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Allelopathic effects of weed *Neanotis montholonii* on seed germination and metabolism of mungbean and rice

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ABSTRACT

The higher concentrations of whole plant aqueous extracts of *Neanotis montholonii* proved most inhibitory to mungbean and rice. The highest concentration (10 %) of aqueous extract reduced the plumule and radicle length of both crops. Fifteen allelochemicals (Phenols, alkaloids, flavonoids, flavonols and glycerol) were identified in its extract, of these five were major allelochemicals [Linoelaidic acid, Glycidyl oleate, 18-Nonadecenoic acid, Palmatic acid and Glycidyl palmitate]. These compounds significantly inhibited the seed germination in mungbean (59.78 %) and rice (58.34 %) at 5 DAS. The inhibitory effects of allelochemicals on seeds germination followed the order: Linoelaidic acid > Glycidyl oleate > 18-Nonadecenoic acid > Palmatic acid > Glycidyl palmitate. The radicle and plumule growth at 11 DAS also followed the same trend. The metabolic changes showed that the aqueous extract reduced the protein, starch, carbohydrates, phenols, tannins, flavonoids and flavonols contents in both mungbean and rice crops and were concentration dependent. The allelopathic potential of *N. montholonii* was due to the presence of 15 inhibitory compounds (detected by GC-MS) present in its extract.

Keywords: Allelochemicals, allelopathic effects, aqueous extract, inhibition, metabolism, mungbean, *Neanotis montholonii*, *Oryza sativa*, plumule, radicle, rice, seed germination, seedling growth, *Vigna radiata*.

INTRODUCTION

In Maharashtra, India the *Neanotis montholonii* (Hook.F) W.H. Lewis syn. *Anotis montholonii* (family Rubiaceae) is major weed in field crops (Groundnut, maize, sorghum, pearl millet, rice, mungbean etc.). It reduces the growth and yield of these crops. Genus *Neanotis* has 33 species distributed in tropical and subtropical countries (India, China and Malaysia). Its 10-species are reported from Maharashtra state including the *N. montholonii* (2,38). Mungbean (*Vigna radiata* L.) is main legume crop in Asian cropping systems, as it is rich source of protein for human consumption (28). Rice (*Oryza sativa* L.) is the staple food in many regions of India.

Allelopathy refers to any direct or indirect, inhibitory or stimulatory effects of one plant on another through the production and release of secondary metabolites into the environment (36). The donor plants may affect the germination, growth and development of recipient plant species (42). The higher concentrations of allelochemicals usually inhibit the growth of recipient plants and soil microorganisms or both (25). In nature, many plants growing together interact with each other by inhibiting or stimulating growth and development of each other through allelopathic interactions (24,32). The different types of allelochemicals present in weeds, reduces the crop yield and quality (22,43). Weeding in crops increases the cost of cultivation and is great problem for the farmers (10,42). Hence, biological control of weeds through understanding the weed-weed and crop-weed interactions is important research area (2,27). The test weed *N. montholonii* grows

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luxuriantly in crops (rice, groundnuts, mungbean, grapes and tomato etc.) in Nashik district, Maharashtra. The field observations showed that this weed inhibited the crops growth and reduces the crop yields. However, there was no attempt to study its allelopathic influence on crops (2,37). This study aimed to (i). Identify the allelochemicals present in this weed and to (ii). Determine their phytotoxic effects on rice and mungbean.

MATERIAL AND METHODS

The *N. montholonii* plants were collected at flowering in September from the crop fields in Nashik (Maharashtra) (19°59’50.8344”N, 73°47’23.2908”E). The specimen voucher number is BSI/WRC/IDEN.CER./ 2016/800.

Aqueous extract preparation

The collected plants were brought to the laboratory, cleaned with distilled water and spread on filter paper for shade drying at room temperature for 48 h. The dry plant material was ground in Wiley Mill and passed through 2 mm sieve. From this 100 g powder was soaked in 1000 ml distilled water for 24 h at 25 ºC and the extract was filtered through Buchner funnel using Whatman filter paper no. 1. The filtered extract was stored in refrigerator in amber coloured bottle to avoid degradation by sunlight. Further desired concentrations (2.5, 5.0, 7.5 and 10 % concentration) of extract for the treatments were prepared by diluting the stock solution of extract with distilled water. T1 was prepared by taking 1.5 ml from stock solution and final volume 100 ml was made with distilled water. Similar procedure was followed to make concentrations of T2 (2.5 ml), T3 (5 ml), T4 (7.5 ml), T5 (10 ml). Distilled water treated plants were used as control. The germination (%) was calculated as under:

Germination % = Number of seeds that germinate/ Number of seeds on the petridish×100.

