Waste and Biomass Valorization
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ORIGINAL PAPER

Valorization of *Zingiber officinale* (Ginger) Leaf Waste by Extraction and Identification of Compounds with High Phytotoxicity

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Abstract

Global consumption of ginger has been growing in recent years resulting in high volumes of ginger root, rhizome, and leaf waste. Valorization of ginger waste is of great importance for a sustainable environment. In this research, ginger leaf waste was considered a valuable source for the extraction of phytotoxic chemicals. Ginger leaf waste from two varieties (*Chinese* and *Sidhdha*) was used for extraction using different organic solvents. The extracts were tested for phytotoxicity against elongation of root and shoot of *Lactuca sativa* seeds, bioassay-guided fractionation as well as spectroscopic and chromatographic techniques. All of the tested phytochemical groups were qualitatively identified in the methanolic extracts of both varieties. It was found that the level of inhibition was concentration-dependent, but the effect of variety on the extraction yield and level of phytotoxicity was nonsignificant. The methanolic extract showed significantly higher elongation inhibitions than the other solvent extracts. Accordingly, methanol was found to be the most effective solvent for extracting phytotoxic chemicals. Two potent phytotoxic compounds, named as R/ZO/1 and R/ZO/2, with 100% elongation inhibition, were recovered from the bioassay-guided fractionation. These compounds were identified as polar organic compounds and modified terpenoids with oxidized and decomposed fatty acid derivatives. The results of this study revealed the phytotoxic potential of methanolic extracts from ginger leaves.

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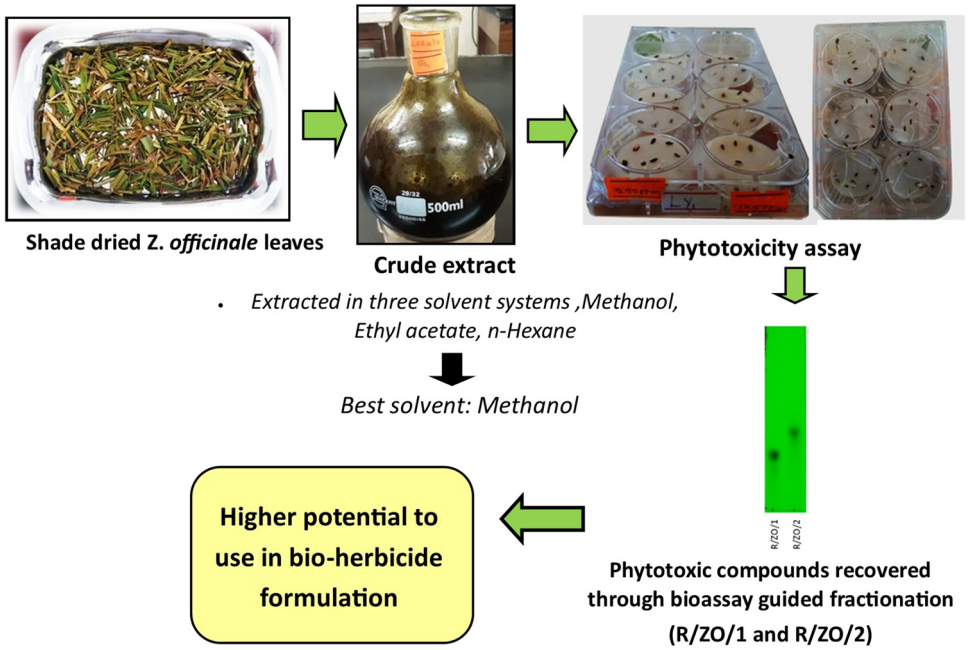
Graphical Abstract



Keywords Waste valorization · Inhibitory effect · *Zingiber officinale* leaf · Herbicides · Phytotoxic compounds

Statement of Novelty

Large amounts of ginger waste need to be valorized efficiently. Valorization of ginger leaf waste to extract compounds with phytotoxic potential was rarely considered by researchers in the past years, making it worth investigating as a novel research.

Introduction

Ginger, scientifically named *Zingiber officinale roscoe*, belongs to the Zingiberaceae family and the Zingiber genus. It was initially cultivated in the southern and eastern parts of Asia and subsequently introduced to numerous regions across the globe [1]. The ginger rhizome, a well-known food flavoring agent, has been utilized for its medicinal properties by traditional pharmaceutical industries for centuries. It has been used effectively to cure various illnesses, such as stomach aches, diarrhea, nausea, asthma, and respiratory disorders, since ancient times [2].

Ginger cultivators primarily focus on the ginger rhizome, which is harvested after 6–12 months of planting when the 1-m-tall leaf sections of the plant have withered off [3]. Although ginger is primarily grown for its rhizome, the other parts of the plant also contribute significantly to its mass. Hence, annually, in different regions of the world, a considerable quantity of ginger leaves is generated and discarded as waste. Currently, alcoholic fermentation, biogas generation, and the formation of briquettes are the conventional uses of ginger leaf waste, which account for only a minor level of monetary benefit [4].

Isolation of compounds from wasted biomass with potential bioactivity has attracted the interest of many researchers. Bio-valorization of ginger waste has been reviewed recently [5]. This study discussed the extraction of bioactive compounds from the ginger peel, their bioactive properties such as phytotoxicity, antioxidant, antimicrobial, and antifungal properties, and their industrial applications such as pharmaceutical, cosmetics, food, and biorefinery. In a recent work, ginger leaves and branches were digested by a composite microbial system to increase the composting efficiency and produce organic fertilizer [6]. Biorefinery approaches were also used to utilize the ginger waste biomass for resource recovery. Valuable compounds such as oils (ginger oil and bio-oil), biopolymers (starch and micro-fibrillated cellulose), and hydro-char were obtained from spent industrial ginger [7].

Despite the extensive research on the ginger rhizome [8], studies on ginger leaves still need to be expanded in the literature in order to efficiently valorize the wasted biomass. Various compounds with potential phytotoxicity such as phenolics [9], flavonoids [10], labdane-type diterpenes, diarylheptanoids, and phenylbutanoids have been identified in

ginger leaves [11]. Leaves of the Etlingera species of ginger (Tribe Alpinieae and family Zingiberaceae) exhibited antioxidant, antibacterial, anti-tyrosinase, and hepatoprotective activities [11]. Accordingly, the significance of exploring the possibilities of using the phytochemicals found in *Zingiber officinale roscoe* (ginger) leaves, for unexplored applications has been recognized.

Extraction of phytotoxic chemicals from ginger leaf was identified as a valuable means of utilizing an often-discarded waste. According to Fujita et al. (1994) [12], a-pyrones from benzene and n-hexane extracts of the leaves of the Zingiberaceae family plant Alpinia species K. *Schum*. (Shell ginger) exhibited inhibitory effects against lettuce seedling elongation. However, to our knowledge, a detailed study has yet to be carried out to investigate the phytotoxic potential of *Zingiber officinale roscoe* (ginger) leaf extracts.

Accordingly, this study aimed to extract and characterize potentially bioactive phytotoxic compounds from ginger leaves, an underutilized agricultural biomass often considered as waste. We employed ultrasonic extraction as a cost-effective and environmentally friendly approach to valorize ginger leaf waste. The constituents of the resulting extract were analyzed qualitatively and quantitatively using analytical methods, and their phytotoxic effects on the shoot and root growth of *Lactuca sativa* (lettuce) seeds were evaluated.

Materials and Methods

Chemicals

All of the chemicals and reagents were obtained from well-known suppliers and used as received. $HgCl_2$, $FeCl_3$, and n-hexane were purchased from Sigma (France). Analytical grade methanol (99%) and ethyl-acetate (99%) were purchased from Fluka (NC, USA). KI was purchased from VWR Chemicals (UK), sulfuric acid (98%) from Daejung (Korea), chloroform (99%) from Ranken (India), and NaOH (98%) from Loba Chemie (India).

Sample Collection and Preparation

Samples of mature ginger leaves, weighing 1 kg each from the 'Chinese' and '*Sidhdha'* varieties, were collected from plant breeders in the North-Western Province of Sri Lanka. This region features a tropical climate, with daytime temperatures ranging between 28 and 30 °C. The collected leaves of each variety were cleaned with tap water to remove dust and other dirt particles. The leaves were cut into smaller pieces and dried for five days in the shade. The dried leaves were then powdered using a domestic grinder, stored in a dark plastic bottle, sealed, and kept at 4 °C.

Crude Extract Preparation and Yield Calculation

Zingiber officinale roscoe leaf powder of the 'Chinese' cultivar and "*Sidhdha*" were extracted separately with pure n-hexane, ethyl-acetate, and methanol in increasing polarity order [13]. A sample containing 100 g leaf in 500 mL of solvent was placed in an ultrasonic extraction apparatus (Rocker, Soner 206 H, Taiwan) operating at 35 °C for 30 min at a 50 kHz frequency, and phytochemicals were extracted. Each solvent was repeated three times for every leaf sample, followed by filtration. The extract was then evaporated by rotary evaporation at 40 °C under vacuum to obtain a dry crude extract. The extracts obtained were dried under vacuum for 24 h. The initial crude extracts obtained through n-hexane, ethyl-acetate (EtOAc), and methanol (MeOH) solvent systems of the 'Chinese' variety and the 'Sidhdha' variety were named RB/ZO/C/Hexane, RB/ZO/C/EtOAc, RB/ZO/C/MeOH and RB/ZO/S/Hexane, RB/ ZO/S/EtOAc, RB/ZO/S/MeOH, respectively, and used for further analysis.

