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Phytotoxic effects of leaf leachates of an invasive weed Cassia uniflora and Characterization of its Allelochemical

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ABSTRACT

Cassia uniflora Mill. Non Spreng. a dominant invasive weed from Ganeshkhind area has affected the phytodiversity of Pune University campus (M.S., India). Its leaf leachates inhibited seed germination and seedling growth in test crops like tomato and brinjal at all the concentrations indicating their phytotoxic nature. However, maximum inhibition was recorded at higher concentrations (17.5 – 20%). The allelopathic leaf leachates had also adversely influenced the seedling physiology in both the vegetables. Hence to investigate its phytotoxic nature, isolation and characterization of allelochemicals in it, was undertaken. The leaf samples of Cassia showed the presence of a branched chain saturated aliphatic ester ($C_{16}H_{32}O_2$) which is Dodecane-**4**yl-butyrate.

Key Words - Cassia uniflora, dodecane-4yl-butyrate, invasive weed, phytotoxicity, seed germination bioassay, seedling physiology.

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INTRODUCTION

The invasive weeds keep encroaching the newer habitats and often replace the native phytodiversity by becoming aggressive outside their natural range and become dominant in the community due to their allelopathic potential [1-3]. Allelopathic potential of these invasive weeds helps in their successful invasion [4]. Allelopathy is involved in positive and negative interference between the plants and micro-organisms. The negative influence prevents seed germination and seedling growth by producing growth inhibiting allelochemicals [5]. Allelochemicals released from such plants causes negative effect on the plant species by affecting their physiological processes [6]. This phytotoxicity may be caused due to high levels of inhibitory allelochemicals / ecochemicals which also affect the physiology of recipient plants [7-9].

Pune University Campus with about 164.8 hectares area is rich in diversity of weeds. The phytosociological studies have indicated that [10] invasive weeds like Cassia uniflora, Synedrella nodiflora, S. vialis and Alternanthera tenella are becoming highly dominant in the campus and threatening the native plants' diversity. It was further reported that Cassia was one of the most dominant invasive weed among all those.

The phytotoxic nature of aqueous leaf leachates of Cassia, affecting the growth of crop plants was well documented [11]. The GC-MS studies of this weed Cassia uniflora Mill. non Spreng, indicated the presence of steam volatile components as 2-pentadecanone, hexadecanoic acid, phytol, isobutyl phthalate, dioctyl phthalate as major compounds and nonpolar n-hexane extract showed the presence of 2-pentadecanone, neophytidiene and phytol as major constituents [12]. Therefore the present investigation was undertaken to explore the novel allelochemicals existing in the same weed.

MATERIALS AND METHODS

Leaf leachate preparation

The fresh leaf samples of Cassia uniflora were collected from the natural sites during the flowering stage, and brought to laboratory, cleaned with distilled water and spread on filter paper for shade drying. These dry leaves were powdered and 100g of it, was soaked in 1000 ml distilled water for 24 h at 25^0C. The leachate (20%) was filtered through Buchner funnel using Whatman filter paper No. 1 and was stored in refrigerator in amber coloured bottle.

Seed Germination Bioassay

The seeds of tomato (Lycopersicon esculentum L. var.Vaishali) and brinjal (Solanum melongena L. var. Manjri gota) were obtained from College of Agriculture, Pune. The healthy seeds were used for bioassay study in seed germination chamber using sterilized petriplates (9

cm diameter) lined with germination papers. The seeds of tomato and brinjal were surface sterilized with 0.02% aqueous $HgCl_2$ for two minutes. Then the seeds were thoroughly washed with distilled water. Seed germination papers were thoroughly moistened with respective concentrations of leaf leachates (5ml.) of Cassia (2.5% to 20%) which were prepared by dilutions with distilled water. The seeds kept on germination paper moistened with only distilled water were considered as control.

Twenty seeds each of tomato and brinjal were uniformly kept in each petri plate. The two ml leachates of respective concentration were added in each petriplate on third day. The petriplates were again moistened with the leachates only once. The treatments were in triplicates. Seed germination (%), root length, shoots length and vigour index were recorded on 7^{th} day [41].

Biochemical analysis of seedlings Randomly selected 0.1g material of seedlings (control and leachate treated) was taken for analysis of starch [42], reducing sugars [43], total sugars [44] and proteins [45] according to standard methods. Total phenolics also were estimated [46]. The absorbances were recorded on UV-visible spectrophotometer (Shimadzu-1601) at respective wavelengths.

