

Effects of allelochemicals from *Melaleuca leucadendra* (L.) tree leaf leachates on seed germination of *Vigna radiata* (L.) R. Wilczek

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ABSTRACT

We studied the influence of leaf leachates and organic extracts of leaves of exotic tree specie *Melaleuca leucadendra* (L.) on some physiochemical properties of *Vigna radiata* (L.) Wilczek seeds. The leaf leachate was extracted with organic solvents of decreasing polarity: Methanol > ethyl acetate > chloroform > hexane). Extracts were prepared with each organic solvent at 3-concentrations (5 %, 10 % and 15 %), by adding 5 g, 10 g or 15 g of dried leaf powder to 100 ml of organic solvent. The germination (%), speed of germination and triphenyl tetrazolium chloride stainability were reduced. T₅₀ values (time required for 50 % of seeds to germinate), were increased. These effects were strictly concentration dependent in both leaf leachate and organic extracts. Increased levels of amino acids, soluble carbohydrates and activity of amylase were found. On the other hand, insoluble carbohydrates, proteins and catalase activity were reduced. Methanol extract (15 %) among all organic extracts showed highest inhibitory effects on pre-treated seeds. Through column chromatography the methanol extract was separated into different fractions based on their polarity. Among the isolated fractions Hexane: Ethylacetate (9:1 v/v) elutes showed the maximum inhibition. The High-Resolution Mass Spectroscopic (HRMS) data confirmed the presence of five major compounds viz., neral, 3-methylbutyl heptanoate, nerolidol, geranyl benzoate and phytol in elute.

Keywords: Agroforestry, Allelochemicals, Bioherbicide, Chromatography, Exotic Tree Plantation, Germination Inhibition, HRMS, Sustainable Agriculture, Tree Allelopathy.

INTRODUCTION

The plantation of exotic tree species in India was started due to their faster growth than indigenous species and to rehabilitate degraded forests. However, these tree species are harmful to the environment and have inhibitory effects on regeneration of natural forest and ecosystems (15). Most of the exotic invasive plants are major threats to biodiversity and some of also cause economic losses due to their allelopathy (28). These plants adversely effects the ecological diversity and agroforestry (1,7,9,28). All higher plants release allelochemicals into their surrounding soil, which may adversely affect the germination, and growth of other plants. This hinders successful establishment of many species. Such harmful effect of allelopathic plants may alter the metabolism of receptor plants by modifying physiochemical parameters. Some indices are used to assess the allelopathic potential of experimental species using bioassays of test species (11). The lack of adequate research in allelopathy allowed us for isolation, purification and identification of allelochemicals from *M. leucadendra* by determining the physiochemical properties-using *V. radiata* as target spp.

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MATERIALS AND METHODS

The study area was located in Midnapore district, West Bengal, India (22°38'27" N, 87°19'18" E). The area spans approximately 553.39 km² and features a deciduous and evergreen forest cover with an elevation of 23-28 m above the sea level. The maximum temperature: 40 °C and the minimum temperature: 10 °C. The mean annual rainfall: 135.09 cm. The study was conducted in 2023 from March to September. Pot experiment was done thrice at different times. Germination bioassay duration was 120 h. The first experiment was started from 20.03.2023 and end up with 25.03.2023. Other bioassay experiments were conducted later on during May to August. Fresh, healthy and mature leaves were collected from *Melaleuca leucadendra* (L.) L. in Salboni (22°38'27" N, 87°19'18" E) area, Paschim Medinipur district, West Bengal, India. Fully viable *Vigna radiata* (L.) R. Wilczek (K75) seeds were procured from Midnapore town and used in bioassays.

Leaf leachates preparation: The leaf leachates of *M. leucadendra* were prepared by immersing 500 g shade dried leaf powder (27,28) in 300 ml double distilled water at room temperature (30 °C) for 48 h. The leachates were filtered with Whatman No.1 filter paper (4) and required amount of distilled water was added to make its volume to 500 ml. Such leachate was considered as the 1:1 (w/v) proportion of leaf leachate and was used as stock solution to prepare dilutions of 1:2 and 1:3 (w/v). Distilled water was used as control (28).

Healthy *V. radiata* seeds were immersed in 0.1 % HgCl₂ solutions for 90s for surface sterilization. Then, seeds were rinsed several times with distilled water to remove HgCl₂ traces. The sterile seed lots (100 seeds each) were pre-soaked for 24 h in stock solution of leaf leachate or one of its dilutions. Then seeds were surface-washed with distilled water.