The root length and shoot length were measured using the scale at 2 days after sowing.

Laboratory Bioassay

The bioassay consisted of 11-Treatments [Control, T1 (2%), T2 (2%), T3 (2%), T4 (2%), T5 (2%), T6 (2%), T7 (10%), T8 (10%), T9 (20%), T10 (20%)] (Table 1). The autoclaved petri dishes, lined with one filter paper were used in bioassays. The seeds of Mungbean (*Vigna radiata* L.) variety ‘Vaibhav’ and rice (*Oryza sativa* L.) variety ‘Phule samrudhi’ were obtained from M.P. Agricultural University, Rahuri, Maharashtra (29). The test crops seeds were surface sterilized with 0.02 % $HgCl_2$ for few secs and washed thoroughly with distilled water. Twenty seeds of mungbean and rice were sown equidistant on filter paper in each petri dish (10 cm dia) and 5 ml aqueous extracts of different concentrations was added (as per treatment). The distilled water was used as control. The treatments were replicated thrice in randomized block design. The experiment was done at room temp 28 to 30 ºC. Five ml aqueous extracts were added to each petri plate daily. The germination (%), length of plumule and radicle were recorded at 3, 5, 7, 9 and 11 days after sowing. A similar method was used for bioassay of 5-major allelopathic compounds found in extract.

Field studies

Field experiments were done in our Botanical Garden, Department of Botany, during June-September 2018. Mungbean seedlings were grown in polythene nursery bags (35 cm x 40 cm x 30 cm), filled with 5.0 Kg soil. While rice seedling were grown on raised beds (1 m x 1 m). The sowing was done on16 June 2018. The treatments were replicated thrice in Randomized Block Design. The required agronomic practices were followed to grow these crops. Treatments were applied at 15 DAS for mungbean, and in rice 7 days after transplanting. The mungbean mature pods were harvested on 15 September 2018, while rice was harvested on 25 September 2018. The mature pods of mungbean and the mature ear heads of rice were harvested and threshed to get grain yield.

Table 1. Details of Experimental Treatments

Treatment	Treatments details
T1	1.5 ml stock solution diluted to 100 ml with distilled water
T2	1.5 ml stock solution diluted to 100 ml with distilled water
T3	1.5 ml stock solution diluted to 100 ml with distilled water
T4	1.5 ml stock solution diluted to 100 ml with distilled water
T5	1.5 ml stock solution diluted to 100 ml with distilled water
T6	1.5 ml stock solution diluted to 100 ml with distilled water
T7	15 ml stock solution diluted to 100 ml with distilled water
T8	15 ml stock solution diluted to 100 ml with distilled water
T9	20 ml stock solution diluted to 100 ml with distilled water
T10	20 ml stock solution diluted to 100 ml with distilled water

Biochemical parameters of recipient crops

Randomly selected leaf samples of field grown mungbean and rice were collected at 30, 60 and 90 DAS and used for the biochemical parameters: (i) Carbohydrates determined by Anthrone method (12), (ii) Starch, by Anthrone Reagent (12,35), (iii) Proteins by Lowry's method (23), (iv) Tannins by Ferric chloride method (31,35), (v) Phenols by Trease and Evans method (23), (vi) Flavonoids by Aluminium chloride colorimetric method (27) and (vii) Flavonols by $AlCl_3$ method (27).

GC-MS analysis

To identify the different bioactive compounds in the extracts of *N. montholonii* weed, GC-MS analysis was done with a Shimadzu TQ 8030 GC system. The HP-5 MS capillary column (30 m×0.25 mm; Film thickness- 25 μm; I.D- 0.2 mm) was used for the sample analysis. As a carrier gas helium was used at a flow rate of 1 ml/min. The temperature programme for GC-MS analysis was as follows: the initial temperature was 50 ºC and was heated for 5 min and then it was heated up to 240 ºC at the rate of 3 °C per min and was increased at a rate of 3 ºC per minute until 178 ºC. This temperature was maintained for 2 min, with a total time of analysis of 50 min. The identification of the compounds was carried out by using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrums of the unknown compounds were compared with the spectrum of the known compounds stored in the NIST library (16).

HPLC analysis

The major compounds detected in GCMS were further isolated to know their allelopathic impacts on crops. The adverse impacts of these weeds on associated crops are due to various bioactive allelocompounds in the weed. Hence to know such compounds, we did the HPLC analysis. 0.5 g dried power was added to 20 ml methanol in a conical flask, extracted by shaking on a rotary shaker for 12 h at 100 rpm, followed by ultra-sonication for 15 min at 60 °C, cooling to room temperature and centrifuging at 5000 rpm for 10 min. The supernatants were filtered using Whatman (grade 1) filter paper. The filtrates were concentrated under vacuum using a rotary evaporator at 30 °C. The residue was dissolved in 50 ml of 50 % v/v of n-hexane and ether and filtered using 0.22 µm cellulose acetate filters. The filtrates were placed in HPLC vials and stored at -20 °C till use. All the five standard compounds were purchased from Sigma-Aldrich Chemie, Steinheim, Germany with more than ≥ 99% purity.