Extraction, Isolation and characterization of allelochemicals

Air shade dried and powdered material was extracted (10g) using acetone as a solvent at room temperature with continuous stirring for 12hrs. The material was then filtered. Acetone was removed under reduced pressure and extract was obtained as a sticky mass (0.438g, 4.38%). Extract was showing mixture of seven compounds of which two were major and both the compounds were UV active with green and pink glow, when the TLC was run in 20% ethyl acetate in toluene as solvent system.

The crude extract was adsorbed (438mg) on silica gel (5g). Broad fractionation was carried out using nonpolar to polar solvents using magnetic stirrer for 4hrs. The fractionation of acetone extract was carried out using n-hexane (250 ml, **A**), acetone (400 ml, **B**), Ethanol (200ml, **C**), and methanol (200ml, **D**) (Table 3).

TABLE 3.- BROAD FRACTIONATION OF ACETONE EXTRACT OF CASSIA UNIFLORA LEAVES

No.	Solvent	Quantity ml.	Net weight of Fractions in grams
A	n-Hexane	250	0.035
B	Acetone	400	0.253
C	Ethanol	200	0.027
D	Methanol	200	0.018

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The hexane extract **A** showed a mixture of five components of which two were major. It was found that one component was UV active and showed green fluorescence. The TLC was carried out in 100% Toluene. The mixture was insoluble in acetone and ethanol.

Purification of A

The compound was purified by repeated crystallization using mixed solvent system of hexane and ethanol to yield pure hygroscopic, amorphous, white powder (8mg, $\mathbf{A_1}$).

Statistical analyses

The data were summarized as means of three replicates with standard deviation as the measure of variability. One way ANOVA was used to compare the mean values followed by DMRT at p= 0.05 to compare the mean differences. Sigma Stat 3.5 and Microsoft Excel 2007 were used for the data analyses.

RESULTS AND DISCUSSION

Seed germination bioassay of tomato and brinjal

The leaf leachates of Cassia uniflora at all concentrations have inhibited seed germination and seedling growth of tomato and brinjal (Tables 1 and 2). The germination percentage was also found to be decreasing with the rise in concentrations. The IC_{50} value in both the vegetables was at 17.5% concentration. Reduction in root and shoot length, root: shoot ratio and vigour index was also recorded with increasing concentrations of leaf leachates. However, in tomato opposite trend was noted for shoot length and vigour index at 2.5% concentration only. For all these parameters, brinjal was more sensitive than tomato to the higher concentrations of leaf leachates. The results were significant at p = 0.05.

TABLE 1 - EFFECTS OF CASSIA UNIFLORA LEAF LEACHATES ON SEED GERMINATION OF TOMATO

Treatment Tomato	% Germination	Root length Mean ± SD	Shoot length Mean ± SD	R : S Ratio Mean ± SD	Vigour index Mean ± SD
Control	90	8.13 a ± 0.12	8.20 a ± 2.46	1.06 ab ± 0.35	738.00 a ± 221.00
2.50%	85	3.43 b ± 0.40	9.63 a ± 1.01	0.36 b ± 0.01	818.83 a ± 85.56
5%	75	3.23 b ± 0.49	6.90 ab ± 1.15	0.48 ab ± 0.15	517.50 ab ± 86.49
7.50%	70	2.47 b ± 0.50	7.60 ab ± 2.08	0.35 b ± 0.14	532.00 ab ± 145.66
10%	65	2.23 b ± 0.15	4.97 bc ± 1.19	0.47 ab ± 0.11	322.83 bc ± 77.55
12.50%	65	3.17 b ± 0.72	4.80 bc ± 1.48	0.72 ab ± 0.32	312.00 bc ± 96.19

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15%	60	2.33 b ± 0.76	4.97 bc ± 1.29	0.52 ab ± 0.31	298.00 bc ± 77.15
17.50%	50	1.97 b ± 0.85	2.30 c ± 0.50	0.94 ab ± 0.58	115.00 c ± 25.00
20%	35	2.37 b ± 0.40	1.87 c ± 0.65	1.36 a ± 0.48	65.33 c ± 22.77
p-value		< 0.001	< 0.001	< 0.012	< 0.001
*Values followed by different letters differ significantly by Duncan's multiple range test at p = 0.05					

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TABLE 2.- EFFECTS OF CASSIA UNIFLORA LEAF LEACHATES ON SEED GERMINATION OF BRINJAL