The crude extract yielded by each solvent system was calculated as a percentage of the initial amount of dried powder of Z. *officinale* leaves used (w/w) as given in Eq. 1 [14].

$$\text{Extraction yield} = \frac{\text{Weight of dried crude extract}}{\text{Weight of the dried leaf powder used}} \times 100\% \quad (1)$$

Qualitative Identification of Phytochemicals in Crude Extracts

To gain an overview of the types of phytochemicals included in the extracts of two varieties of ginger leaves, categories of phytochemicals were assessed qualitatively. The qualitative phytochemical tests for detecting alkaloids, flavonoids, phenols, steroids, terpenoids, saponins, tannins, and anthraquinone were conducted as follows according to the standard tests described in [15–17]

Testing of Alkaloids

Mayer's reagent for testing alkaloids was prepared by dissolving 335 mg of $HgCl_2$ in 60 mL of distilled water and combining it with 5 g of KI diluted in 20 mL of distilled water. Then, Mayer's reagent was added to 2 mg of each crude extract, followed by a few drops of concentrated H_2SO_4. The development of a white precipitate represented the availability of alkaloids in crude extracts.

Testing of Flavonoids

The alkaline reagent test was performed to test the flavonoid content. A mixture of 1 mg of the extracts and a few drops of NaOH solution was shaken to test the flavonoids. The development of a bright yellow color that became colorless when diluted acid was added indicated that flavonoids were present.

Testing of Phenols

The Ferric Chloride test was performed by mixing 0.5 mg of the extracts with 1 mL of 10% $FeCl_3$ solution to test the phenol content. Phenols were present if the color shifted from reddish to blue.

Testing of Steroids and Terpenoids

The steroids were examined using the Liebermann-Burchard test. The crude extracts were dried and mixed with chloroform (0.2 g of extract and 2 mL of chloroform). A few drops of acetic anhydride and 2 mL of concentrated H_2SO_4 were added, and the formation of a violet-to-blue ring indicated the presence of steroids.

To test terpenoids, 0.5 mg crude extracts were mixed with 2 mL of concentrated H_2SO_4 and 2 mL of chloroform. The formation of a red-brown color between the two layers denoted the terpenoids' presence in crude extracts.

Testing of Saponin

The presence of saponins in the extracts was assessed using a frothing test. A mixture of 2 mg of crude extract was shaken with 2 mL of distilled water to test for any presence of saponin. Next, the mixture was examined to see if a soapy coating formed. In this case, saponin was present.

Testing of Tannins

The presence of tannins in the extracts was evaluated using Braymer's test. A notable development of a dark blue complex upon the addition of 1 mg of each crude extract to 2 mL of 5% ferric chloride indicated the presence of tannins within the extracts.

Testing of Anthraquinone

The presence of anthraquinone was assessed using a modified version of Borntrager's Test. Initially, 1 mg of the crude extract was combined with 5 ml of dilute HCl, followed by boiling and subsequent cooling. The resultant mixture was then filtered to obtain the filtrate, which was shaken with an equal volume of benzene. After the benzene layer was separated, the remaining filtrate was treated with a 10% ammonia solution. The emergence of a light red coloration indicated the presence of anthraquinone in the extract.

Phytotoxicity Assay for the Initial Crude Extracts

Phytotoxicity against *Lactuca sativa* (Lettuce) seed germination inhibition was tested for the initial crude extracts (RB/ZO/C/Hexane, RB/ZO/C/EtOAc, RB/ZO/C/MeOH, RB/ZO/S/Hexane, RB/ZO/S/EtOAc, and RB/ZO/S/MeOH) following the method described in previous research with fewer modifications [18, 19]. Initially, floating seeds were removed from the L. *sativa* seeds after they had been rinsed with tap water. Three replicates of each concentration of the crude extract diluted in distilled water were used to assess the germination of L. *sativa* seeds over a concentration range of 0–1000 mg/L. The assay was conducted in trays with 3 mm diameter wells, and each well was lined with filter papers at the bottom before adding 400 µL of the test solution. Then, ten L. *sativa* seeds were arranged in each well, lined with filter paper. In the control experiment, 400 µL of distilled water was added to trays lined with filter paper, and the seeds were kept to germinate. Trays with lids sealed with parafilm to retain moisture were kept in the dark at room temperature for five days. After five days, the lengths of both shoots and roots were recorded, and the percentage of inhibition of root and shoot elongation was calculated using Eq. 2 [20].

$$\text{Inhibition of root elongation} = \frac{\text{Control root length} - \text{Test sample root length}}{\text{Control root length}} \times 100\% \quad (2)$$

Shoot elongation inhibition was also calculated according to the above equation by replacing root length with shoot length (shoot and root lengths were calculated as the mean value of three replications of ten seedlings each). Figure 1 shows the step-wise procedure followed from sample collection to phytotoxicity assay.

Bioassay-Guided Fractionation of the Crude Extract

Based on the phytotoxicity assay results, the solvent fractions that showed significant inhibitory levels compared to the control were selected for bioassay-guided fractionation to recover highly potent phytotoxic constituents from the ginger leaf extract. Bioassay-guided fractionation of the active compounds was carried out following the methodology described in previous publications [19, 21] with slight modifications. Accordingly, the methanolic extract, which showed the highest phytotoxicity, was chromatographed

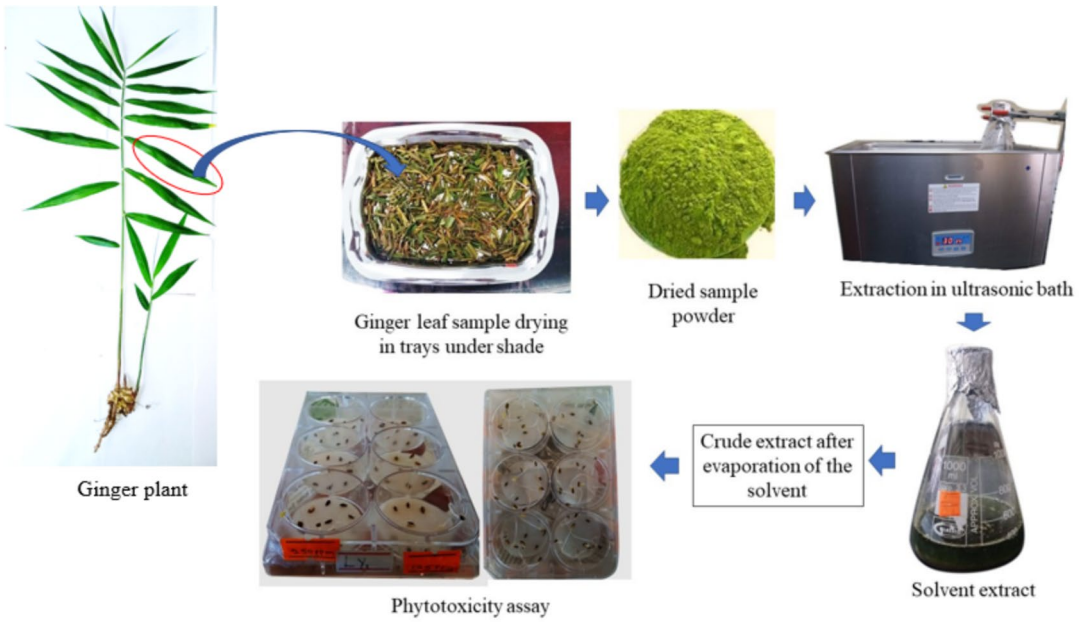


Fig. 1 The step-wise procedure followed from sample collection to phytotoxic assay of crude extracts

over silica gel (70–230 mesh size) in a 6 cm diameter glass column with hexane, EtOAc, and MeOH in increasing order of polarity. Compounds were collected into test tubes, and thin-layer chromatography (TLC) was performed for all of the compounds. The TLC patterns were observed under an ultraviolet (UV) lamp, and anisaldehyde spraying was performed to observe the patterns in compounds that were not UV-sensitive. The compounds with comparable TLC patterns were combined into a single pool. The silica gel column chromatography of crude extract (RB/ZO) resulted in 80 test tubes. Pooling of test tubes into fractions based on the TLC spot patterns resulted in 17 pooled fractions. As mentioned in Sect. ''1H NMR, LC–MS, and FTIR Spectral Analysis of Recovered Compounds'', phytotoxicity tests were conducted independently for the crude extracts of every pooled fraction. According to the phytotoxicity assay results, fractions that showed higher inhibitory percentages (> 80%) were combined.

