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CHEMICAL ECOLOGY OF ALFALFA: LC-HRMS IDENTIFICATION OF ALLELOCHEMICALS AND THEIR INFLUENCE ON EARLY GROWTH OF INDIAN MUSTARD

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Abstract: Allelopathy plays an important role in plant-plant interactions through the release of secondary metabolites that can influence the germination and growth of neighbouring species. The present study was conducted to evaluate the allelopathic effects of aqueous whole-plant extracts of *Medicago sativa* on the germination and early seedling growth of *Brassica juncea*. Seeds of *B. juncea* were exposed to different concentrations (10, 20, 50, 70 and 100%) of the extract and germination percentage, root length, shoot length, and biomass accumulation were assessed after five days. Germination percentage and biomass accumulation showed a gradual decline with increasing extract concentrations, whereas root and shoot elongation exhibited a stimulatory response. The highest extract concentration resulted in the greatest reduction in germination and biomass accumulation. To explore the possible chemical basis of these effects, the extract was subjected to LC–HRMS analysis. The phytochemical profiling revealed the presence of several metabolites belonging to phenolic acids, flavonoids, anthraquinones, phenolic amides, diterpenoid phenolics and lignan-type compounds. Major constituents included sinapic acid, quinizarin, isopongaflavone, pongaflavone, and triptophenolide. The findings suggest that *M. sativa* possesses measurable allelopathic activity and contains diverse secondary metabolites that may contribute to plant-plant interactions.

Keywords: Allelopathy, *Brassica juncea*, Flavonoids, LC–HRMS, *Medicago sativa,* Phenolic compounds.

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INTRODUCTION

Allelopathy is a biological phenomenon in which plants influence the growth, survival, germination, and development of neighbouring organisms through the release of biologically functional secondary metabolites known as allelochemicals (Inderjit and Duke, 2003; Singh, 2019). These compounds are released into the environment through various pathways, including root exudation, volatilization, leaching from aerial plant parts, and decomposition of plant residues (Rice, 1984; Einhellig, 1995; Singh, 2021). Allelopathic interactions play a significant role in shaping plant communities and regulating ecological processes in both natural and agricultural ecosystems (Singh, 2021). The effects of allelo-chemicals may be either inhibitory or stimulatory depending on their concentration, environmental conditions, and the sensitivity of the target species (Einhellig, 2002).

In recent decades, allelopathy has attracted considerable attention due to its potential applications in maintaining sustainable agriculture, integrated weed management, and the reduction of synthetic agrochemical inputs (Weston and Duke, 2003; Jabran *et al.,* 2015). Understanding allelopathic interactions can contribute to the development of environmentally friendly crop production systems and improve the efficiency of crop rotations and intercropping practices.

Medicago sativa L. (alfalfa or lucerne) is one of the most widely cultivated perennial forage legumes in the world owing to its high nutritional value, adaptability, and nitrogen-fixing ability (Radović *et al.,* 2009). In addition to its agronomic importance, alfalfa synthesizes a wide range of secondary metabolites, including phenolic acids, flavonoids, saponins, coumarins, and other bioactive compounds that have been reported to possess allelopathic activity (Chon, 2004; Scavo *et al.,* 2018). These compounds can influence seed germination, root elongation, nutrient uptake, and physiological processes of neighbouring plants. *Brassica juncea* (L.) Czern. & Coss., commonly known as Indian mustard, is an economically important oilseed crop extensively cultivated in Asia, particularly in India. The crop is valued for its edible oil, medicinal properties, and industrial applications. Since mustard is frequently grown in cropping systems where forage legumes or their residues may be present, understanding potential allelopathic interactions between alfalfa and mustard is essential for optimizing crop productivity and management practices.

A number of studies have demonstrated that allelochemicals released from alfalfa residues and root exudates may affect the germination and early growth of several crop species (Chon, 2004; Scavo *et al.,* 2018). Such effects may include inhibition of seed germination, reduction in root and shoot growth, alteration of chlorophyll synthesis, modification of enzymatic activities, and disruption of nutrient acquisition. Conversely, low concentrations of certain allelochemicals may exert stimulatory effects, a phenomenon commonly referred to as hormesis (Belz *et al.,* 2011). Therefore, investigating the response of *Brassica juncea* to allelochemicals produced by *Medicago sativa* is important for understanding plant–plant interactions and their implications for agroecosystem sustainability.

