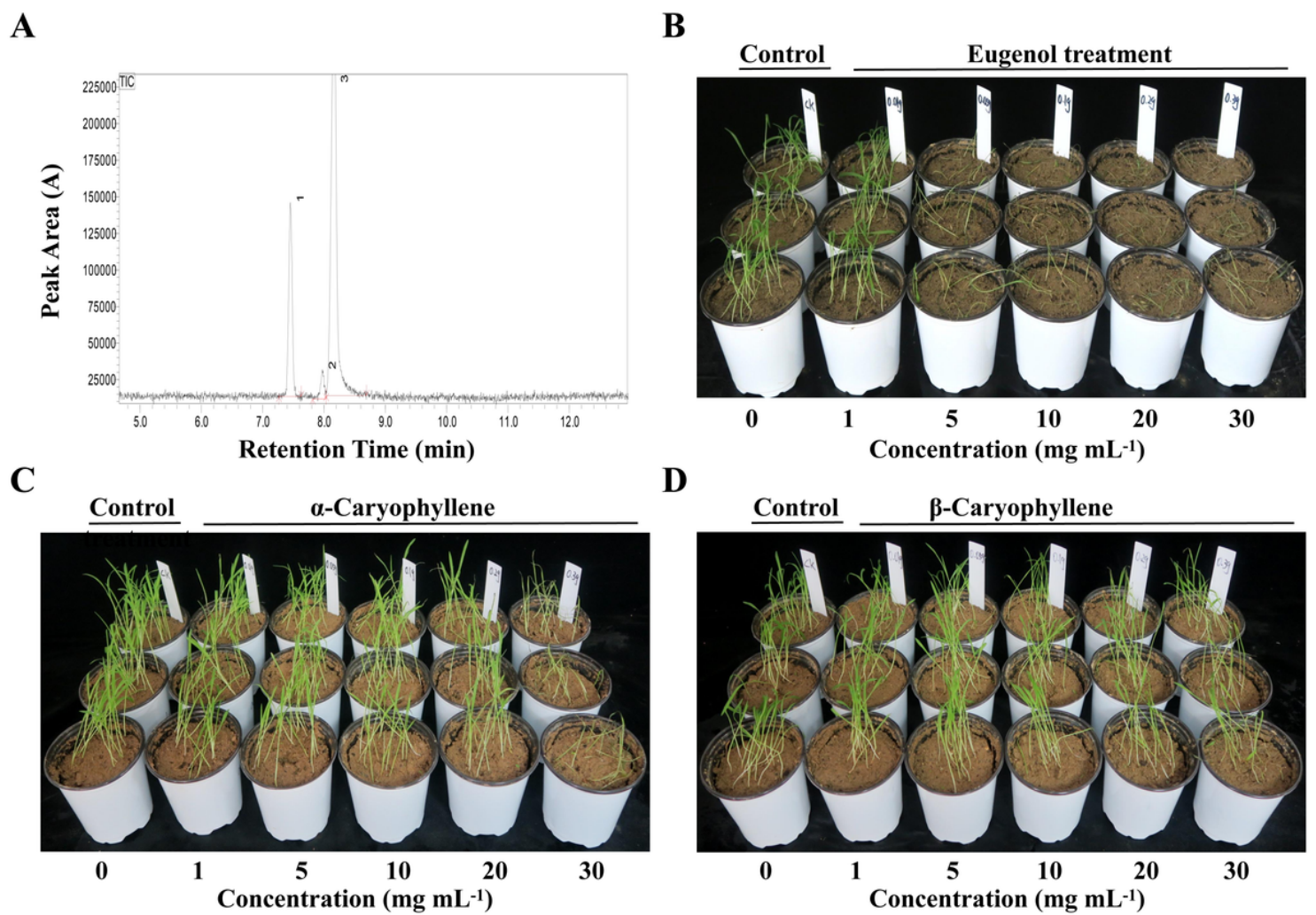


Figure 2

Main compounds identified in SAEO-165 by GC-MS and their inhibitory effects. **(A)** Diagram of GC-MS. **(B)** Inhibitory effect of different concentrations of eugenol (0, 1, 5, 10, 20, 30 mg mL⁻¹) on *E. crus-galli*. **(C)** Inhibitory effect of different concentrations of α-caryophyllene (0, 1, 5, 10, 20, 30 mg mL⁻¹) on *E. crus-galli*. **(D)** Inhibitory effect of different concentrations of β-caryophyllene (0, 1, 5, 10, 20, 30 mg mL⁻¹) on *E. crus-galli*.