Treatment Brinjal	% Germination	Root length Mean ± SD	Shoot length Mean ± SD	R : S Ratio Mean ± SD	Vigour index Mean ± SD
Control	90	3.23 a ± 1.08	6.03 a ± 0.68	0.55 ab ± 0.23	543.00 a ± 61.26
2.50%	85	2.53 abc ± 0.45	5.53 a ± 0.68	0.46 ab ± 0.05	470.33 a ± 57.68
5%	80	1.53 b ± 0.06	3.37 b ± 1.06	0.49 ab ± 0.17	269.33 bc ± 84.79
7.50%	75	1.40 b ± 0.53	4.17 ab ± 0.47	0.34 b ± 0.15	312.50 b ± 35.44
10%	75	2.80 ab ± 0.92	3.37 b ± 0.93	0.93 ab ± 0.57	252.50 bc ± 69.69
12.50%	65	1.07 c ± 0.12	4.47 ab ± 0.50	0.24 b ± 0.01	290.33 b ± 32.72
15%	60	1.30 b ± 0.17	2.90 bc ± 0.53	0.46 ab ± 0.08	174.00 bc ± 31.75
17.50%	55	1.50 b ± 0.61	2.93 bc ± 0.60	0.55 ab ± 0.30	161.33 c ± 33.15
20%	30	1.30 b ± 0.52	1.23 c ± 0.50	1.10 a ± 0.33	37.00 d ± 15.10
p-value		0.002	<0.001	0.018	<0.001
*Values followed by different letters differ significantly by Duncan's multiple range test at p = 0.05					

To Be Inserted On Page 4 Or 5 With Seed Germination Bioassay

The seed germination bioassay is an important test to screen the stimulatory and inhibitory allelopathic effects of donor plant species on the recipient one. The laboratory bioassays and field studies are integral part of allelopathy research, because they are fast and repeatable tools for investigating the potential for different types of interactions [24]. It was further stated that for successful demonstration of allelopathic interactions, ecological, chemical and physiological analysis is highly essential. Considering this, to prove the hypothesis of allelopathic interactions between test crops like tomato and brinjal and invasive weed like Cassia, present investigation was focused on seed germination bioassay and physiological as well as biochemical of the seedlings.

Emergence of root and shoot is a primary phase in seed germination, which determines the future plant growth. Many workers have reported [13] [14] positive as well as negative impact of aqueous leachates on seed germination and development of root and shoot while working on rice, cowpea, green gram and pigeon pea. The different types of allelochemicals present in leachates of Cassia might have caused the retardation in seed germination percentage by acting negatively on the process. With increasing concentrations of leachates the allelochemicals present in them might have acted more negatively, leading to complete inhibition of seed germination of tomato and brinjal.

The roots are more sensitive to allelochemicals than shoots. The allelopathic impact of leachates was therefore more harmful to radical [21] as it was coming in close contact with the leachates / extracts. The similar type work on lettuce also supported this fact [22]. The favorable or adverse impact of allelochemicals on processes like seed germination is concentration **/**dose dependant which is explained as "Homosis" [23]. This means stimulation of growth at lower concentration and inhibition at higher concentrations of leachates. The delay in seed germination can have important biological and ecological implications, because it can affect the ability of the seedlings to establish in natural conditions.

The root and shoot length is the important parameter of seedling growth, which determine the growth of future plant. Many workers have reported positive and negative impact of aqueous leachates and different allelochemicals existing in them on root and shoot length. The root**:** shoot ratio is very confirmatory and reliable indicator of morphological, physiological and biochemical changes in germinating seeds and developing seedlings. In the present investigation it was found that all the treatments slightly decreased the root**:** shoot ratio as compared to control. The decreased values of R: S ratio indicated more shoot length as compared to less root length. This may be due to direct contact of roots with allelopathic extracts with roots resulting in to inhibition of root length. The rate of root growth might have retarded drastically due to their contact with allelochemicals, resulting in to reduced root length as compared to shoot length. The shoot is not directly coming in contact with the allelochemicals and hence its growth rate might be higher than the root at low concentration.

The vigour index is a vital parameter to determine the efficiency of any germinating seed to utilize and mobilize the reserved food materials. VI indicates the allelopathic effects on seedling establishment. The reductions in root length, shoot length, root**:** shoot ratio and vigour index are also in agreement [11] [15 - 17]. The phytotoxic effects of various weeds on seed germination and growth of different crops was reported [18 - 20].

Seedling physiology

The results represented (Figures 1 and 2) indicated that lower concentrations of C. uniflora leaf leachates have exhibited very less reduction in total sugar contents in tomato and brinjal seedlings, as compared to control, which increased along with the concentrations. The results were significant.