The combined fraction from the silica column chromatography was chromatographed over Sephadex in a column using 100% MeOH. Column fractions were combined based on TLC patterns. The crude extract of pooled fractions was analyzed for phytotoxicity, and active fractions were combined and chromatographed over Sephadex in a column using 100% MeOH. The process continued for a series of Sephadex column phases, followed by phytotoxicity assays and TLC until the TLC spots of the active fraction became distinguishable enough to be separated by Preparative Thin Layer Chromatography (PTLC). Then, the active fraction selected for PTLC was used to isolate the identified phytotoxic compounds. The target compounds were recovered by removing the sorbent layer from the plate and eluting the separated material from the sorbent using a 10% MeOH solution. Finally, the solution was filtered, and the solvent was evaporated to recover the compounds. The recovered compounds were tested for phytotoxicity following the same procedure described in Sect. ''Phytotoxicity Assay for the Initial Crude Extracts''. The detailed procedure followed in recovering the phytotoxic substances is illustrated in Fig. 2.

Proton Nuclear Magnetic Resonance (^{1}H-NMR) Analysis

The NMR spectra were recorded with a Jeol JMN-AL300, Tokyo, Japan (300 MHz for 1H) spectrometer in CDCl3-CD3OD (10:1). ^{1}H chemical shifts are reported based on the internal TMS.

Liquid Chromatography-Mass Spectroscopy (LC–MS) Analysis

LC–MS (Agilent Technologies -LC/MSD XT, USA) involved dissolving 1 mg of each isolated compound in 2.5 mL of methanol and injecting a 1 µL sample into the column. A gradient solvent, comprising 0.1% (v/v) formic acid in water and acetonitrile plus 0.1% (v/v) formic acid, was employed for the elution procedure starting at a ratio of 95:5, changing to 60:40 from 1.00 to 8.00 min, then to 0:100 from 8.00 to 13.00 min, and ending with a ratio of 95:5. The flow rate was 0.3 mL/min. The instrument settings were as follows: Start mass

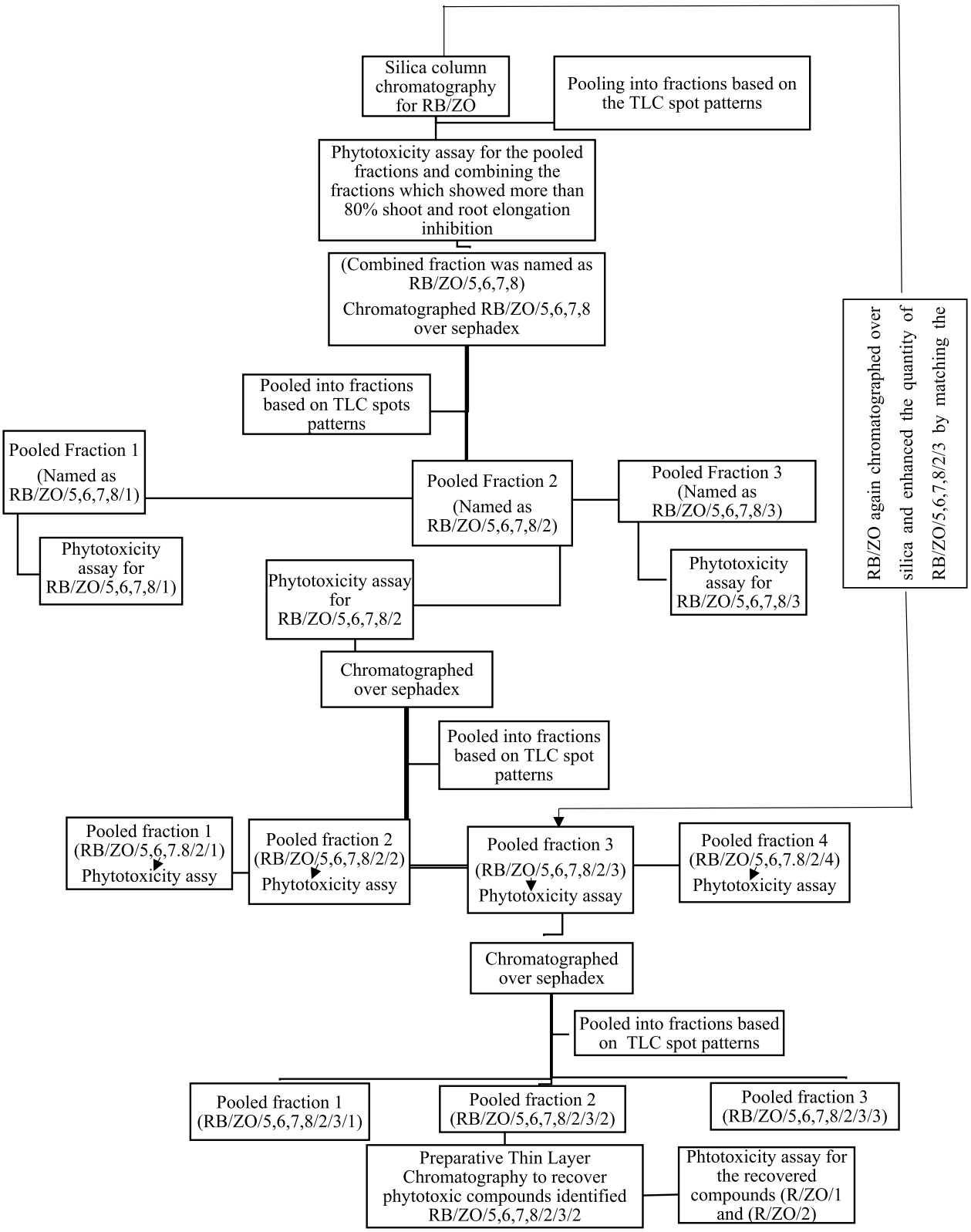


Fig. 2 Step-wise process followed to recover the phytotoxic compounds

50.0–1200.0 m/z, acquisition time 0–16 min, low CE 6 eV, high CE 10–40 eV, scan time 0.1 s, cone voltage 30 V, cone gas flow 50 L/h, acquisition mode ESI (+), capillary voltage 2 kV, collision energy 6 eV, source temperature 120 °C, desolvation temperature 500 °C and desolvation gas flow 1000 L/h, sample temperature 20 °C and column temperature 40 °C [22].

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The functional groups in the compounds obtained were identified using FTIR (Thermo Nicolet iS50 FTIR, Madison, WI, USA). For analysis, 10 mg of dry extract was transferred to an agate mortar and mixed with 100 mg KBr (pellet), forming a sample disk (translucent). The spectrum was obtained by scanning the disk from 4000 to 400 cm^{-1} wavenumbers.

Statistical Analysis

The findings of phytotoxicity testing were analyzed statistically using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). An ANOVA (analysis of variance) was performed to assess variations in means, followed by a Tukey's test with a significance level of 0.05.

Results and Discussion

Yield Percentage of Crude Extracts

According to the results shown in Table 1, a significantly higher yield of crude extract was obtained when using methanol as the solvent for extraction compared to ethyl acetate and n-hexane. The yield of the extract was significantly increased with the increase of solvent polarity. However, the variety factor did not significantly affect the weight yield of crude extracts. Previous studies have also demonstrated that the yield of phytochemical extraction from herbal plants relies on the type of solvent utilized and its polarity [23]. Previous research by Ghasemzadeh et al. (2011) [24] investigated the impact of different solvents, specifically methanol, acetone, and chloroform, on the phenolic, flavonoid, and antioxidant activities of two young Malaysian varieties of ginger known as Halia Bara and Halia Bentong. They found that the extraction solvent significantly impacted the overall phenolic, flavonoid, and antioxidant recovery, with the methanolic extracts of the ginger rhizome, stem, and leaf exhibiting the highest concentration of each component. Similar findings have also been recorded by Ezez and Tefera, (2021) [25] in their efforts to investigate the effect of solvents on the total phenolic content and antioxidant capacity of ginger extracts. According to their research, of the four solvents trialed (ethanol, methanol, ethyl acetate, and acetone), the methanol extract showed the highest phenolic content and DPPH radical scavenging activity. In contrast, the acetone extract recorded the least. Furthermore, Zorrilla et al. (2024) [26] investigated the phytotoxicity of ginger root extract and found that methanol extracts resulted in higher yields of phytotoxic compounds compared to ethyl acetate extracts. This observation implies that ginger roots also contain a significant proportion of highly polar metabolites, which may contribute to their phytotoxic potential. This research also proved that methanol is more efficient than solvents with low polarity in extracting phenolics and flavonoids from ginger.

A one-way ANOVA was conducted. Post hoc by Tukey's test. The values that are given by the same letter are not significantly different.