Preparation of organic extracts: The leaf leachate was extracted with organic solvents of decreasing polarity: Methanol > ethyl acetate > chloroform > hexane. Extracts were prepared with each organic solvent at 3-concentrations (5 %, 10 % and 15 %), by adding 5 g, 10 g or 15 g of dried leaf powder to 100 ml of organic solvent. Then, extracts were stirred with magnetic stirrer (in 1000 g) at room temperature (32 °C) for 24 h (22,33). The extractions were filtrated with Whatman No.1 filter paper. Then organic extracts were tested for their effects on seed germination (%), germination inhibition, speed of germination and T₅₀ values [Times required (h) for 50 % germination] for further screening of suitable solvent system. Concentrated extracts were used for isolation and purification purpose (29).

SEED GERMINATION

(i). **Seed Germination:** Germination (%) was determined as per ISTA rules (21) and T₅₀ values (time required in h for 50 % seed germination) were calculated using the methods of Coolbear et al., (10).

(ii). **Speed of germination:** The speed of germination of treated seeds was calculated by analyzing seeds germination (%) at 24 h intervals up to 120 h. The speed of germination was calculated as under:

$$N_1/T_1 + N_2/T_2 + N_3/T_3 \quad (17)$$

Where, N: Number of germinated seeds observed at time, T: time in h.

(iii). Germination inhibition: Germination inhibition (%) was calculated as per formula of Sundra and Pote (40) as under:

$$I = 100 - (E_2 \times 100/E_1);$$

Where, I: Inhibition (%), E₂: Response of control seeds and E₁: Response of treated seeds.

(iv). Seed viability and vigour: TTC stainability was analysed by 2, 3, 5-triphenyl tetrazolium chloride (TTC) Stainability test the method of Halder (20). Here 100 dehusked seeds were immersed in 0.5 % TTC solution (w/v) in Petridishes for 24 h in dark. The TTC (%) stained (red colour) seeds were calculated from the total number of seeds of each treatment (20).

Soluble carbohydrates, insoluble carbohydrates, amino acids and proteins: To find the content of soluble carbohydrates, insoluble carbohydrates, amino acids and proteins, 20 seeds each were separately treated with 3 leaf leachates [1:1(w/v), 1:2(w/v), 1:3(w/v)] of *M. leucadendra* and another 20 seeds were treated with distilled water as control (26,27). Then seed kernels were used to estimate soluble carbohydrates, insoluble carbohydrates, amino acids and proteins. The soluble carbohydrates and insoluble carbohydrates contents were estimated following the method of McCready *et al.*, (30), the amino acids were quantified as per the method of Moore and Stein (24) and protein was analysed using the method of Lowry *et al.* (25).

Amylase and catalase activity: Catalase and amylase activities were estimated using the method of Snell and Snell (37); modified by Biswas and Choudhuri (8) and by Khan and Faust (23), respectively.

All experiments were repeated thrice and mean values were taken for calculation.

Column chromatography: The concentrated (15 %) methanolic extract was used as a sample for column chromatography and the allelochemicals present were separately isolated through chromatographic method (silica column). The dimension of column was 4-5 mm and the size range of silica gel mesh was 60-120 (19,32). The methanolic extracts were purified by running sequentially through 6-solvents (mobile phase) [hexane (100 %), hexane : ethyl acetate (19:1 v/v), hexane : ethyl acetate (9:1 v/v), hexane : ethyl acetate (4:1 v/v), hexane: ethyl acetate (1:1 v/v), DCM : methanol (25:1 v/v) (last elute)] based on the polarity (non-polar to polar). The elutes of each fraction were collected in labelled test tubes (25 ml size) and used for bioassay tests to recognize the fraction showing highest inhibitory effects due to presence of putative allelochemicals (33).

High resolution mass spectrometry (HRMS): The High resolution mass spectrometry (HRMS) was recorded on a thermo specific Q-Extractive, Accela 1250 pump (Quadrupole-Orbitrap MS system) HRMS spectrum of hexane: ethylacetate (9:1 v/v) elutes of 15 % concentrated methanol extract isolated through column chromatography. The m/z values were analysed to understand the molecular weight and molecular formula of putative allelochemicals, and from which the probable name of the putative allelochemicals were determined (39).