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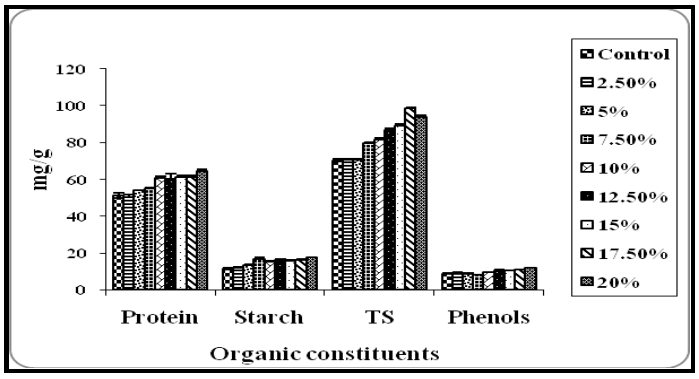
At lower concentration treatments of Cassia leaf leachates there was very less reduction in starch content of seedlings of both the vegetables over control. However, it increased with higher concentrations. Similar observations were recorded for protein content of the seedlings of both the test crops. There was also rise in phenolic contents, with treatments of higher concentrations (Figures 1 and 2). The results of seedling physiology have indicated the sensitiveness of brinjal to leaf leachates of Cassia than tomato. This corroborates also with the results of seed germination bioassay.

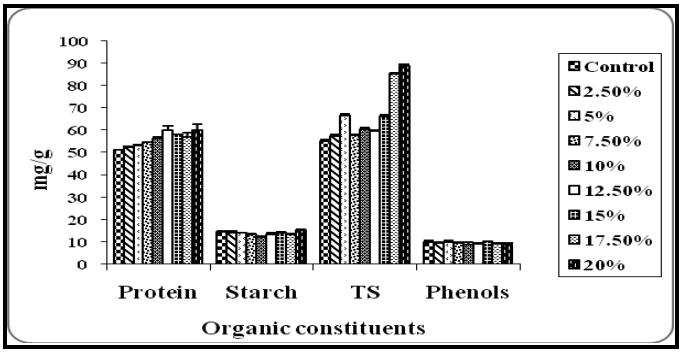
The importance of the physiological studies on seed germination as affected by allelopathy has been given lot of prominence [24]. The physiological analysis of seedlings will illustrate their metabolic status and capability to mobilize and effectively utilize the reserve food materials, resulting into successful seedling development, growth and establishment. Hence investigation on physiology and biochemistry of germinating seeds was under taken. The results (Figures 1 and 2) on seedling physiology indicated lower mobilization efficiency of the reserve food materials during seed germination which are treated with leaf leachates.

Fig. 1: Effect of Cassia Uniflora Leaf Leachates on Seedling Physiology Tomato



TS = Total sugars[#] Data columns are the pooled means of three replicates with Standard deviation as error bars.

Fig. 2: Effect of Cassia Uniflora Leaf Leachates on Seedling Physiology of Brinjal



[#] Data columns are the pooled means of three replicates with Standard deviation as error bars.

Carbohydrates' status is reflected in seed germination, growth, development and yield

[25]. The allelopathic influence may cause reduction or increase in the contents of carbohydrates [26 - 27]. This increase might be due to non-utilization of total sugars by leachate treated seeds of both the test plants, as a result of this the germination % was decreased (Tables 1 and 2). Similar was the explanation given by some researchers [28]. According to them, the carbohydrates, proteins, lipids and fats stored in the seeds are oxidized during the process of seed germination for supplying the energy to the developing seedlings. As a result of this these organic constituents get reduced, but opposite trend was observed in leachate treated seeds, as there was inhibition of seed germination. Increase in amount of starch in germinating seeds of test crops with higher concentration treatments of leachates was also noted in wheat by [29 - 30].

The alterations in protein metabolism plays an important role in seed germination and seedling growth, which is influenced by treatments of leachates, extracts and residues of different allelopathic plant species. Germinating seeds of tomato and brinjal showed increase in protein content, which may also be due to non-mobilization and non-utilization of reserved foods. The positive or negative impact of allelobiogenesis stress induced due to the treatments of leachates and extracts of Eucalyptus, Dalbergia, Parthenium and Andrographis on protein content in germinating seeds of mung bean and rice is well documented [31-32]. The different allelochemicals present in the leaf leachates might be synergistically acting on the processes of seed germination, seedling growth and their physiology.