HPLC analyses were done using the HPLC-1200 infinity series system (Agilent Technologies, Waldbronn, Germany), on a Symmetry C18 column (0.25 m x 4.6 mm x 5 pm containing silica gel with a pore size of 6 nm). Mobile phase: 2- Propanol R1, Hexane R (1:99 v/v), Flow rate: 1 ml/minute. Detection: Spectrophotometer at 210 nm, Injection: 50 µl and total running time 35 min. (6).

Statistical analysis

All data were statistically analyzed using ANOVA and Duncan's Multiple Range Test (DMRT). The data were analyzed by using (SYSTAT) SPSS Statistics software.

RESULTS AND DISCUSSION

Seed Germination

The inhibitory effects of aqueous extracts on seed germination and seedling growth of test crops were concentration dependent (Fig. 1). The treatments T_4 to T_7 delayed the seed germination. The inhibitory effect was more pronounced in T_8 to T_{10} treatments at 3 and 5 DAS respectively (Fig. 1). Similar was the finding of Demissie *et al.* (13) and Chopra *et al.* (11) in rice, soyabean and bean (*Phaseolus vulgaris* L.). The inhibition in seed germination may be due to imbibed allelochemicals present in extracts of *E. colona* L. and *C. iria* L. Similarly, Mawal *et al.* (26) also noted reduction in germination and radical growth of maize due to *Parthenium* leachates. Our results are in confirmation with above findings.

Seedlings growth

The extent of adverse effects of extract on crops depended on the concentration of allelochemicals in leachates (4, 28). In the present investigation, the aqueous extracts were inhibitory to the plumule and radicle length over the control at all concentrations and the reduction was concentration dependent in both test crops. Treatments T_1 to T_7 decreased the plumule and radicle length at 3, 5, 7 and 9 DAS respectively. While in treatments T_8 to T_{10} the seed germination was completely inhibited at 3 DAS onwards (Fig. 1).

Baber *et al*. (5), Wakjira (39) and Arzoo *et al.* (3) demonstrated the same trend. They revealed significant reduction in seed germination of various crops due to higher concentration of leaf extract and leachates of *Ageratum, Hyptis suaveolens* L. and *Parthenium* sp. Ghayal *et al.* (17) also reported inhibitory effects of higher concentrations of leachates / extract of *Cassia uniflora* Mill. weed on root and shoot length of radish and mustard. The negative influence of leachates of different weeds on seed germination and seedling growth of various crops had been documented by several workers like Wu *et al*. (40) and Afridi and Khan (1). It was due to the allelochemicals present in the respective leachates and extracts of donor plants. El-Khatib *et al*. (15) reported that retarded seedling elongation might be due to the direct interference of allelochemicals in cell division that alters the balance of different growth hormones. The difference in the activity of these extracts in suppressing the seedling growth may be due to variations in the type and concentration of allelochemicals present in extracts (4,39).