Table 1 Percentage weights yield of crude extracts

	n-Hexane	Ethyl-Acetate	Methanol
Chinese variety	6.86 ± 0.04% ^{c}	8.43 ± 0.08% ^{b}	15.03 ± 0.06% ^{a}
'*Sidhdha*' variety	6.85 ± 0.06% ^{c}	8.44 ± 0.04% ^{b}	14.92 ± 0.33% ^{a}

Qualitative Examination of the Phytochemicals in Crude Extracts

The phytochemicals in the n-hexane, ethyl acetate, and methanolic crude extracts of the two types of ginger leaves were analyzed qualitatively and the results are presented in Table 2. All phytochemicals, except steroids, were present in the crude extracts of each solvent. Only the crude extracts of methanol from both ginger varieties were found to contain detectable levels of steroids. In a similar study, Edo et al. (2023) [15] analyzed the phytochemicals of crude extracts of Z. *officinale* leaves in water, ethanol, and n-hexane. They found that the ethanol extract of ginger contained alkaloids, anthraquinone glycosides, cardiac glycosides, saponins, tannins, phenols, flavonoids, steroids, terpenoids, protein, and carbohydrates. However, only carbohydrates were identified in water and n-hexane extracts, and steroids were not detected, which is consistent with our findings. Moreover, according to Arawande et al. (2018) [14] the ethyl acetate extract of the Z. *officinale* rhizome contained no steroids in qualitative phytochemical screening tests. However, their study obtained negative results for saponins and tannins in ethyl acetate, contrasting our findings. Accordingly, this suggests that the types of phytochemicals detected through qualitative tests are influenced by the plant part, the polarity of solvents, and the polarity of specific compounds that belong to different phytochemical classes included in those plant parts. However, according to the findings of Jayasundara and Arampath, (2021) [27], who studied the impact of variety (based on the three varieties of Sidhdha, Rangoon, and Chinese cultivars in Sri Lanka), geographical location, and stage of maturity on the chemical composition of essential oils extracted from Z. *officinale* rhizomes, the individual

Table 2 Qualitative examination of the phytochemicals in Z. *officinale* leaf extract from the Chinese and "*Sidhdha*" varieties

Solvent	n-Hexane		Ethyl Acetate		Methanol	
Variety	Chinese	'*Sidhdha*'	Chinese	'*Sidhdha*'	Chinese	'*Sidhdha*'
Alkaloids	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Phenol	+	+	+	+	+	+
Steroids	–	–	–	–	+	+
Terpenoids	+	+	+	+	+	+
Saponin	+	+	+	+	+	+
Tannin	+	+	+	+	+	+
Anthraquinone	+	+	+	+	+	+

Key: Presence of phytochemicals: '+', absence of phytochemicals: '–'

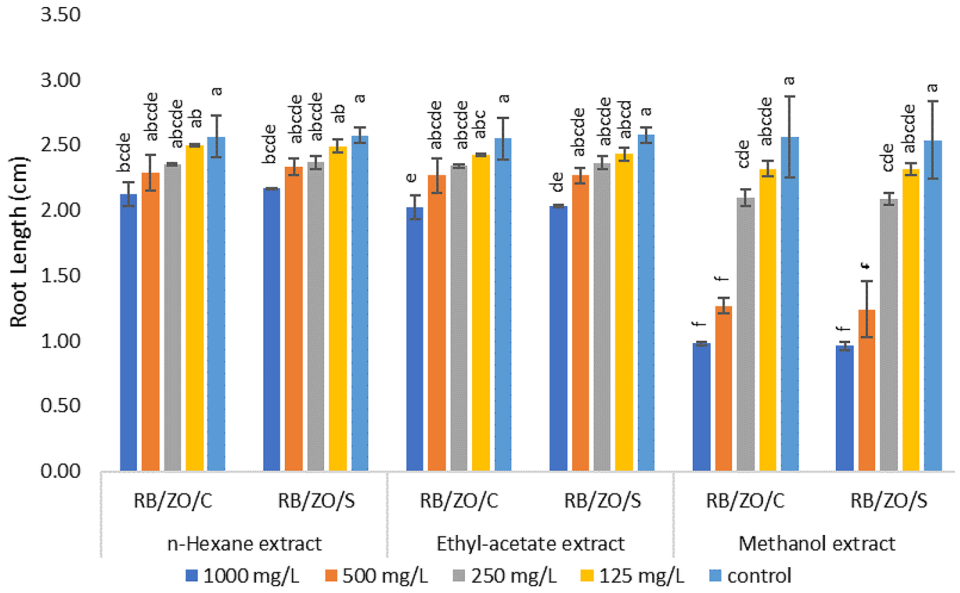
effect of variety was not significant on the phytochemical composition. Nonetheless, the composition of phytochemicals varied significantly with the change in geographical location and the maturity stage. Similarly, in our study, a difference in phytochemical composition between varieties was not observed from the qualitative phytochemical analysis. In addition, previous research demonstrated that seasonal variations can significantly alter the phytochemical profiles of numerous plant species, particularly affecting the concentrations of secondary metabolites [28]. For instance, a study on *Piper cernuum* leaves, revealed that seasonality notably influenced both its antimicrobial properties and the composition of its essential oils [29]. Similarly, extracts from *Phillyrea angustifolia* collected in winter displayed marked phytotoxicity toward *Triticum ovatum*, correlating with increased levels of oleuropein and enhanced biological activity [30]. However, it is crucial to consider that the plant samples used in this study originated from Sri Lanka, a tropical region characterized by minimal seasonal fluctuations, suggesting that the impact of seasonal changes on phytochemical composition might be limited in this context.

Phytotoxicity Results

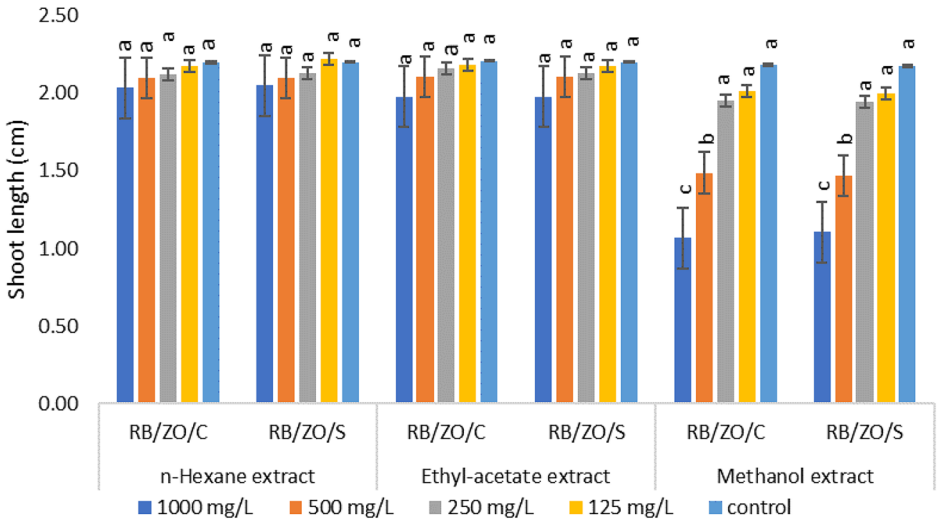
This section reports the effect of the initial crude extracts of ginger leaf on the elongation of the shoot and root of *Lactuca sativa*. Figure 3 shows the outcomes of the shoot and root elongation of *Lactuca sativa* at various concentrations of the crude extracts of the ginger leaf (prepared by evaporating the solvent extracts of n-hexane, EtOAc (ethyl-acetate), and methanol) compared to the control. Also, each examined concentration level showed reduced root and shoot elongation. Consequently, this research suggested that the phytochemicals present in ginger leaf extracts inhibit the elongation of roots and shoots of the test plant species. As the crude extract content increased, the elongation of the *Lactuca sativa* root and shoot decreased. Our findings align with earlier findings that showed concentration-dependent growth inhibitory effects in response to various plant extracts [31, 32]. Further, studies have found that increasing phytotoxic extract concentrations significantly inhibits water uptake by germinating seeds, leading to lower germination rates. Phytotoxic constituents in these extracts develop defense mechanisms [33]. The studies indicate that allelochemicals present in plant extracts can impede cell division, thus hindering the germination process of plants [34]. Further, plant growth inhibition also results from the disturbance of peroxidase activities, alpha-amylase, and acid phosphates [35]. According to previous studies on essential oils extracted from the ginger rhizome, α-zingiberene, β-sesquiphellandrene, ar-curcumin, and β-bisabolene showed strong inhibitory effects against the radicle and hypocotyl growth and germination of the *Portulaca oleracea*, *Lolium multiflorum*, and *Cortaderia selloana* weed species in *in-vitro* conditions [36].

The present study's findings proved that ginger leaves also contain phytotoxic chemical substances that inhibit the elongation of the roots and shoots of *Lactuca sativa* plants.