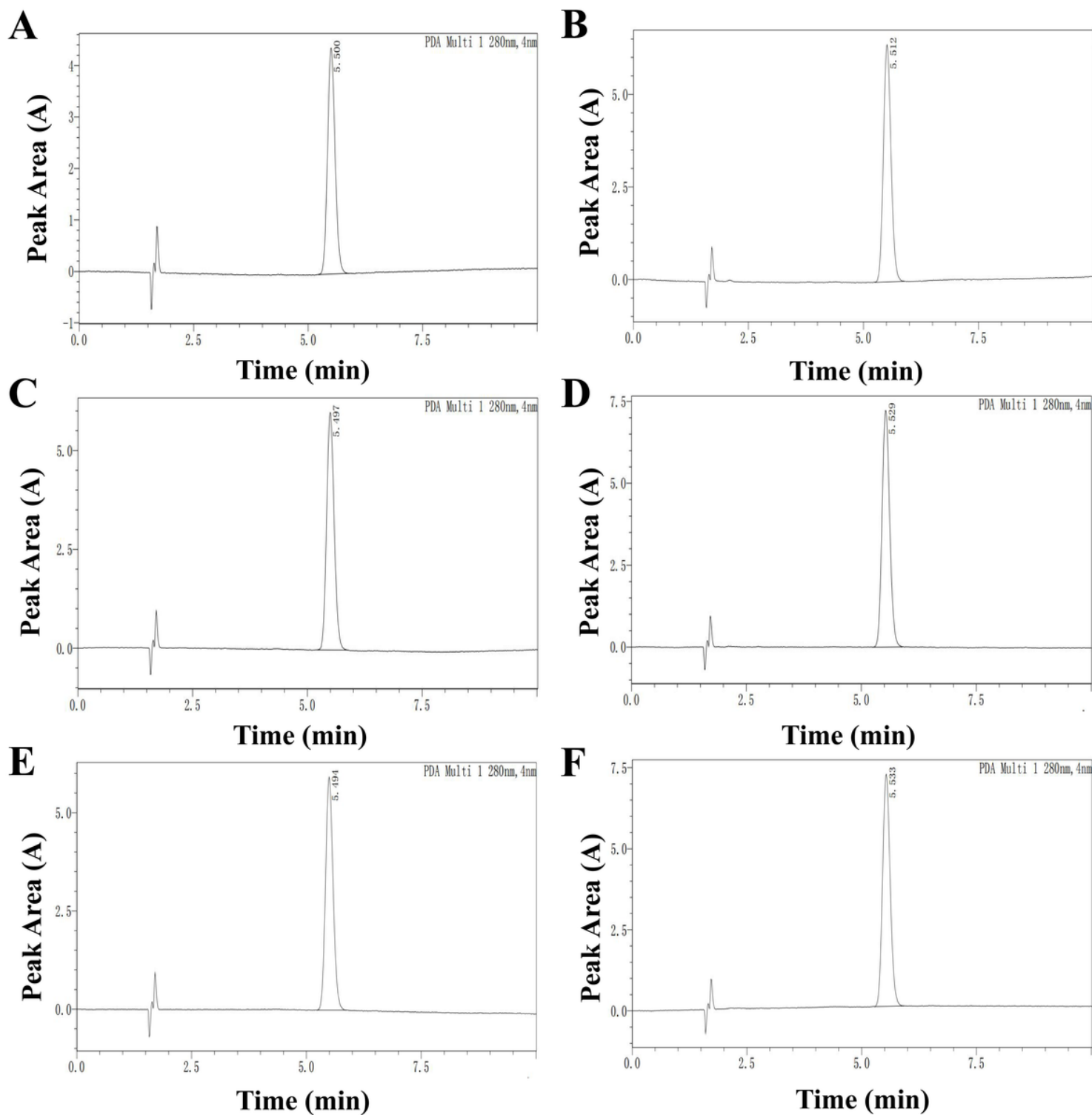


Figure 3

The eugenol chromatlog in SAEO conditions from HPLC analysis. (A)164°C. (B)165°C. (C)169°C. (D)170°C. (E)175°C. (F) 180°C.