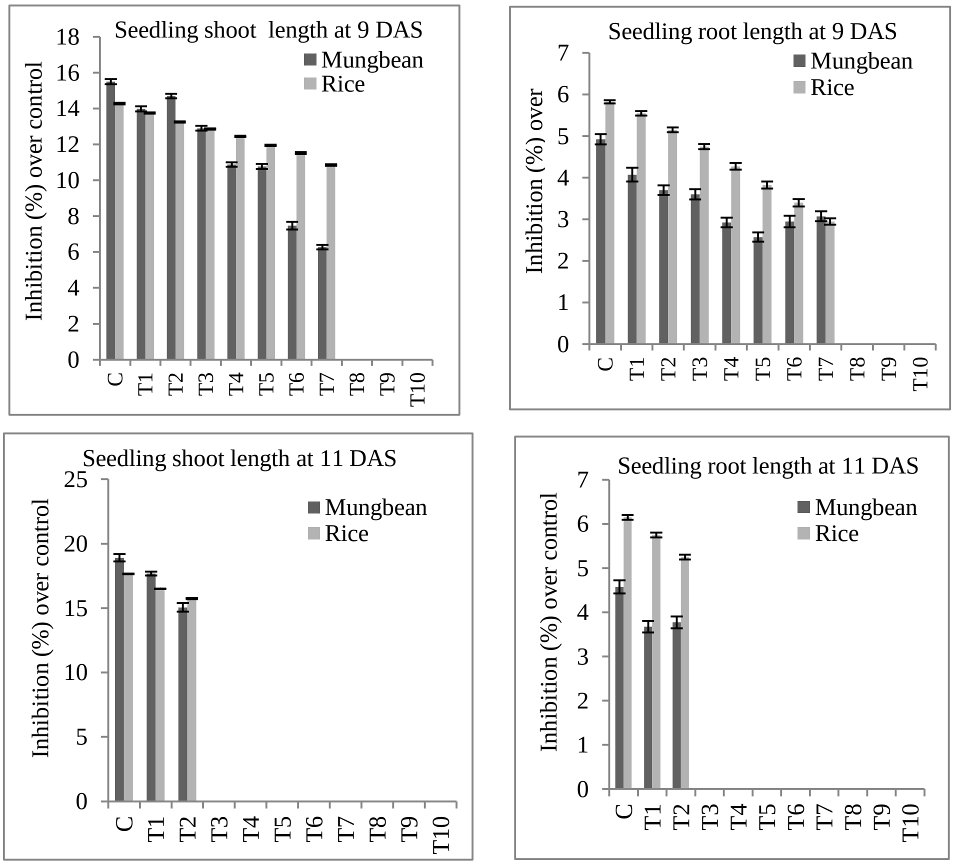


Figure 1. Inhibitory effects of *N. montholonii* extract concentration on seedling length of Mungbean and Rice

T1 (2 % : 1.5 ml stock solution diluted to 100 ml for 4 h), T2 (2 % : 1.5 ml stock solution diluted to 100 ml for 8 h), T3 (2 % : 1.5 ml stock solution diluted to 100 ml for 12 h), T4 (2 % : -1.5 ml stock solution diluted to 100 ml for 16 h), T5 (2 % : 1.5 ml stock solution diluted to 100 ml for 20 h), T6 (2 % : 1.5 ml stock solution diluted to 100 ml for 24h), T7 (10 % : 15 ml stock solution diluted to 100 ml for 12h), T8 (10 % : 15 ml stock solution diluted to 100 ml for 24 hrs), T9 (20 % : 20 ml stock solution diluted to 100 ml for 12 h), T10 (20 % : 20 ml stock solution diluted to 100 ml for 24 h)]

Inhibition of seed germination by major allelochemicals

The results of 5-bioassay of allelochemicals isolated from *N. montholonii* on seed germination indicated that seed germination (%) inhibition at 5 DAS in mungbean was: 65.34 % (Linoelaidic acid), 62.45 % (Glycidyl oleate), 49.67 % (18-Nonadecenoic acid), 47.56 % (Palmatic acid) and 45.45 % (Glycidyl palmitate). The seed germination (%) inhibition in rice was: 65.67 % (Linoelaidic acid), 63.45 % (Glycidyl oleate), 51.89 % (18-Nonadecenoic acid), 49.56 % (Palmatic acid) and 45.67 % (Glycidyl palmitate) (Fig. 2). The order of seed germination (%) followed the order: Glycidyl palmitate > Palmatic acid > 18-Nonadecenoic acid > Glycidyl oleate > Linoelaidic acid (Fig. 2, Table 2). The trend of radicle and plumule growth at 3, 7 and 11 DAS was similar (Fig. 2). The seed germination, radicle and plumule length were inhibited due to non-utilization of reserved food material in the seeds of test crops.

Table 2. Chemical composition of ethanol extracts of *N. montholonii* by GC-MS (Concentrations based on calibration curve of authentic compounds) and the Quantity of Major compounds

S. No.	Name of the compound	Formulae	RT	Area (%)*	Total amount (μg/g)
1	Tetradecanoic acid	$C_{14}H_{28}O_2$	16.55	0.16	-
2	(10Z)-10-Heptadecenoic acid	$C_{17}H_{32}O_2$	18.462	2.11	-
3	Palmatic acid	$C_{16}H_{32}O_2$	18.678	11.6	6.12 ± 0.1
4	Linoelaidic acid	$C_{18}H_{32}O_2$	20.364	27.72	17.11 ± 0.1
5	18-Nonadecenoic acid	$C_{19}H_{36}O_2$	20.395	14.96	8.15 ± 0.0
6	Stearic acid	$C_{18}H_{36}O_2$	20.538	3.27	-
7	Glycidyl palmitate	$C_{19}H_{36}O_3$	21.66	5.51	-
8	13Z,16Z-docosadienoic acid	$C_{22}H_{40}O_2$	22.011	0.31	-
9	Glyceryl monolinoleate	$C_{21}H_{38}O_4$	22.784	0.31	-
10	Oleic anhydride	$C_{36}H_{66}O_3$	22.817	0.02	-
11	Glycidyl oleate	$C_{21}H_{38}O_3$	23.325	22.68	14.08 ± 0.0
12	Glycidyl palmitate	$C_{19}H_{36}O_3$	23.555	1.68	5.09 ± 0.0
13	(R)-(-)-14-Methyl-8-hexadecyn-1-ol	$C_{17}H_{34}O$	25.952	0.31	-
14	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	$C_{19}H_{34}O_2$	26.275	1.59	-
15	γ-Sitosterol	$C_{29}H_{50}O$	28.409	0.33	-