The present study aimed to evaluate the allelopathic potential of *Medicago sativa* against *Brassica juncea* by examining its effects on seed germination and early seedling growth. Furthermore, the study sought to quantify the inhibitory or stimulatory responses induced by alfalfa-derived allelochemicals and to profile the bioactive metabolites present in *M. sativa* through LC–HRMS-based phytochemical analysis.

MATERIALS AND METHODS

Plant material collection

Whole plants of *Medicago sativa* L. were collected from healthy populations growing in agricultural fields of Balrampur, Uttar Pradesh, India. The collected plant material was washed thoroughly with tap water followed by distilled water to remove soil particles and debris. The plant samples were shade-dried at room temperature for 10-15 days and subsequently ground into a fine powder using an electric grinder.

Preparation of aqueous extract

The dried whole-plant powder of *M. sativa* was used for the preparation of aqueous extracts. One hundred gram of powdered material were soaked in 1 L of distilled water for 24 h at room temperature with intermittent shaking. The extract was filtered through muslin cloth followed by Whatman No. 1 filter paper. The filtrate obtained was considered as 100% stock extract. Different concentrations (10, 20, 50, 70 and 100%) were prepared by diluting the stock extract with distilled water.

Seed material and germination bioassay

Certified seeds of *Brassica juncea* (L.) were obtained from a local agricultural seed supplier. Uniform and healthy seeds were selected for the experiment. The seeds were surface sterilized with 1% sodium hypochlorite solution for 2 min and rinsed thoroughly with sterile distilled water. The germination bioassay was conducted in sterile Petri dishes (90 mm diameter) lined with Whatman No. 1 filter paper. Ten seeds were placed in each Petri dish and treated with 5 mL of different concentrations (10, 20, 50, 70 and 100%) of *M. sativa* aqueous extract. Distilled water served as the control treatment. Each treatment was maintained in triplicate. The Petri dishes were incubated under laboratory conditions at 25 ± 2°C with a 12 h light/12 h dark photoperiod.

Assessment of germination and seedling growth

Seed germination was recorded after two days of incubation. A seed was considered germinated when the radicle protruded at least 2 mm. Germination percentage was calculated using the following formula:

Germination Percentage (%) = (Number of germinated seeds / Total number of seeds) × 100

After five days, seedling growth parameters including root length, shoot length, and fresh biomass accumulation were measured. Root and shoot lengths were recorded using a digital ruler, while fresh biomass was determined using an analytical balance.

LC–HRMS analysis

The aqueous extract of *M. sativa* was subjected to liquid chromatography–high resolution mass

spectrometry (LC–HRMS) analysis for metabolite profiling. Chromatographic separation was performed using an Accucore C18 column (150 × 4.6 mm, 2.6 µm particle size). The chromatographic run generated a base peak chromatogram (BPC), and major peaks were selected for compound identification. Mass spectral data were acquired in both positive and negative electrospray ionization modes. Identification of compound was effectively carried out by comparing accurate mass measurements, molecular formulae, fragmentation patterns, and available spectral databases. The identified metabolites were classified according to their chemical classes and evaluated for their reported allelopathic significance based on published literature.

Statistical analysis

The experiment was arranged in a completely randomized design (CRD). All data were expressed as mean ± standard error (SE) of five replicates. Statistical differences among treatments were analyzed using one-way analysis of variance (ANOVA), and treatment means were compared at $P \leq 0.05$. Graphical representation of the data was prepared using Microsoft Excel.

RESULTS

The germination percentage of *B. juncea* seeds gradually declined with increasing concentrations of *Medicago sativa* extract (Fig. 1D). The highest germination (approximately 97%) was observed in the control treatment, whereas the lowest germination (about 76%) was recorded at 100% extract concentration. This reduction shows an inhibitory influence of alfalfa-derived allelochemicals on seed germination. Root length increased progressively with increasing extract concentration (Fig. 1A). The shortest roots were observed at 10% extract concentration (approximately 0.50 cm), while the maximum root length (about 1.70 cm) was recorded at 100% extract concentration. Similarly, shoot length showed a significant increase with increasing extract concentration (Fig. 1B), reaching its highest value at 100% extract concentration. These results suggest that although germination was suppressed, the seedlings that successfully germinated exhibited enhanced elongation growth. In contrast, seedling biomass decreased markedly in response to the extract treatments (Fig. 1C). Fresh biomass declined from approximately 0.20 g in the control to nearly 0.04 g at 100% extract concentration. The reduction in biomass indicates that the extract adversely affected seedling vigor and dry matter accumulation despite promoting elongation growth.