Statistical Analysis: The data were expressed as mean ± SEM and analyzed by one-way analysis of variance (ANOVA) using the software GraphPad Prism. P<0.5 represented significant difference between means (Dunnett's multiple range test) (3,12,22).

RESULTS AND DISCUSSION

The allelopathic effects of different concentrations of aqueous leaf leachates of *M. leucadendra* inhibited all the parameters analyzed viz., seed germination to metabolism (Tables 1 and 2). Experimental Results show inhibition in percent seed germination and speed of germination but increased T₅₀ values in *V. radiata* seeds pretreated with leaf leachates of *M. leucadendra* (Table 1) than control samples. The leaf leachates treatment on *V. radiata* seeds shows differential TTC stainability and the effect was strictly concentration dependent. The speed of germination was decreased in all treatments. Seeds pretreated with 1:1 (w/v) concentration of leaf leachates show maximum inhibition.

Table 1. Effects of aqueous leaf leachates of *M. Leucadendra* on germination parameters of *V. radiata* seeds.

Parameters	Leaf leachates concentrations			
	Control	1:1 Ratio	1:2 Ratio	1:3 Ratio
Germination (%)	93.33±1.45	11±3.00****	53.33±2.90****	77.33±3.52****
T ₅₀ value	22.67±0.57	63.33±2.08****	48.33±2.51****	44.66±1.52****
TTC stainability	96.00±0.02	48.33±1.52****	60.33±1.52****	77.66±0.57****
Speed of germination	2.054±0.02	0.32±0.15****	1.20±0.04****	1.60±0.07****

****= at P <0.0001 probability level the results are significantly different. *M.L.L. = *M. leucadendra* leaf leachate.

Table 2. Effects of aqueous leaf leachate of *M. leucadendra* on the contents of amino acids, soluble carbohydrates, insoluble carbohydrates, proteins and activities of amylase and catalase in *V. radiata* seeds.

Parameters	Leaf leachates concentrations			
	Control	1:1 Ratio	1:2 Ratio	1:3 Ratio
Soluble carbohydrates (mg/g. fr.wt.)	0.46±0.05	0.68±0.04*	0.58±0.006*	0.56±0.012*
Insoluble carbohydrates (mg/g. fr. wt.)	0.20±0.03	0.05±0.01**	0.06±0.01**	0.09±0.02**
Amino acid (mg/g. fr. wst.)	0.24±0.03	0.67±0.01***	0.45±0.03***	0.30±0.05***
Protein (mg/g. fr. wt.)	138±3.60	112.67±1.2***	122.00±2.30***	130.67±1.85***
Amylase (Unit/h/g fr.wt.)	0.20±0.22	1.10±0.05****	0.79±0.03****	0.44±0.05****
Catalase (Unit/h/g fr.wt.)	1.53±0.02	0.87±0.03****	1.33±0.03****	1.49±0.03****

****= at P <0.0001 probability level the values differsignificantly, # ***= at P <0.001 probability level the values differsignificantly, # **= at P <0.01 probability level the values differsignificantly, # *= at P <0.1 probability level values differsignificantly, †M.L.L. = Aqueous leaf leachate of *M. leucadendra*.

Amino acids, soluble carbohydrates, insoluble carbohydrate, amylase and catalase activity: Higher concentrations of leaf leachates released higher levels of amino acids and carbohydrates in pretreated seeds. Magnitude of increase of amino acid and soluble carbohydrate levels was less in lower concentrations of leaf leachates treatments. Results

indicated that, the amino acids and soluble carbohydrates levels were increased with concomitant reduction of insoluble carbohydrates and proteins in *V. radiata* seeds. Leaf leachates pretreated seeds decreased catalase enzyme activity, while increased the amylase activity in a dose-dependent manner. Total insoluble carbohydrates and proteins decreased in pretreated seeds than control.

Furthermore, different solvent extract treatments reduced germination (%) (Figure 1) and speed of germination (Figure 2), while, they increased inhibition of germinability (Figure 3) and T_{50} values (Figures 4) on pretreated seeds. When *V. radiata* seeds pretreated with methanolic extract (15 % w/v) compared to other different concentrations of methanolic extracts show the maximum inhibitory effect (Figure 2).