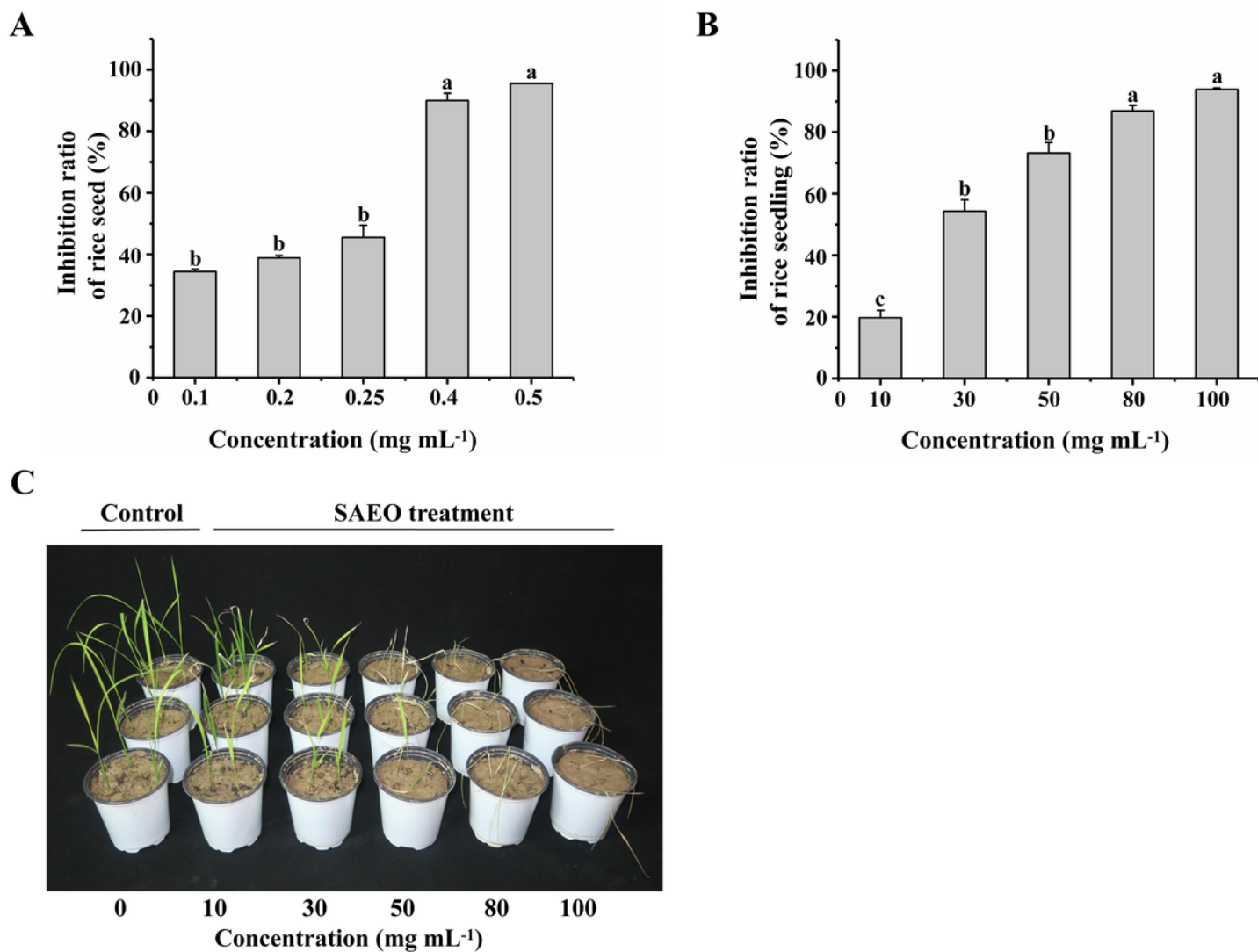


Figure 4

Safety evaluation of SAEO on rice. **(A)** Inhibitory effect of SAEO (0.1, 0.2, 0.25, 0.4, 0.5 mg mL⁻¹) on rice seeds. **(B)** Inhibitory effect of SAEO (10, 30, 50, 80, 100 mg mL⁻¹) on rice seedlings. **(C)** Inhibitory effect of different concentrations of SAEO (0, 10, 30, 50, 80, 100 mg mL⁻¹) on rice.