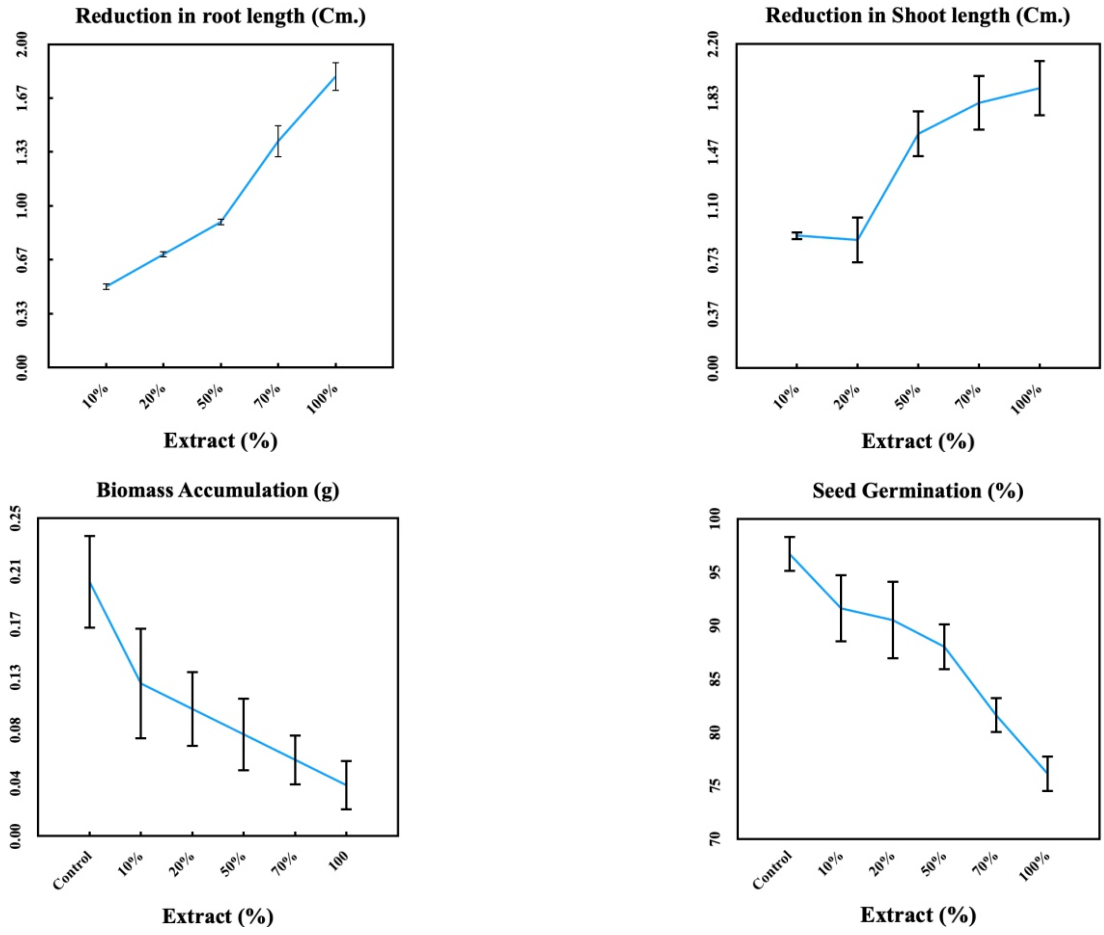

Fig. 1: The germination and growth assay of the *Brassica juncea* L. seedling plants grown under *Medicago sativa* whole plant aqueous extract after fifth day of germination. The data represents mean SE.

LC–HRMS analysis of the whole-plant aqueous extract of *Medicago sativa* revealed the presence of several secondary metabolites belonging to different chemical classes, including phenolic acids, flavonoids, anthraquinones, diterpenoid phenolics, phenolic ketones, phenolic amides, and lignan-type phenolics (Table 1; Fig. 2).