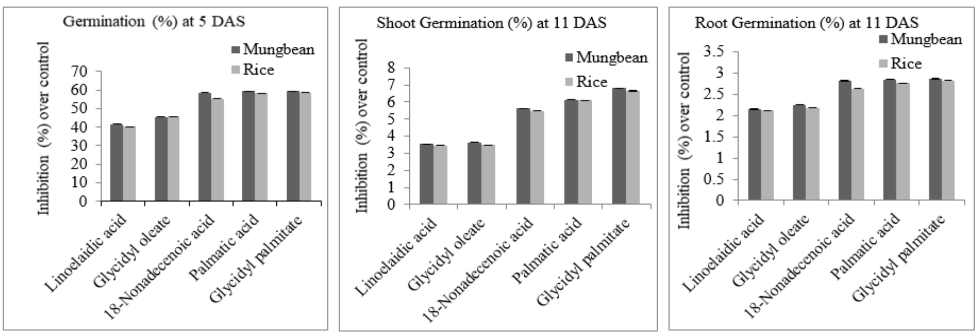


Figure 2. Inhibitory effects of *N. montholonii* allelochemicals applied at 1 μg/100 ml on seed germination and seedling growth of mungbean and rice

Metabolic changes in test crops

The foliar applications of aqueous extracts of *N. montholonii* on field grown test crops showed that low concentration (1.5 %) was slightly inhibitory to protein (11 %), carbohydrates (8 %) and starch (8.5 %) in mungbean (Fig. 3, 4 and 5). While in rice, the inhibition was 2, 4 and 5 % respectively at 30 DAS (Fig. 3, 4 and 5). But the inhibition in seeds germination was drastic at 10 % concentration in above metabolites was 60, 81 and 77 %, respectively in mungbean. The trend was similar in rice at 60 DAS over the control (Fig. 3, 4 and 5). However in 10 % aqueous extract concentration at 90 DAS; there was complete wilting of both field grown plants. Bhakat *et al.* (9) and Jash *et al.* (21) also reported a decrease in carbohydrates, protein and starch contents in mungbean, rice and grass pea due to 5 and 10 % concentrations of leaf extract/ leachate of *Parthenium* sp. and *Chromolaena odorata* L. The higher concentrations of leachates/ aqueous extracts retarded the photosynthesis and thereby decreased the contents of proteins and carbohydrates (8, 21). Singh *et al*. (36) also documented similar effects on protein content in germinating seeds of mungbean and rice due to harmful effects of leachates and extracts of *Eucalyptus* and *Parthenium.* The influence of leachates during seed germination physiology might have reduced the seed germination and seedling growth of mungbean and rice.

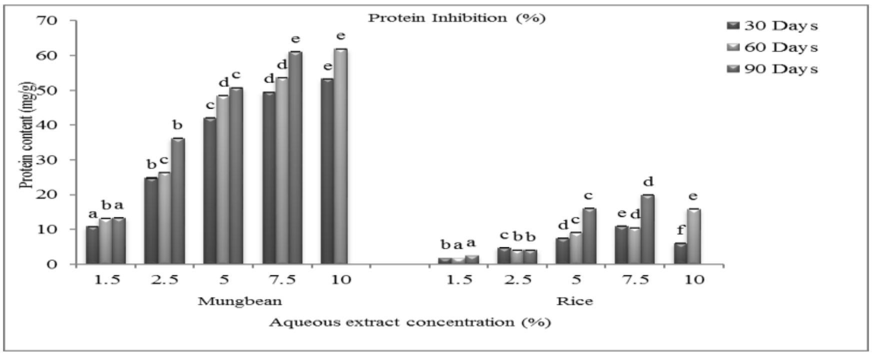


Figure 3. Effects of *N. montholonii* aqueous extract (%) on Protein content of mungbean and rice. Vertical bars show standard errors. Bars with different letters show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test.