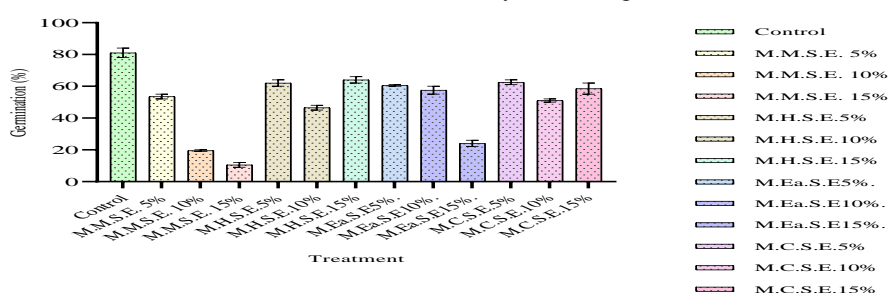


Figure 1. Effects of solvent extracts of *M. leucadendra* on germination (%) of *V. radiata* seeds (value represented as \pm SEM). *SEM= Standard Error Means. *SEM= Standard Error Means. *M.M.S.E. = *Melaleuca* methanolic solvent extract; M.H.S.E. = *Melaleuca* hexanic solvent extract; M.Ea.S.E. = *Melaleuca* ethylacetate solvent extract; M.C.S.E. = *Melaleuca* chloroform solvent extract.

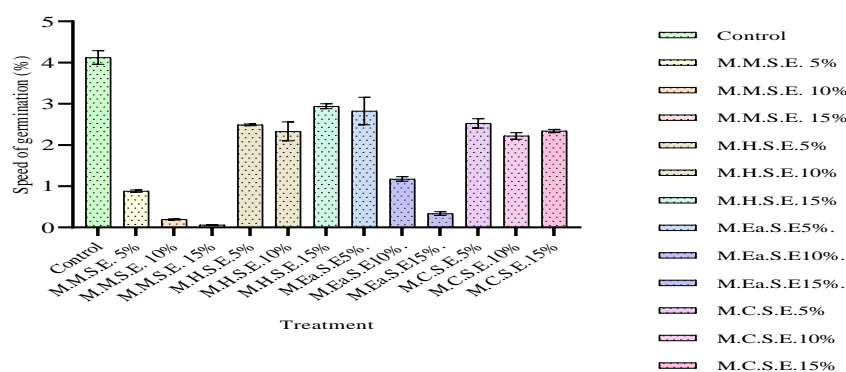


Figure 2. Effects of solvent extracts of *M. leucadendra* on speed of germination of *V. radiata* seeds (value represented as \pm SEM) *SEM= Standard Error Means. *M.M.S.E. = *Melaleuca* methanolic solvent extract; M.H.S.E. = *Melaleuca* hexanic solvent extract; M.Ea.S.E. = *Melaleuca* ethylacetate solvent extract; M.C.S.E. = *Melaleuca* chloroform solvent extract.

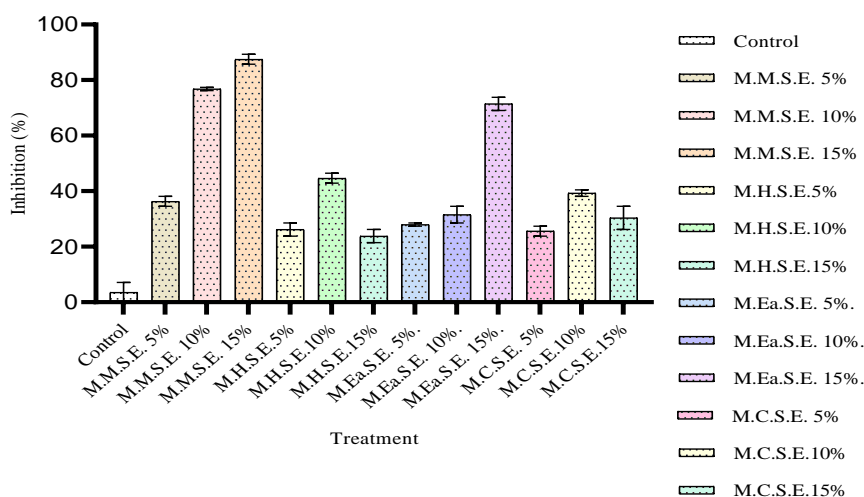


Figure 3. Effects of solvent extracts of *M. leucadendra* on seeds germination inhibition (%) of *V. radiata* (value represented as \pm SEM). *SEM= Standard Error Means. *M.M.S.E. = *Melaleuca* methanolic solvent extract; M.H.S.E. = *Melaleuca* hexanic solvent extract; M.Ea.S.E. = *Melaleuca* ethylacetate solvent extract; M.C.S.E. = *Melaleuca* chloroform solvent extract.