A total of nine major compounds were identified, *namely*:

1. sinapic acid
2. 1,4-dihydroxyanthraquinone (quinizarin),
3. isopongaflavone,
4. pongaflavone,
5. triptophenolide,
6. 3-(3,4-dihydroxyphenyl)-5,7-dihydroxy-6,8-bis(3-methylbut-2-enyl)chromen-4-one,
7. 1-[4-hydroxy-3-(3-methylbut-2-enyl)phenyl] ethanone,
8. (E)-N-[2-hydroxy-2-(4-hydroxyphenyl)ethyl]-3-(4-hydroxy-3-methoxyphenyl)prop-2-enamide,
9. 2,3-bis[(4-hydroxy-3-methoxyphenyl) methyl] butane-1,4-diol.

Among the detected compounds, isopongaflavone exhibited the highest peak area (~100,000), indicating its abundance in the extract, followed by pongaflavone (~40,000). Sinapic acid and quinizarin were also detected with appreciable peak areas (~10,000 each) (Fig. 2). Several other metabolites were identified qualitatively and recorded as detected. The identified compounds are widely recognized for their ecological functions in plant defence, phytotoxicity, and allelopathic interactions.

The LC–HRMS profile demonstrated that flavonoids constituted a major group of metabolites in *M. sativa*, followed by phenolic compounds and anthraquinone derivatives. The occurrence of these compounds suggests a chemically diverse allelopathic system capable of influencing neighbouring plant species through multiple physiological mechanisms.