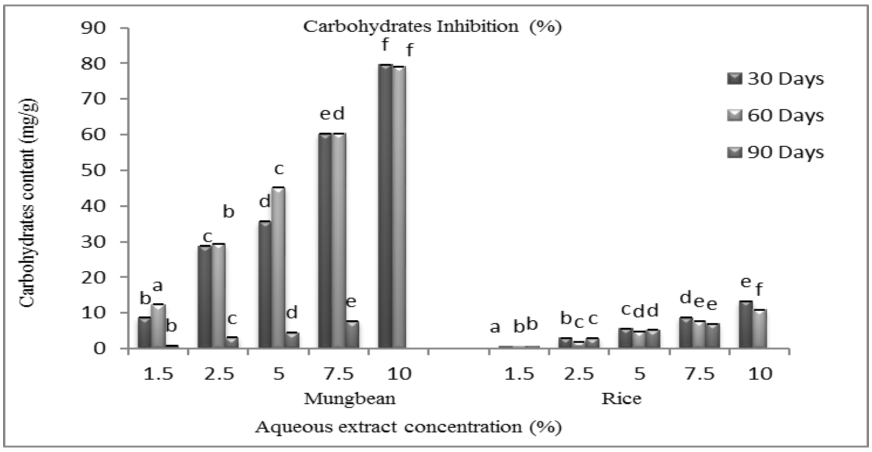


Figure 4. Effects of N. montholonii aqueous extract (%) on carbohydrates content of mungbean and rice. Vertical bars show standard errors. Bars with different letters show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test.

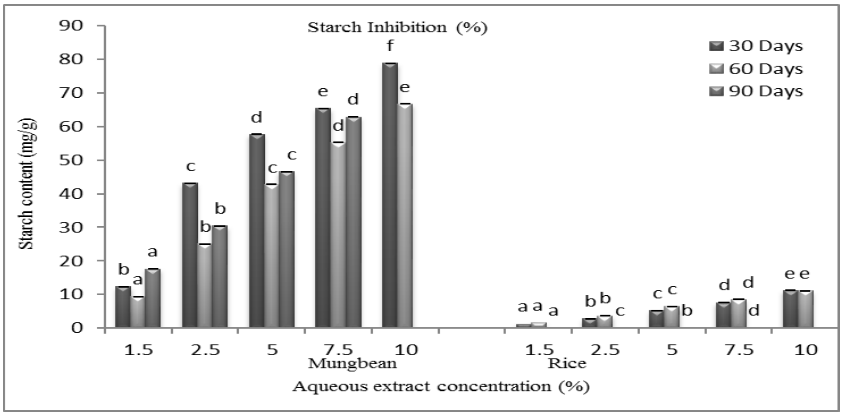
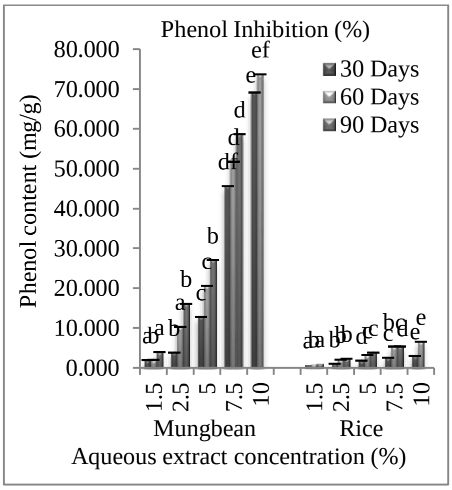


Figure 5. Effects of N. montholonii aqueous extract (%) on Starch content of mungbean and rice. Vertical bars show standard errors. Bars with different letters show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test. C: Control



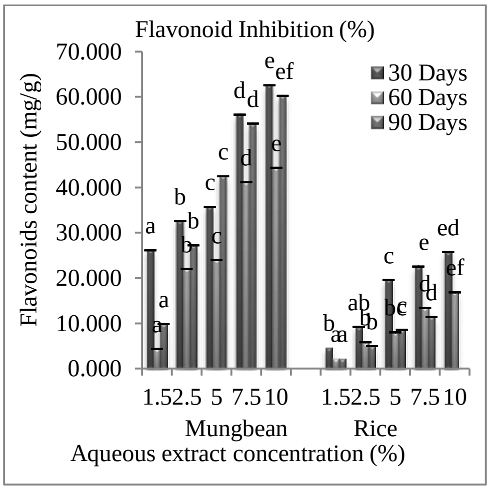


Figure 6. Effects of *N. montholonii* aqueous extract (%) on Phenol content of mungbean and rice. Vertical bars show standard errors. Bars with different letters show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test