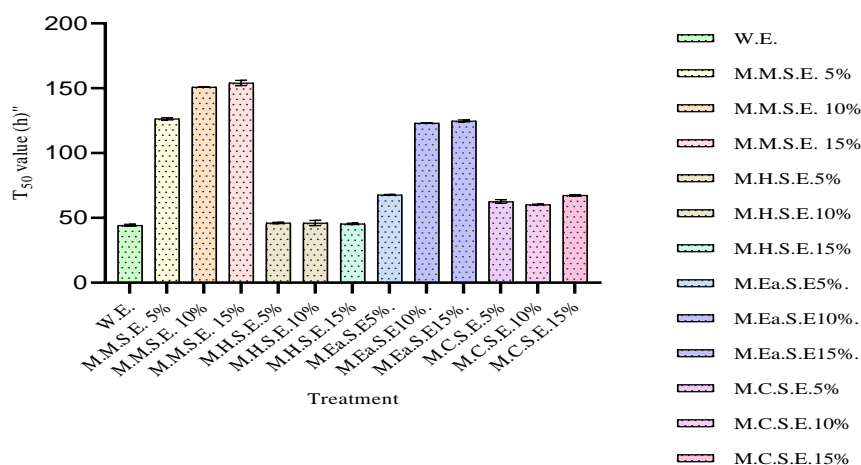


Figure 4. Effects of solvent extracts of *M. leucadendra* on T₅₀ values (time required for 50% seed germination) of *V. radiata* seeds (value represented as \pm SEM). *SEM= Standard Error Means. *M.M.S.E. = *Melaleuca* methanolic solvent extract; M.H.S.E. = *Melaleuca* hexanic solvent extract; M.Ea.S.E. = *Melaleuca* ethylacetate solvent extract; M.C.S.E. = *Melaleuca* chloroform solvent extract.

The fractions of methanolic extracts (15 %) obtained through column chromatography showed significant allelopathic effects in terms of seed germination and percent inhibition (Figures 5,6). But, the leaf leachates showed a more pronounced effect compared to the fractionized compounds. Here elutes 10C and 10D (Figures 5) from the fraction hexane: ethylacetate (9:1 v/v) showed maximum effects than other elutes.

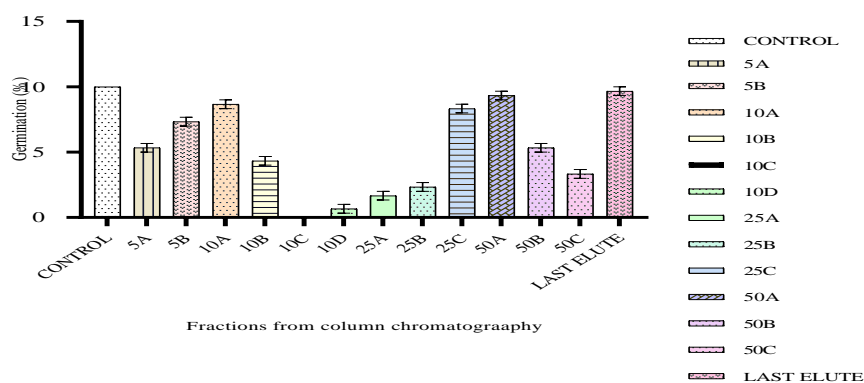


Figure 5. Effects of elutes from different fractions from column chromatography of *M. leucadendra* on seed germination of *V. radiata*. (Value represented as \pm SEM). *SEM= Standard Error Means. *M.M.S.E.= *Melaleuca* methanolic solvent extract; M.H.S.E.= *Melaleuca* hexanic solvent extract; M.Ea.S.E.= *Melaleuca* ethylacetate solvent extract; M.C.S.E.= *Melaleuca* chloroform solvent extract.