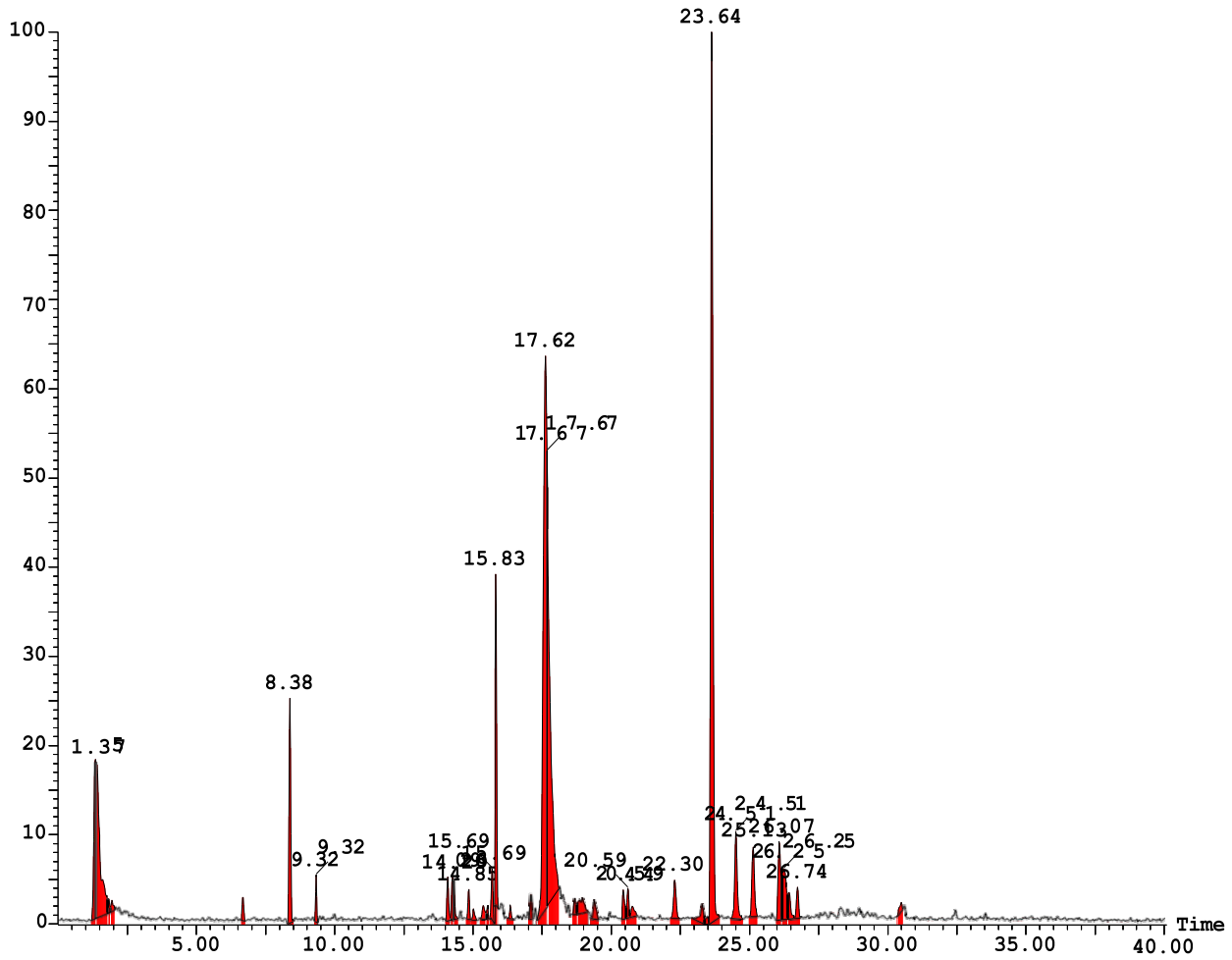

Fig. 2: Representative LC–MS base peak chromatogram (BPC) of the *Medicago sativa* whole-plant aqueous extract analyzed using an Accucore C18 column (150 × 4.6 mm, 2.6 μm). The major peaks that were subsequently subjected to LC–HRMS analysis for compound identification and characterization of potential allelochemicals.

Table 1: Allelochemicals were identified through LC-HRMS analysis in *Medicago sativa* L.

Compounds	Molecular formula	Chemical Class	Peak Area*	Allelopathic / Ecological Relevance	References
Sinapic Acid	$C_{11}H_{12}O_5$	Phenolic acid	~10,000	Regulates seed germination, affects ABA homeostasis, and is widely recognized as a phenolic allelochemical.	Bi *et al*., 2017
1,4-Dihydroxyanthraquinone (Quinizarin)	$C_{14}H_8O_4$	Anthraquinone	~10,000	Anthraquinones are important phytotoxic compounds involved in plant interference and allelopathy.	Li *et al.*, 2010
Isopongaflavone	$C_{21}H_{10}O_4$	Flavonoid	~100,000	Flavonoids may suppress root elongation, alter auxin signalling, and participate in plant–plant interactions.	Patil *et al*., 2024
Pongaflavone	$C_{21}H_{10}O_4$	Flavonoid	~40,000	Plant defence metabolite; flavonoid-type compounds are frequently reported as allelochemicals.	Patil *et al*., 2024
Triptophenolide	$C_2H_{24}O_4$	Diterpenoid phenolic compound	Detected	Terpenoid phenolics often exhibit phytotoxic and growth-inhibitory activity toward neighbouring plants.	Li *et al*., 2010
3-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-6,8-bis (3-methylbut-2-enyl) chromen-4-one	$C_2H_{24}O_6$	Prenylated flavonoid	Detected	Polyhydroxylated flavonoids are known to influence seed germination and root growth.	Patil *et al*., 2024
1-[4-Hydroxy-3-(3-methyl but-2-enyl)phenyl] ethanone	$C_{13}H_{10}O_2$	Phenolic ketone	Detected	Phenolic derivatives commonly function as allelochemicals released through litter decomposition and root exudation.	Li *et al*., 2010
(E)-N-[2-hydroxy-2-(4-hydroxyphenyl)ethyl]-3-(4-hydroxy-3-methoxyphenyl)prop-2-enamide	$C_{1}H_{1}NO_4$	Phenolic amide	Detected	Phenolic amides possess phytotoxic and defence-related activities associated with allelopathic interactions.	Li *et al*., 2010
2,3-bis[(4-hydroxy-3-methoxyphenyl)methyl] butane-1,4-diol	$C_2H_2O_6$	Lignan-type phenolic	Detected	Lignan and phenylpropanoid derivatives are recognized sources of allelopathic activity in several plant families.	Li *et al*., 2010

DISCUSSION

The present study demonstrated that aqueous extracts of *Medicago sativa* significantly influenced the germination and early seedling growth of *Brassica juncea*. Authors found that germination percentage and biomass accumulation decreased progressively with increasing extract concentrations, whereas root and shoot elongation exhibited a stimulatory response. These findings indicate that *M. sativa* possesses considerable allelopathic potential and that its phytotoxic effects are concentration-dependent. The reduction in germination percentage suggests that allelochemicals present in the extract interfered with physiological and biochemical processes essential for seed germination, including enzyme activation, water uptake, respiration, and hormonal regulation.