In the methanolic extracts of the leaves of both ginger varieties, shoot elongation was significantly decreased at concentrations of 1000 mg/L and 500 mg/L compared to the control. Similarly, root elongation was reduced significantly compared to the control at concentration levels of 1000 mg/L, 500 mg/L, and 250 mg/L. The other solvent extracts showed no significant differences in shoot elongation compared to the control at any of the concentration levels tested. However, root elongation was significantly lower at the 1000 mg/L concentration level in crude extracts prepared using n-hexane and EtOAc solvents. When comparing the three types of solvents at the 1000 mg/L concentration level, a significantly lower root length was observed with methanolic extracts, compared to EtOAc and n-hexane extracts. Moreover, at 1000 mg/L, root elongation was not significantly different between EtOAc and n-hexane extracts, although they showed significantly lower root growth compared to the control. The root elongation was not significantly different in methanolic extracts at 1000 mg/L and



(a)



(b)

Fig. 3 **a** *Lactuca sativa* root elongation inhibition and **b** shoot elongation inhibition by the crude extracts of the two varieties of ginger leaves extracted with MeOH, EtOAc, and n-Hexane. (The initial crude extracts obtained through hexane, ethyl-acetate (EtOAc), and methanol (MeOH) solvent systems of the 'Chinese' variety and the 'Sidhdha' variety were named RB/ZO/C/Hexane, RB/ZO/C/EtOAc, RB/ZO/C/MeOH and RB/ZO/S/Hexane, RB/ZO/S/EtOAc, RB/ZO/S/MeOH, respectively)

500 mg/L concentration levels. However, the shoot elongation with methanolic extracts was significantly lower at 1000 mg/L compared to the 500 mg/L concentration level.

Moreover, concerning the variation of shoot elongation between solvents, a significant difference was not observed between the EtOAc and n-hexane extracts at any of the concentrations tested. The methanolic extracts showed no difference with EtOAc and n-hexane extracts at 250 mg/L and 125 mg/L concentration levels in terms of shoot growth of *Lactuca sativa*. Further, a significant difference was not observed in root and shoot elongation compared to the control based on the variety factor (Fig. 3). The results of this study determined that methanol is more effective than n-hexane and EtOAc solvents in extracting phytotoxic compounds from ginger leaves. Since biologically active compounds occur in minimal concentrations, it is crucial to select a suitable solvent. Solvents with different degrees of polarity could be used for the extraction process. However, solvents

with high polarities provide higher extraction yields, which was also proved in our study [37]. Further, based on the outcomes of the phytotoxicity assays conducted in this study, it was proven that the highly potent phytotoxic chemicals in ginger leaves are polar. Additionally, the results support previous research findings [38], claiming that lettuce seeds are highly responsive to inhibitory and stimulatory chemical compounds.

The mean ± standard deviation of two independent experiments from three replications using ten seedlings each ($n = 30$) are shown. Means denoted by distinct letters are significantly different at ($p < 0.05$), (One-way ANOVA, post hoc by Tukey's mean separation).

Further, the results showed that root elongation inhibition was higher than shoot elongation inhibition at each level of the tested concentrations (1000 mg/L, 500 mg/L, 250 mg/L, and 125 mg/L) (Figure S1). Similar results have been documented in previous studies on the phytotoxicity of plant extracts [13, 39]. The reason for this phenomenon is the higher permeability of the roots compared to shoots in absorbing compounds. Roots are the first to emerge during germination, and as a result, roots are in direct contact with the extracts at their peak concentrations [40]. Moreover, the higher metabolic rates of roots make them more susceptible to environmental stresses such as phytotoxic chemicals [13]. Table 3 shows the percentage elongation inhibitions of roots and shoots of *Lactuca sativa* seeds and the IC_{50} (extract concentration corresponding to the 50% root/shoot elongation inhibition, calculated from the graph in Figure S1) results of the crude extract (RB/ZO) of ginger leaf waste.

The results of the phytotoxicity assay for the crude extract of ginger leaf indicated that it contains phytotoxic chemicals, making it a potential bioherbicide for agricultural use through compound isolation. The study also showed that methanol is the most suitable solvent for extracting highly potent phytotoxic chemicals from the ginger leaf. The variety factor between the two varieties, '*Sidhdha*' and 'Chinese,' did not significantly impact the results. Therefore, further extraction and bioassay-guided fractionation were performed by combining both leaf varieties and using 100% methanol as the solvent.

Table 3 Percentage elongation inhibitions of root and shoot of *Lactuca sativa* at each concentration and IC_{50}

Elongation Inhibition	Shoot (%)					Root (%)				
Concentration	1000 mg/mL	500 mg/mL	250 mg/mL	125 mg/mL	IC_{50}	1000 mg/mL	500 mg/mL	250 mg/mL	125 mg/mL	IC_{50}
RB/ZO/C/n-Hexane	7.44	4.55	3.49	1.06	> 1000 mg/mL	17.14	10.77	8.44	2.72	> 1000 mg/mL
RB/ZO/S/n-Hexane	6.82	4.70	3.33	−0.76	> 1000 mg/mL	15.67	9.19	7.90	3.10	> 1000 mg/mL
RB/ZO/C/EtOAc	10.42	4.53	2.27	1.06	> 1000 mg/mL	20.62	11.22	8.35	4.96	> 1000 mg/mL
RB/ZO/S/EtOAc	10.15	4.39	3.18	1.21	> 1000 mg/mL	21.18	12.14	8.39	5.68	> 1000 mg/mL
RB/ZO/C/MeOH	51.07	31.80	10.40	7.80	< 1000 mg/mL	61.73	50.39	17.96	9.37	605 mg/mL
RB/ZO/S/MeOH	50.92	32.52	10.43	8.13	< 1000 mg/mL	62.07	51.04	17.84	8.79	615 mg/mL

Bioassay Guided Fractionation of Phytotoxic Compounds in Ginger Leaf Extract

Repeated fractionation of the methanolic crude extract of ginger leaves by a series of chromatographic phases over silica and Sephadex, followed by a phytotoxic assay and TLC, resulted in a single fraction with clearly distinguishable spots on TLC (named 'final fraction') with 100% elongation inhibition of roots and shoots of *Lactuca sativa* seeds at each level of concentration tested. Further, the separation

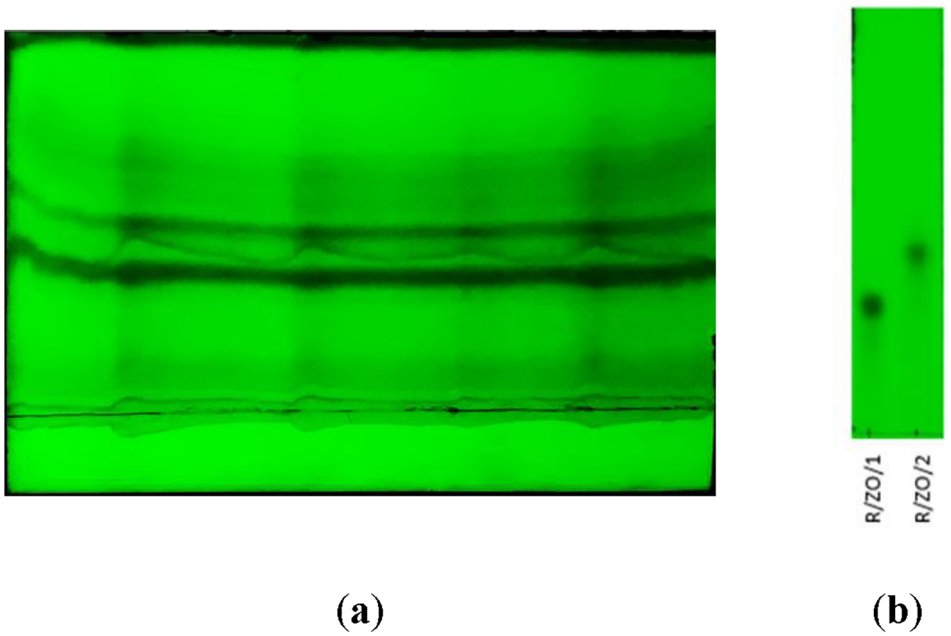


Fig. 4 **a** PTLC (10% MeOH/$CHCl_3$) profile of the final fraction, **b** TLC (10% MeOH/$CHCl_3$) profile of the recovered compounds

of the compounds in the final fraction through PTLC (10% MeOH/$CHCl_3$) recovered two compounds (R/ZO/1 (20 mg) and R/ZO/2 (15 mg)) (Fig. 4).

According to the phytotoxicity assay test results of the two recovered compounds (Figure S2)., R/ZO/1 and R/ZO/2, the root and shoot elongation inhibition of the two compounds was not significantly different. Both compounds (R/ZO/1 and R/ZO/2) significantly reduced the germination of *L. sativa* seeds at each level of concentration tested compared to the control. The calculated percentage inhibition at each concentration tested was 100%, proving that R/ZO/1 and R/ZO/2 were potent inhibitors of *L. sativa* root and shoot elongation even at low concentrations (125 mg/L) (Table 4).