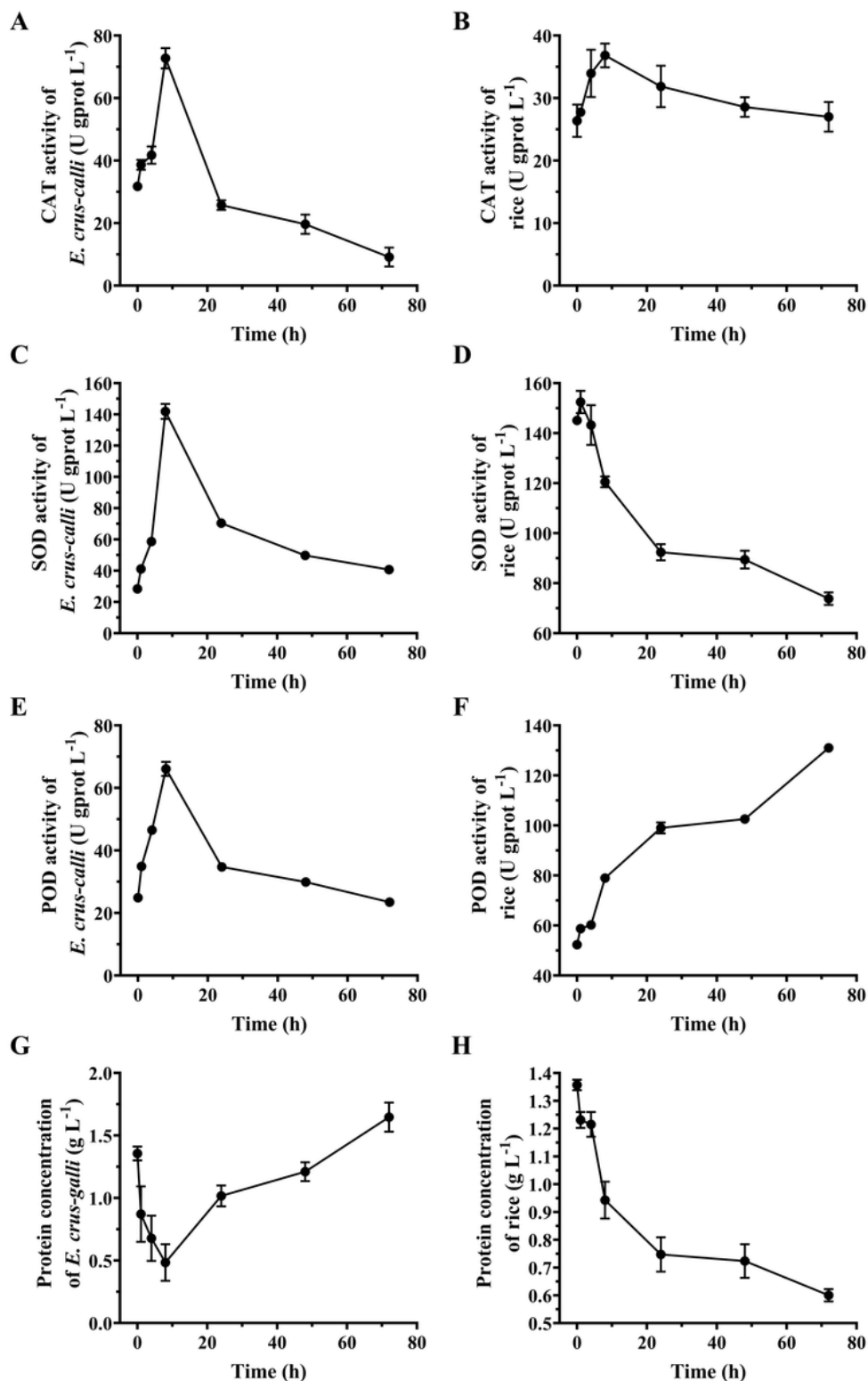


Figure 5

Inhibitory effect of SAEO on the activities of defense enzymes in *E. crus-galli* and rice. Effects of SAEO on (A) CAT activity in *E. crus-galli* (B) CAT activity in rice (C) SOD activity in *E. crus-galli* (D) SOD activity in rice, (E) POD activity in *E. crus-galli* (F) POD activity in rice (G) TP activity in *E. crus-galli* (H) TP activity in rice.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [CATofE.crusgallidata.xlsx](#)
- [CATSODPODTPofrice.xlsx](#)
- [HPLCanalysis.xlsx](#)
- [SUPPORTINGINFORMATION3.19.docx](#)
- [TPofE.crusgallidata.xlsx](#)
- [biologicaltestdata3.19.xls](#)
- [fractiondistillationsbiologicaldata.xlsx](#)
- [podofE.crusgallidata.xlsx](#)
- [sodofE.crusgallidata.xlsx](#)
- [GraphicalAbstractImage.png](#)