The LC–HRMS analysis revealed the presence of several bioactive metabolites, including sinapic acid, quinizarin, isopongaflavone, pongaflavone, triptophenolide, phenolic ketones, phenolic amides, and lignan-type compounds. These metabolites belong primarily to phenolic acids, flavonoids, anthraquinones, and terpenoid phenolics, which are well-known classes of allelochemicals. The inhibitory effect on germination observed in the present study may be attributed to the combined action of these compounds. Sinapic acid has been reported to regulate abscisic acid

metabolism and suppress seed germination, while anthraquinones such as quinizarin are known to induce oxidative stress and disrupt cellular metabolism (Li *et al.,* 2010). Similarly, phenolic compounds released from plant residues and root exudates have been shown to inhibit germination and seedling establishment in numerous crop species (Rice, 1984).

Interestingly, root and shoot length increased with increasing extract concentration. Such stimulatory responses have been reported previously and are often associated with hormesis, a phenomenon in which low to moderate concentrations of allelochemicals stimulate certain known growth parameters while simultaneously causing physiological stress. The high abundance of flavonoids, particularly isoponga-flavone and pongaflavone, detected through LC–HRMS analysis may be associated with altered auxin transport and cell elongation processes. Flavonoids are known to influence plant growth regulators and can promote elongation growth under specific conditions.

However, the simultaneous reduction in biomass indicates that the elongated seedlings were physiologically weaker and accumulated less structural material, suggesting that the observed elongation represents a stress response rather than enhanced growth. The reduction in biomass accumulation further supports the phytotoxic nature of the alfalfa extract. Phenolic acids, flavonoids, and terpenoid compounds can interfere with nutrient uptake, photosynthesis, protein synthesis, and energy metabolism, ultimately reducing biomass production. The presence of multiple classes of allelochemicals in *M. sativa* suggests that these compounds may act synergistically, producing stronger inhibitory effects than individual metabolites alone.

The results of the present study are in agreement with the study of Chon and Kim (2004), who reported that alfalfa extracts containing phenolic compounds significantly, inhibited the germination and growth of several crop species. Similarly, Xuan *et al*. (2005) concluded that phenolic acids and flavonoids are among the major allelochemicals responsible for growth suppression in agricultural systems. Li *et al.* (2010) also reported that phenolics, anthraquinones, and related secondary metabolites contribute significantly to plant interference and allelopathic interactions. However, the stimulatory effect on root and shoot elongation observed in the present investigation contrasts with some studies that reported growth inhibition by alfalfa extracts. Such differences may be attributed to species-specific sensitivity, extract concentration, environmental conditions, and variations in the composition and abundance of allelochemicals.

Overall, the growth assay and LC–HRMS results collectively demonstrate that *M. sativa* contains a diverse array of allelopathically active compounds capable of influencing seed germination and seedling development in *B. juncea*. The observed inhibition of germination and biomass, together with the altered seedling growth pattern, can be attributed to the combined effects of phenolic acids, flavonoids, anthraquinones, and other phytotoxic metabolites identified in the extract.

CONCLUSIONS

The present study demonstrated that aqueous whole-plant extracts of *Medicago sativa* influence the germination and early seedling growth of *Brassica juncea*. Increasing extract concentrations resulted in a gradual reduction in germination percentage and biomass accumulation, indicating an inhibitory effect on seedling establishment. In contrast, root and shoot elongation showed a stimulatory response, suggesting a differential effect of the extract on various growth parameters. LC–HRMS analysis revealed the presence of diverse secondary metabolites such as phenolic acids, flavonoids, anthraquinones, phenolic amides etc. which are known to participate in allelopathic interactions. The observed biological responses may be associated with the combined action of these metabolites. Overall, the findings suggest that M. sativa possesses allelopathic potential and contains bioactive compounds capable of influencing the growth and development of neighbouring plant species. Further investigations under greenhouse and field conditions are needed to confirm these effects and to understand the ecological and agricultural implications of alfalfa-derived allelochemicals.

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