Phytotoxins are naturally produced chemical compounds in plants resulting from secondary metabolic pathways. They are also referred to as plant toxins, allelochemicals, and phytochemicals. Phytotoxins can be synthesized in various parts of the plant, such as leaves, roots, shoots, and flowers, and they exhibit bioactivities against plant pests like weeds, pathogens, and insects [41]. However, this study focused on their efficacy against weed growth. Accordingly, based on the in vitro phytotoxicity assay results, the germination percentage of L. *sativa* seeds was significantly reduced ($p \leq 0.05$) in response to almost all extract concentrations of the recovered compounds compared to the control.

Phytochemicals can inhibit seed germination by interfering with the oxidative pentose phosphate and mitochondrial respiration pathways. Furthermore, the synergistic effects of various phenolic compounds in the plant extract may increase phytotoxicity [42]. As reported by previous research studies, the growth inhibition by extracts varies with the target plant species owing to differences in absorption mechanisms, translocation, and site of action of phytochemicals in various plant species. Seed germination also depends on seed parameters like size, shape, seed coat permeability, and structure [43]. Additionally, the susceptibility of a plant species to phytotoxicity when tested in a laboratory depends on

Table 4 Phytotoxicity assay test results of recovered compounds R/ZO/1 and R/ZO/2

Compound	Concentration (mg/L)	Elongation Inhibition %		Elongation (Mean Value)	
		Shoot	Root	Shoot	Root
R/ZO/1	125	100	100	0.00 *	0.00 *
	250	100	100	0.00 *	0.00 *
	500	100	100	0.00 *	0.00 *
	1000	100	100	0.00 *	0.00 *
R/ZO/2	125	100	100	0.00 *	0.00 *
	250	100	100	0.00 *	0.00 *
	500	100	100	0.00 *	0.00 *
	1000	100	100	0.00 *	0.00 *
Control (R/ZO/1)				1.04 ± 0.56	1.11 ± 0.65
Control (R/ZO/2)				1.12 ± 0.52	1.21 ± 0.62

The mean ± standard deviation from three replications using ten seedlings each (n = 30) is indicated. The asterisk indicates a significant difference between the control and treatment groups. * $p < 0.05$ (One-way ANOVA conducted separately for each concentration level, post hoc by Tukey's test)

the biochemical and physiological characteristics associated with that particular species [13].

This study selected the species *Lactuca sativa,* typically employed in phytotoxicity assays, due to its benefits over other species, including rapid germination, uniform early development, and high susceptibility to toxic substances [44]. According to the qualitative phytochemical screening tests (Table 2), methanolic extracts of ginger leaves were positive for all the phytochemical classes tested. Based on the previous literature, phenols, alkaloids, and terpenoids, including saponins and steroids extracted from several plant species, have shown strong inhibitory effects against the germination and root and shoot elongation of *Lactuca sativa* species [45]. Accordingly, Matsumoto et al. (2010) [46], studied the effect of the ethanol extract of the leaves of *Annona glabra* on the root and shoot elongation of *Lactuca sativa* and identified triterpenes, tannins, and flavonoids as the most responsible components for growth inhibition. Furthermore, dammarane-type triterpene 11-α-acetylbrachycarpone- 22(23)-ene, extracted from *Cleome arabica* L, was identified as being toxic for the seedling growth of *Lactuca sativa* [35]. Similarly, Boonmee et al. (2018) [20], isolated methyl gallate, a methyl ester of gallic acid, from leaf and stem extracts of *Caesalpinia mimosoides* Lamk, which was found to exert high inhibitory effects on *Lactuca sativa* seed germination.

The phytotoxic mechanism of different phytochemicals present in ginger, specifically, the terpenes and terpenoids, can be related to inhibiting plant growth by impairing the biosynthesis pathway of gibberellin, altering the mitotic process, and causing anatomical and physiological changes in plant seedlings. These mechanisms result in an accumulation of lipid globules in the cell cytoplasm, a reduction in membrane permeability and respiration, and possibly, inhibition of DNA and RNA synthesis [47, 48]. According to a study by Chen et al. (2022) [49], the toxicity mechanism of alkaloids involves altering enzyme activity, which affects the division of plant cells and DNA synthesis, while saponin-induced phytotoxicity results from its interaction with membrane cholesterol, destabilizing the membrane [50]. Further, many polyphenols have been identified among the phytotoxins. Tannins, anthraquinone, and flavonoids, also identified in ginger leaf extract through qualitative phytochemical tests, have been proven to inhibit the seed germination and seedling growth of weed plants. Scientists have predicted that the allelopathy of potential polyphenolic compounds can be due to the degradation of enzymes and other bioactive compounds produced by weed plants, altered metabolic processes of plants due to the effect of phytotoxins, disruption of photosynthesis and respiration, suppression of protein synthesis or due to an unknown mechanism that has not yet been discovered[51–53]. Therefore, further studies are needed to study the specific mode of action of these phytotoxic compounds.

Their on-site efficacy is the main factor preventing bioherbicides from being widely used. Bioherbicides are essential for controlling weeds, increasing farmer profits, and providing food for a growing population, but using bioherbicides is more challenging than it might appear. Numerous parameters, including the concentration of phytotoxic compounds, plant growth stage, type of formulation, spray preparation, application technique, type of soil, and environmental factors, can affect the efficacy of biological herbicides [34]. Further, when considering the ecological implications of ginger extracts, previous literature has stated that stem, rhizome, and leaf extracts of ginger are heterotoxic. Hence, it may inhibit non-target species during field applications [54]. Therefore, extensive field trials, target application, and dosage optimization are crucial when formulating bioherbicides using recovered compounds. However, it is notable that the ecological implications under natural environmental conditions are challenging to study through laboratory experiments [55].

In the present study, several chromatography techniques, including silica gel column chromatography and size exclusion chromatography (Sephadex LH-20), were used to extract phytotoxic compounds from crude extracts of Z. *officinale* leaf waste. Accordingly, two inhibitory compounds (R/ZO/1 and R/ZO/2) were identified through fractionation guided by bioassay.

^{1}H NMR, LC–MS, and FTIR Spectral Analysis of Recovered Compounds

The use of ^{1}H NMR for metabolic fingerprinting analysis to determine authenticity has gained significant interest lately because of its excellent reproducibility, ability to produce impartial structural information, and capacity to detect fraudulent substances in a sample [56]. Accordingly, ^{1}H NMR spectral analysis of the two recovered compounds, R/ZO/1 and R/ZO/2, proved that the two compounds are polar organic compounds (Fig. 5). In TLC analysis, these two compounds moved with 10% MeOH: $CHCl_3$ as the eluent, indicating the compounds to be polar.

The most abundant active constituents of ginger rhizomes are phenolic and terpene compounds. The main phenolic substances in ginger are gingerols, paradols, and shogaols. Gingerols, including 10-gingerol, 8-gingerol, and 6-gingerol, are abundant polyphenols in fresh ginger. The terpenes are monoterpenes, sesquiterpenes, and sesquiterpene alcohols [57, 58]. Further, prior research studies have reported that even though ginger rhizomes are high in phenolic content, mature ginger leaves contain lower amounts of phenols than rhizomes [59]. Since mature ginger leaf waste was used in this study, it may have lower phenolic levels.