7. Effects of *N. montholonii* aqueous extract (%) on Flavonoids content of mungbean and rice. Vertical bars show standard errors. Bars with different letters show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test

Our findings indicated noticeable changes in secondary metabolites (phenols (Fig. 6), flavonoids (Fig. 7), Flavonols (Fig. 8), tannins (Fig. 9), etc.) in mungbean and rice. These compounds in the treated crops were analyzed to confirm the impact of foliar spraying of weed extracts on the test crops. The growth, yield and other morpho physiological attributes are governed by physiological processes. The anomalies in normal metabolism are introduced in the test crops by treating them with weed extracts. The physiological process ultimately lead to yield, hence, it is very essential to record the negative impact of weeds on associated crops through physiological / biochemical parameters like phenols, tannins, flavonoids and flavonols. The aqueous extracts at lower concentrations (1.5 and 2.5 %) reduced the secondary metabolites over the control. However, at medium and higher concentrations (5, 7.5 and 10 %), there was significant inhibition in phenols, tannins, flavonoids and flavonols at 30, 60 and 90 DAS than control. In mungbean, the inhibition in phenols ranged from 67-74%, tannins (68-73 %), flavonoids (60-63 %), and flavonols (75-80 %) (Fig. 6, 7, 8 and 9). Similar trend was recorded in inhibition of these secondary metabolites in rice (Fig. 6, 7, 8 and 9).

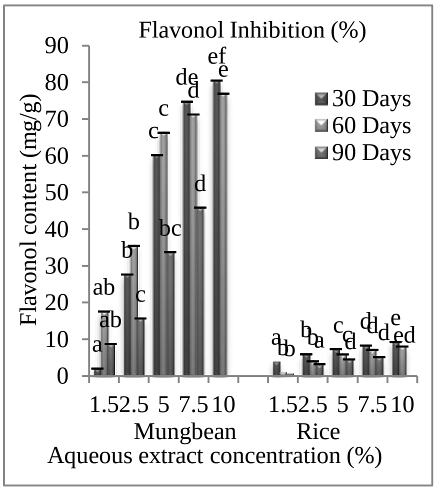


Figure 8. Effects of *N. montholonii* aqueous extract (%) on Flavonol content of mungbean and rice. Vertical bars show standard errors. Bars with different letters show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test

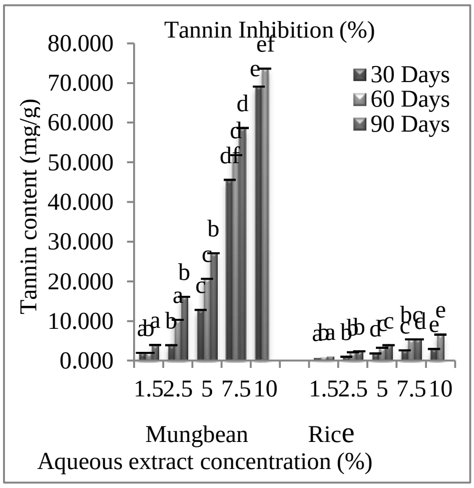


Figure 9. Effects of *N. montholonii* aqueous extract (%) on Tannin content of mungbean and rice. Vertical bars show standard errors. Bars with different letters show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test

1.5 % : (1.5 ml stock solution diluted to 100 ml), 2.5 % : (2.5 ml stock solution diluted to 100 ml), 5 % : (5 ml stock solution diluted to 100 ml), 7.5 % : (7.5 ml stock solution diluted to 100 ml), 10 % : (10 ml stock solution diluted to 100 ml) for 12 h.

The feasible cause of inhibition may be the presence of allelochemicals in aqueous extract of *N. montholonii*. Gomaa *et al*. (19), Ghayal *et al*. (18) and Devi *et al*. (14) also noted a significant reduction in polyphenols, tannins and flavonoids in rice, sorghum and mungbean due to the applied leaf leachates/ aqueous extract of *Parthenium*, *Cassia* and *Synedrella.* Inderjit and Duke (20) have specified that in nature, foliar leachates and root exudates contain diverse types of secondary metabolites (phenolic, terpenoids, polyacetylenes and flavonoids), which cause phytotoxicity in recipient plants. However opposite trend was documented by Batish *et al*. (7), she noted increased phenolic contents in various crops due to higher concentration in leaf leachates of *Parthenium* and *Eucalyptus*. She claimed that an increase in phenolic contents decreased the seed germination rate and seeding growth.