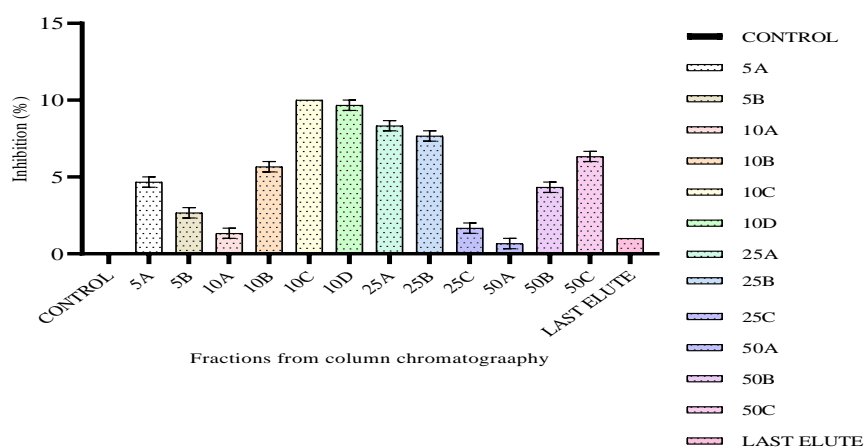


Figure 6. Effects of elutes from different fractions from column chromatography of *M. leucadendra* on inhibition (%) in *V. radiata* seeds. (Value represented as \pm SEM) *SEM= Standard Error Means. *SEM= Standard Error Means. *M.M.S.E.= *Melaleuca* methanolic solvent extract; M.H.S.E.= *Melaleuca* hexanic solvent extract; M.Ea.S.E.= *Melaleuca* ethylacetate solvent extract; M.C.S.E.= *Melaleuca* chloroform solvent extract.

The HRMS data identified and analysed elutes from hexane: ethylacetate (9:1 v/v) which contains allelochemicals. The chemicals were neral (34) shown peak $[M+H]^+$ 153, 3-methylbutyl heptanoate shown peak $[M+H]^+$ 201, nerolidol (6) shown peak $[M+H]^+$ 223, geranyl benzoate shown peak $[M+H]^+$ 259 and phytol (6, 13) shown peak $[M+H]^+$ 297 (Figure 7; Figure 8).

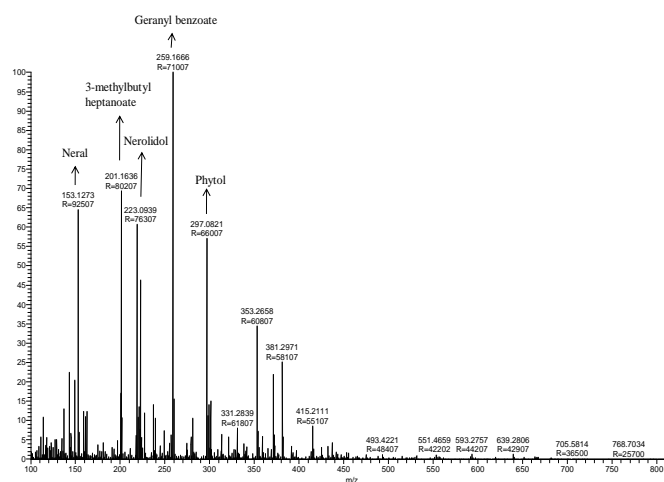


Figure 7. Different peaks of phytochemicals with molecular mass (HRMS authentic graph).