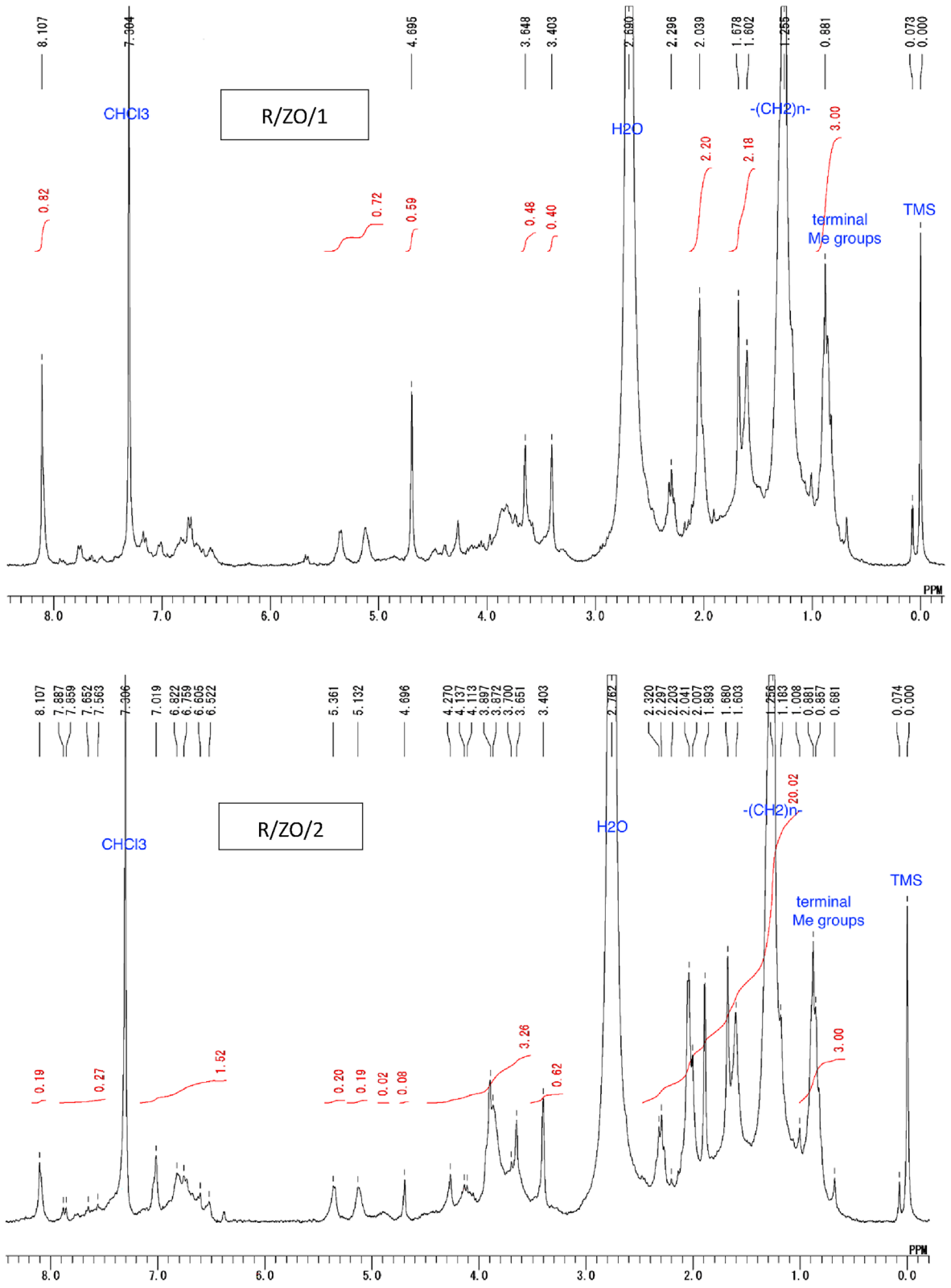


Fig. 5 ^{1}H NMR (CDCl3-CDOD) of R/ZO/1 and R/ZO/2

Moreover, previous studies on the Alpinia species of ginger leaves found that this type contains 6% alkaloids, 6.7% flavonoids, 0.8% saponins, and 0.4% tannins [12]. Further, alkaloids and terpenes are the most potent classes of phytotoxins in plant extracts [60]. Terpenes are abundant in ginger with high phytotoxicity and enzyme inhibition potential [61, 62]. Moreover, it has been found that enzymes such as proteases, lipases, and α-amylases play a crucial role in seed germination. Previous studies have proven that phytotoxins inhibit the enzyme activity of plants [63]. Moreover, the literature states that *Lactuca sativa* germination correlates directly with the activity of the α-amylase enzyme [64]. To further support the argument, it has been recognized that terpenes exhibited potent inhibition of α-amylase enzyme activity [65]. Further, studies have discovered that the sesquiterpene β-selinene (edema-4 (14),11-diene) is found at detectable levels in the ginger rhizome but not in ginger leaf. The (E)-caryophyllene is the most prevalent sesquiterpene in ginger leaves, and studies suggest that the amounts of α-humulene in ginger leaves are low [66]. Terpenes and terpenoids (modified family of terpenes) comprise various compounds with different polarities. The primary factors determining polarity are the polar functional groups such as aryl and acyl, the structure (non-polar, linear, or cyclized hydrocarbons), and the addition of less polar groups (hydroxyl or methyl) from its isoprene-based skeleton. Methanol can extract the polar compounds of terpenes, such as terpenoid glycosides, from crude extracts of plant species [67]. Further, it has been proven that polar terpenes have high levels of phytotoxicity against *Lactuca sativa* seed germination, while less polar terpenes do not play a crucial role [68]. Moreover, it has been found that terpenoids are highly soluble in $CHCl_3$, which was used in this study (10% MeOH: $CHCl_3$) [67]. Furthermore, it has been documented that in ^{1}H NMR spectra, the peaks in the regions 0.5–3 ppm are mainly terpenoids (modified triterpenoids with the tetracyclic ring structure of lanosterol) [69].

In the LC–MS (Fig. 6) analysis of R/ZO/1 and R/ZO/2, peaks were found at m/z 954.46 and m/z 166.99, respectively. These peaks were observed at retention times of 42.64 min and 32.93 min, respectively. Based on the comprehensive review of 1H-NMR, LC–MS, and FTIR results (Fig. 5), it was predicted that R/ZO/1 is composed of saponins. Saponins are a group of high molecular weight phytochemicals that consist of aglycone linked to sugar moieties. Saponins are further categorized into triterpenoids and steroidal saponins [70]. This identification has been supported by (Mroczek et al. 2012) [71], who demonstrated that saponins with an m/z value of 954 could be separated between 40 and 55 min retention time.

It has been reported that saponins exhibit significant phytotoxicity effects on several test plant species. Pérez et al. (2015) [72], identified three triterpenoid saponins that showed growth inhibitory effects against lettuce seedling growth from the aerial parts of the *Trifolium argutum* Sol. plant species. The researchers also noted that the phytotoxicity of triterpenoid saponins can be significantly increased with minor changes in aglycone or a glycoside chain structure.

Similarly, based on the comprehensive overview of ^{1}H NMR, LC–MS, and FTIR results, it was predicted that R/ZO/2 is an auto-oxidized fatty acid derivative. According to the LC–MS results, a significant peak was observed between 30 and 35 min retention time with m/z 166.99. Furthermore, Karlova et al. (2022) [73], identified an unsaturated fatty acid anion called geranate ($C_{10}H_{15}O_2$), a conjugate base of geranic acid (belonging to the class of terpenoids) with m/z 167.10, which could be closely related to the compound that was recovered as R/ZO/2. Moreover, previous research has proven the presence of geraniol and its derivative components in Z. *officinale* plant parts and the potency of geraniol-related compounds to integrate into bioherbicidal production due to their proven phytotoxic activity [74, 75], which further supports our prediction on R/ZO/2.

Accordingly, based on the literature, chemical shifts of ^{1}H NMR spectral peaks, and LC–MS analysis, the closely related structures suggested for the two recovered compounds, R/ZO/1 and R/ZO/2, are given in Table 5.

The FTIR analysis of the two recovered compounds (Fig. 7) showed peaks at 3263–3483 cm^{-1}, 2250–2500 cm^{-1}, 1700–1750 cm^{-1}, and 1640–1655 cm^{-1} in R/ZO/1 and peaks at 3466–3535 cm^{-1}, 2250–2500 cm^{-1}, 1700–1750 cm^{-1}, 1330–1420 cm^{-1}, and 1645–1650 cm^{-1}in R/ZO/2. The functional groups corresponding to each peak are presented in Table 6. The FTIR peaks prove the functional groups in modified terpenoids and oxidized fatty acid derivatives.

For the exact identification of the phytotoxic substances, further purification and in-depth analysis are needed. Characterization of compounds R/ZO/1 and R/ZO/2 suggests they are a terpenoid saponin and a monoterpene, respectively. The phytotoxic effects of these compounds can largely arise from their disruption of microtubule dynamics and compromise of membrane integrity. At the cellular level, these phytotoxins induce lipid peroxidation and alter specific enzymatic activities, while rapidly causing depolarization of root cell membranes. This results in increased membrane permeability, which impairs the plant's ability to absorb nutrients. Given the critical role of the root system in connecting the plant to its environment and facilitating water and nutrient uptake, any impairment in root development can adversely affect the plant's physiological status, thereby stunting plant growth. This mechanism could be efficiently utilized in weed management. The most pronounced allelopathic suppression is likely to occur when the peak concentrations of phytotoxins coincide with the