To understand the allelopathic influence of phenolics present in the leaf leachates of Cassia, detailed investigation was carried out in tomato and brinjal from seed germination process up to seedling growth. The phenols as secondary metabolites have a predominant role in ecophysiological investigations [33] and cause immense adverse effect on seed germination, growth, development and metabolic functioning of plants. Hence phenolics have been a major focus in chemical ecology. Phenolic compounds are the major allelochemicals, through which higher plants interact with each other [34]. To understand the influence of phenolics present in the leachates of Cassia on tomato and brinjal, efforts were made to study the process of seed germination. The Allelopathy researchers [3537] also noted increased phenolic contents in leachate treated mung bean, black gram, corn, sorghum, radish, mustard, maize and rice at higher concentrations of Terminalia, Parthenium and Eucalyptus.

Identification and Characterization of allelochemicals

Compound **A_1** was obtained as highly hygroscopic, white, amorphous solid. It showed molecular formula $C_{16}H_{32}O_2$, as derived by LCMS **(**m/z 256 $[M]^+$**)** as it showed 255amu in negative mode of mass spectrometer. IR spectrum showed characteristic bands 1739.85cm^{-1} (ester carbonyl) and 1215.19cm^{-1} (C- O stretching). ^{1}HNMR spectrum (Table 4) showed upfield triplet at 0.90 δ for nine protons of three methyl groups (t, J = 6 Hz, C-1, C-12 and C-4'), a broad singlet at 1.28 δ for methylene proton envelope. It also depicted a broad singlet at 1.60 δ for methylene protons (br, s, 8H for C_3, C_5, C_6 and C_3'), a triplet at 2.31 δ (t, J=6Hz, 2H) for C_2' methylene protons attached to ester carbonyl group. A downfield broad singlet appeared at 3.83 δ, for methyne proton, at C4 attached to oxygen atom.

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TABLE 4: ^{1}H-NMR SPECTRAL DATA OF COMPOUND A_1 (500 MHZ, $CDCL_3$)

Atom No.	δ ppm
C-1, C-12 and C-4' methyl protons	0.90, t, J= 6Hz
C-11, C-10, C-9, C-8 and C-7 methylene protons	1.28 br. s
C-3, C-5, C-6 and C-3'	1.60 br. s
C-2' (methylene protons attached to ester carbonyl)	2.31, t, J = 6Hz
C-4 methyne proton	3.83 br. s

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TABLE 5: ^{13}C-NMR SPECTRAL DATA OF COMPOUND A_1

Atom No.	δ ppm
C-1' ester carbonyl	163.01, s
C-4 methyne carbon	77.22, d
C-3, C-5 and C-2' methylene carbons	31.93, t
C-1, C-12, C-4' methyl carbons	14.12, q

To be inserted on page 9 with identification and characterization of allelochemicals

The ^{13}CNMR spectrum (Table 5) of compound A_1 showed presence of 16 carbon atoms. DEPT experiment showed that it contains ester carbonyl function and one methyne carbon atom. The spectrum showed signals at 163.01 δ (s, ester carbonyl, C_1'), 77.22 δ (d, C_4) methyne carbon, 31.93 δ (t, C_3, C_5 and C_2' methylene carbons), a strong peak at 29.70 δ (t, methylene envelope), 22.70 δ (t, C_2, C_6, C_{11} and C_3') for methylene carbons and 14.12 δ (q, C_1, C_{12} and C_4') methyl carbons. This data revealed that the compound A_1 is to be Dodecane -4 – yl butyrate. This compound has been isolated for the first time from this plant.

The allelochemicals present in such plants exuded or leached in the environment and cause adverse effect on recipient plants. The invasive weed C. uniflora may also be leaching out allelochemicals which had inhibitory effect. This allelopathic potential was confirmed by seed germination bioassay. To know and confirm the nature of such allelochemicals in Cassia leaves, NMR spectral analysis was conducted. This analysis revealed that the allelochemicals was aliphatic ester Dodecane-4–yl butyrate. This was identified and characterized for the first time in this plant. The study of individual and specific allelopathic action of this compound is in progress by screening different sensitive crops. Similar characterization of novel allelochemicals in ferns like Tectaria and Pteridium was confirmed [38]. Similarly it was reported [39] that polyphenols like catechin, epicatechin and epigallocatechin in Croton lechleri. Characterization of bioactive compounds from potamogetonaceae was done by same method and their activity was studied against aquatic organisms [40].

CONCLUSIONS

Seed germination bioassay studies have clearly thrown light on the allelopathic influence of Cassia uniflora as the leachates of this weed inhibited the seed germination, root and shoot length, root: shoot ratio and vigor index. Not only that but it also influenced the seedling physiology. The allelopathic potential and phytotoxic nature of Cassia might be due to the complex mixture of components, which might be strong inhibitors for the growth and development of associated plants due to presence of different allelochemicals.

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