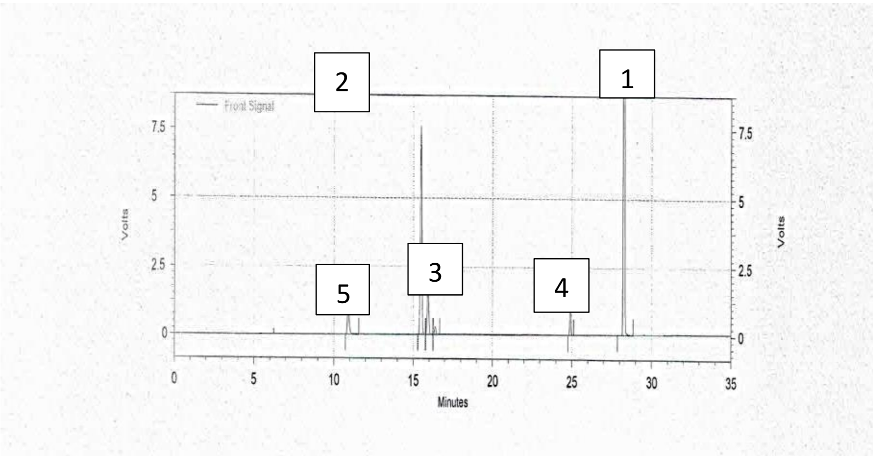


Figure 10. HPLC chromatogram of standards compounds

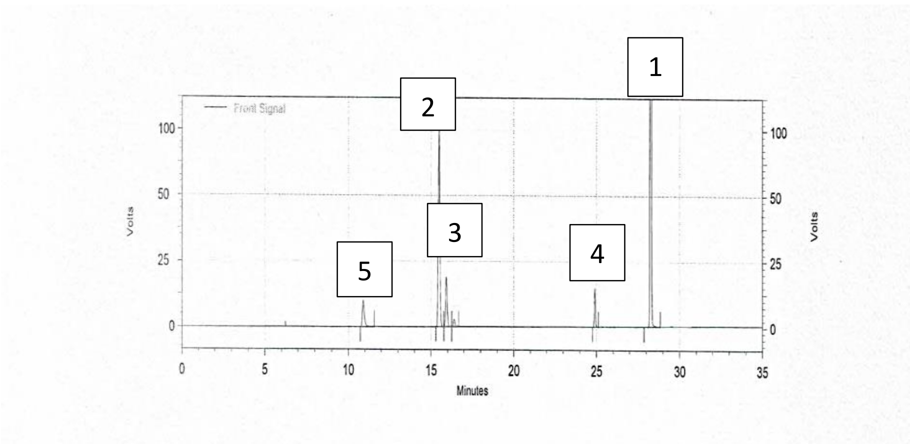


Figure 11. HPLC chromatogram of *N. montholonii* extract

Eucalyptus globulus Labill. had strong inhibitory and suppressive effect on germination, growth of shoots and roots of *Lactuca sativa* L. and *Agrostis stolonifera* L. (30). It also reduced the protein contents and chlorophyll concentrations than control, due to allelochemicals like chlorogenic, two ρ-coumaric derivatives, ellagic, hyperoside, rutin, quercitrin, and kaempferol 3-O-glucoside detected by HPLC.

The reduced growth and development of receiver plants viz., mungbean and rice in our present studies demonstrated that allelochemicals released from the weed *N. montholonii* affected the test crops in different ways. Mungbean was more sensitive than rice to aqueous extract of *N. montholonii* weed. The inhibitory effects may due to the presence of various classes of secondary metabolites (Linoelaidic acid, Glycidyl oleate, 18-Nonadecenoic acid, Palmatic acid and Glycidyl palmitate) detected by GC-MS (Table 2) and HPLC (Fig.10, 11) in *N. montholonii* aqueous extract. These allelochemicals may be the potential source for the strong allelopathic effects detected in mungbean and rice. These allelochemicals might be acting synergistically on seed germination, seedling growth and physiology of mungbean and rice. These allelochemicals are reported for the first time in this weed.

CONCLUSIONS

The weed *N. montholonii* had strong allelopathic effects on seed germination of mungbean and rice. Its aqueous extract phytotoxicity increased with concentration. The inhibitory effects on seed germination, seedling growth and physiology confirmed allelopathic of potential weed *N. montholonii*. Fifteen allelochemicals (Phenols, alkaloids, flavonoids, flavonols and glycerol) were identified in its extract. The five major allelopathic compounds (Linoelaidic acid, Glycidyl oleate, 18-Nonadecenoic acid, Palmatic acid and Glycidyl palmitate) isolated from the extract negatively affected the seeds germination and seedling growth. The foliar applications of aqueous extract of *N. montholonii* were also harmful to field grown test crops. The aqueous extract negatively affected the growth and yield of both test crops. Further research is in progress to determine the herbicidal and pesticidal activities of allelochemicals.

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