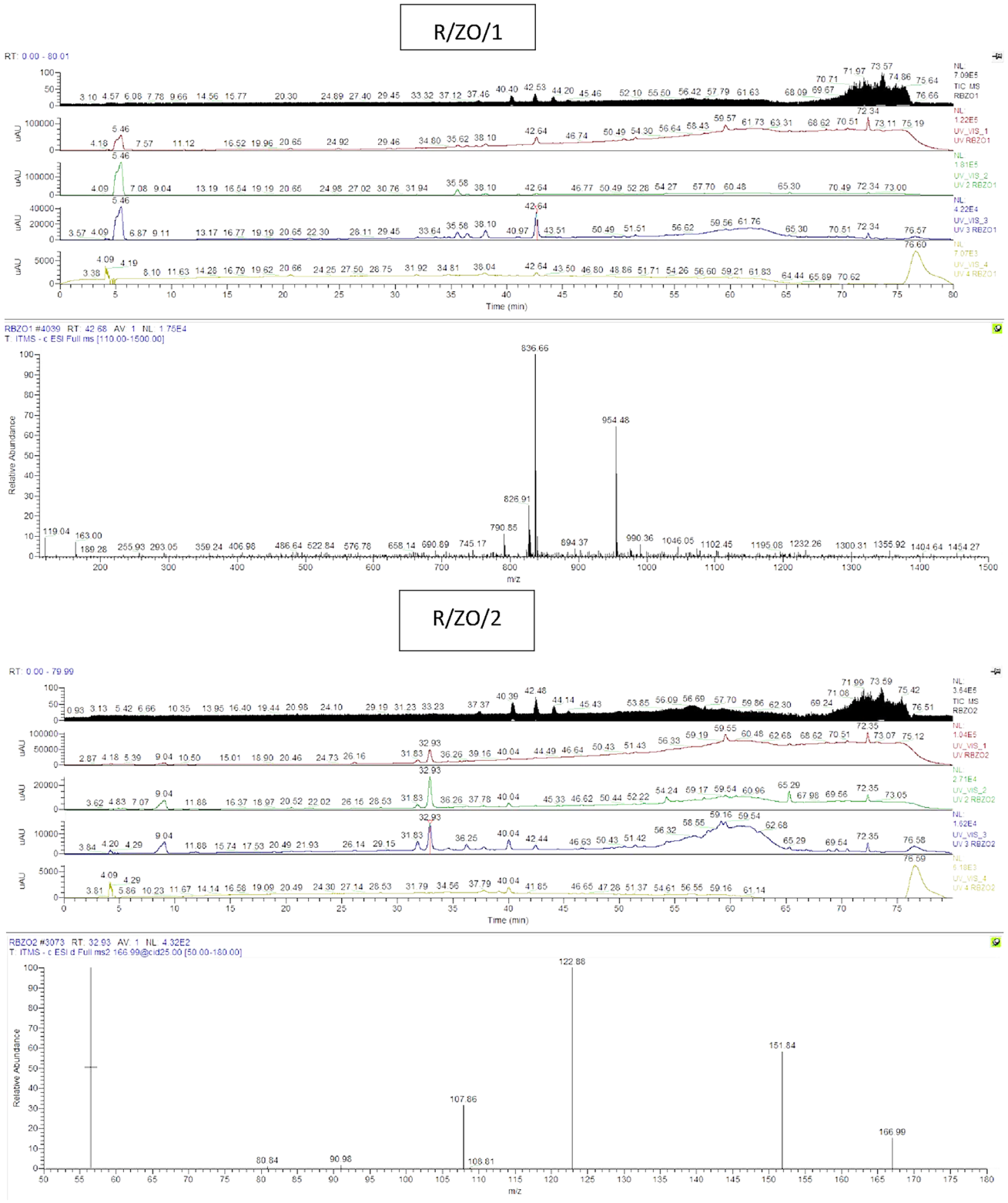
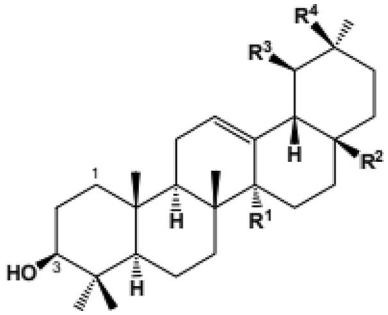
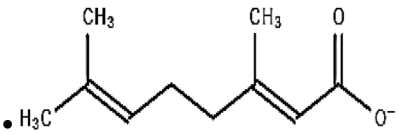


Fig. 6 LC–MS of R/ZO/1 and R/ZO/2

Table 5 Possible compounds to be represented in recovered compounds R/ZO/1 and R/ZO/2 based on the LC–MS and H-NMR spectral data

Recovered Compound	Predicted Phytochemical Class and Closely Related Structures Identified Through Spectroscopic and Literature-Supported Data	H-NMR Chemical Shifts of the Recovered Compound (PPM)	m/z [M-H] − Value and Molecular Weight	References
R/ZO/1	• Triterpenoid saponin  • Generalized triterpenoid saponin structure predicted for R/ZO/1	0.88 1.255 1.602 1.678 2.039 2.296 3.403 3.648 4.696 5.1 5.4	*m/z* = 954.44	[76, 77]
R/ZO/2	 Monoterpene • An unsaturated fatty acid anion called geranate ($C_{10}H_{15}O_2$), a conjugate base of geranic acid	1.60–1.68 (terminal CH_3) 2.007 (CH_3) 2.041 (-CH-) 5.132(-C = CH-) 5.36(-CH-COO)	*m/z* = 166.99 *m/z* of geranate = 167.10	[78]

early developmental stages of the target plants. Hence, the methanolic extracts from ginger leaves identified through this study may represent potent regulators of weed growth, offering promising prospects for sustainable weed management in agricultural practices.

In this study, the active compounds were only fractionated using *Lactuca sativa* as the test plant species. However, it would be beneficial to conduct future studies to investigate the efficacy of these compounds on various other weed species and to evaluate their effectiveness in field conditions. This expansion would enable a more comprehensive understanding of the potential applications of these active compounds. The low yield of recovered active compounds was identified as a significant limitation of this study, and to conduct further trials and purify recovered compounds, the extraction processes should be optimized with novel technologies.

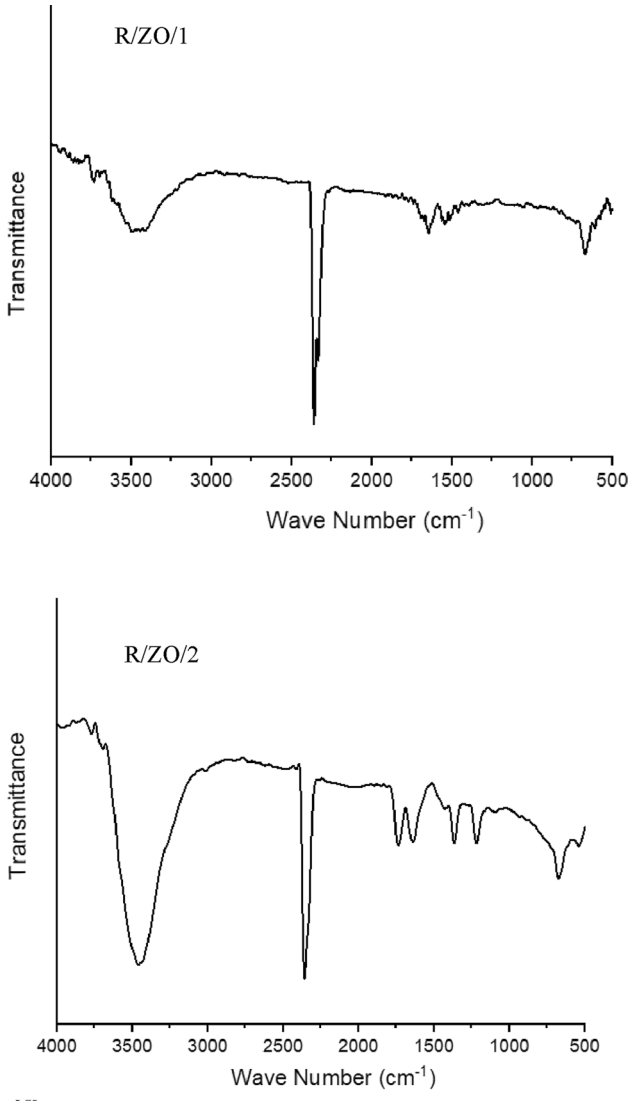


Fig. 7 FTIR spectra of R/ZO/1 and R/ZO/2

Table 6 Functional groups correspond to FTIR spectral peaks of R/ZO/1 and R/ZO/2

Wavenumber (cm^{-1}) (R/ZO/1)	Wavenumber (cm^{-1}) (R/ZO/2)	Functional groups
3263–3483	3466–3535	OH stretch /C-H stretching vibration of methyl groups
2250–2500	2250–2500	A peak due to CO_2
1700–1750	1700–1750	C = O
–	1330–1420	O–H bending of carboxylic acid
1640–1655	1645–1650	C = C stretch/COO^-

Conclusions

Ginger leaf waste is a valuable source of bioactive chemicals with phytotoxic effects. Methanol proves to be the most effective solvent for extracting these compounds from *Zingiber officinale* leaves. Phytotoxicity assays show that ginger leaf extracts inhibit root and shoot growth in *Lactuca sativa*. Notably, compounds R/ZO/1 and R/ZO/2 demonstrated 100% growth inhibition and are identified as polar organic compounds, including a triterpenoid saponin and an unsaturated fatty acid anion related to geranic acid. Further purification and analysis are required for confirmation, indicating the potential of ginger leaf waste as a bioherbicide.

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Author Contributions B.G.R. Rangadharee Bandara: Data curation; Writing—original draft; Investigation; Lalith Jayasinghe: Supervision;Validation; Writing—review & editing; Mojtaba Koosha: Project administration; Writing—review & editing; Xiaodeng Yang: Writing—review & editing; Resources; Tianduo Li: Supervision; Methodology; Writing—review & editing; Hiroshi Araya: Resources; Formal analysis; Yoshinori Fujimoto: Data curation; Validation; All authors contributed to the study conception and design. All authors read and approved the final manuscript.

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Data Availability The datasets generated or analyzed during this research are not publicly available as the sample preparation and characterizations were performed in different laboratories and different countries but will be made available on a reasonable request from the corresponding author(s).

Declarations

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

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