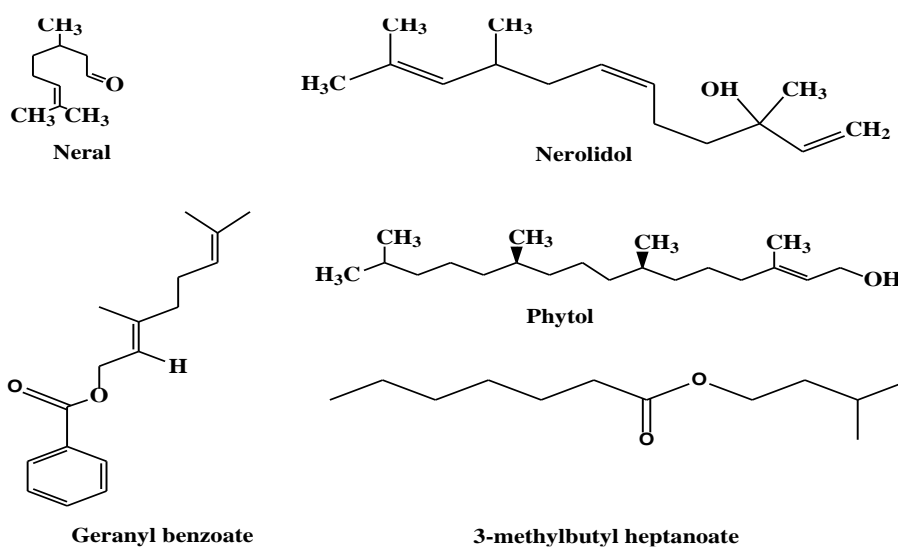


Figure 8. Different phytochemicals identified and their chemical structure.

Thus, we studied the effects of leaf leachates and solvent extract of *M. leucadendra* on *V. radiata* seeds, the results adversely altered the germination behaviour and biochemical parameters. Numerous reports suggest that inhibition of germination and growth parameters might be due to the inhibition in synthesis of various enzymes and other growth hormones under the influence of allelochemicals (35). The permeable membrane of the tonoplast and plasma membrane which retains solute within the cell normally, loses their integrity and hence loses their activity as retentive barrier (28). Leaf leachates and solvent extracts in this study possibly impaired membrane structure when seeds were soaked for 24h with different concentrations. In the present study, increased level of amino acids and soluble carbohydrates may cause possible damage of plasma membrane of pretreated seed samples experiencing with leaf leachates and solvent extract of *M. leucadendra*. We also found similar trends of results in many reports by other researchers in different plants (*viz.*, *Casuarina equisetifolia*, *Lantana camara*, *Eupatorium odoratum*, *Ipoemea pes-carpae*, *Hyptis suaveolens*) of leaf extracts and leachates (18, 26). The impairment of seed germination and growth occurs due to alteration in metabolism and metabolite transport controlled by various enzymes activity from the seeds (28).

A gradual increase in the amount of total amino acids in treatment may be due to the increased biosynthesis of amino acids or degradation and/or decreased biosynthesis of proteins in the plant to overcome environmental stresses. The synthesis of carbohydrates reduced due to the inhibition of biosynthesis of protein, normally protein may be used as respiratory substrate, when the supply of carbohydrates found inadequate due to allelochemicals stress. The interaction of these allelochemicals imparts enzymatic activities that regulate the impairment of metabolism and consequently impairs the germination behavior and metabolism of test seeds. These studies indicate the potential of using allelopathic species suppresses the germination and growth of others.

The effects of allelochemicals neral, nerolidol and phytol from different plant sources have been reported (5,13,14,16,24,34,36). These, three chemicals are also present in *M. leucadendra* leaf (6). The allelopathic effects of these chemicals from *M. leucadendra* leaf leachate were not investigated earlier. But further investigation of allelopathic effect of 3-methylbutyl heptaoate and geranyl benzoate were not reported. Therefore, among these putative allelochemicals, neral, nerolidol, phytol perhaps altered seed germination behaviour and various biochemical consequences and metabolism due to change in the metabolic processes of *V. radiata* seeds.

CONCLUSIONS

The leaf leachate and methanolic solvent extracts of *M. leucadendra* leaf had allelopathic effects on seeds germination and growth due to presence of putative allelochemicals. In petri-plate bioassays, these leaf leachate and solvents extracts reduced seed germination, early growth rate, metabolism and selectively increased atrophy in *V. radiata*. Among the putative allelochemicals, *viz.*, neral, nerolidol, phytol might be responsible for the restricted growth of other co-existing plant species in their vicinity. Thus, plantation of *M. leucadendra* in natural and agroecosystems can interfere with species richness and crop productivity. Furthermore, these putative allelochemicals could serve as

the template to develop new natural herbicide for sustainable weed management in agriculture. There is an urgent need to control the plantation of *M. leucadendra* as agroforestry tree specie.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration with all authors. All authors finally approved and drafted the manuscript.

DECLARATION

We declare that all authors of this Ms. have made substantial contributions. We did not exclude any author who substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

ETHICAL STATEMENT

This is to inform you that in this study, we have not been involved in any animal and human studies.

CONFLICT OF INTERESTS

The authors declare there are no conflicts of interest.

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