

Allelopathic effects of phenolics and terpenoids extracted from *Gmelina arborea* on germination of Black gram (*Vigna mungo*) and Green gram (*Vigna radiata*).

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ABSTRACT

The allelopathic influence of *Gmelina arborea* was determined on the seed germination and seedling growth of green gram and black gram. Their dried leaves were collected and the active principles were extracted. A Thin Layer Chromatography was performed which indicated the presence of polyphenols and terpenoids. The extract was subjected to FTIR analysis and GC-MS. FTIR spectrum showed a -OH stretch at 3416 cm⁻¹ C-O stretch and C-H stretch at 1127 cm⁻¹ & 2924 cm⁻¹ respectively. Indication of the presence of phenolic and terpenoid based compounds which was confirmed by MS. GC- Mass Spectrometric analysis showed characteristic peaks corresponding to terpenoids like Shiromodiol diacetate, 2 Butyl methacrylate, Brusatol and Menthofuran and phenolic compounds like Coumaric acid, Gallic acid, Syringic acid and Vanillic acid. In laboratory bioassay, the effects of extracts were determined on the seed germination and seedling growth of test crops. The *G. arborea* extracts inhibited the germination, seedling growth and the total protein content of both test crops. The PASS (Prediction of Activity Spectra for Substances) prediction showed that the compounds present in *G. arborea* extracts inhibited the proteolytic enzymes [Phosphatase and Propyl aminopeptidase (Pa>70%)], important for seed germination. Thus allelochemicals inhibited the activity of these enzymes and thereby either retarded the seed germination. Hence some preventive measures may be taken to minimize the water drainage from these tree orchards grown for wood, pulp and fuel wood.

Key words: Allelochemicals, Black gram, FTIR, GC-MS, *Gmelina arborea*, Green gram, PASS, Phosphatase and Propyl aminopeptidase inhibitors, pulses, *Vigna mungo*, *Vigna radiata*.

INTRODUCTION

Woody plants are often combined with annual or perennial crops or live stock to increase the biophysical and/or socio-economic productivity of an agricultural enterprise (4). But this often raises concern about the allelopathic effects of trees on cultivated lands and standing crops. The allelopathic effects are selective and vary with different trees and the amount of secondary metabolites released (23). Allelopathy is defined as the direct or

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indirect harmful or beneficial effects of one plant on another through the release of chemical compounds into the environment. Several phytotoxic substances causing germination and growth inhibitions have been isolated from the plant tissues and soils (1). These substances collectively known as allelochemicals are usually secondary plant products of metabolic pathways of plants (25). These are released into the environment through root exudation, leaching through dew and rains volatilization and decaying plant tissue (18). Generally trees are the most potent source of allelochemicals and the decomposition of leaf litter and litter leachings are the major modes of release of allelochemicals in to the soil (17). Phenolic and terpenoids are principle allelochemicals (secondary plant metabolites) present in leaves, fruits, vegetables and legumes. The release of phenolic compounds adversely affects the germination and growth of plants through their interference in cell division, mineral uptake and several other biosynthetic processes.

Tree allelopathy causes bare forest with poor or no vegetation under canopy of some trees, regeneration problem etc. In the tropics, agro-forestry systems allelopathy influences both positive and negative effects in the crop-soil interactions. The mulches and prunings of trees may release allelochemicals that suppress growth and the leaf extracts from 5-years old Multipurpose trees (*Gliricideseppium*, *Gmelina arborea*, *Eucalyptus sp.*, *Terminalia superba* etc.) adversely effects the early growth of cowpea (11).

Gmelina arborea Roxb. (family Verbenaceae) occurs naturally in 11 countries in tropical and subtropical regions (5° to 30° N and 70° to 110° E) of Asia (6,8) in tropical deciduous forests with *Tectona grandis*, *Melia azadiaract*, *Lagerstromia spp.* and *Pterocarpus macrocarpus*. Its fast growth and suitability for pulp making have made it popular. It can be propagated from seeds, cuttings and stumps and may grow 35 m tall and 3 m in diameter in Asia. Allelochemicals responsible for the allelopathic effects of *G. arborea* have not been characterized. Hence, we analyzed its leaf litters for chemical composition and determined the bioactivity of aqueous extracts on seed germination and seedlings growth of *Vigna mungo* (Black gram) and *Vigna radita* (Green gram).

MATERIALS AND METHODS

Fresh leaves and leaf litters of *Gmelina arborea* were collected from 5-years old plantation and stored in polythene bag in a moisture free atmosphere. The leaves were dried in shade. Seeds of Green gram (Co. 6) and Black gram (Co. 5) for the bioassay were procured from Department of Seed Science and technology, Tamil Nadu Agricultural University, Coimbatore.

Extraction of allelochemicals:

Fifty g fresh leaf litters were soaked in 150 mL of distilled water for 24 h at 25°C to extract the allelochemicals. The extract was filtered through two layers of Whatman No.1 filter paper and further extracted as per Harborne *et al* (9) for identification of phenolic compounds. The aqueous leachates were mixed with an equal volume of methanol acidified with 1 mL of HCl. This methanolic extract was mixed with 100 mL peroxide free diethyl ether in a separating funnel. The upper organic phase was separated in a 100 mL Erlenmeyer flask. To the aqueous phase twice the volume of ethyl acetate was added and the organic phase was separated using a separating funnel. The process was

repeated 3 times. All these organic phases were mixed together and to it 3 g sodium sulfate was added and filtered. The final extract was dried thoroughly in a rotary evaporator and stored in air tight vials.

Samples were spotted on pre-coated plates of silica gel G and separated using chloroform: ethyl-acetate: formic acid (5:4:1, v/v/v) as mobile phase. The Phenolic compounds were visualized by spraying the plates with FeCl_3 (2% in ethanol).

Characterization by FT-IR: The phenolic extract was examined through FTIR (FTIR 8400S- Shimadzu) and the absorbance peak was analyzed. The sample was mixed with KBr in the ratio of 1:100 and pressed to form a pellet at 4 t pressure and the sample was analyzed using the IR Solution Software with 20 second scan at a resolution factor of 4.0.

Characterization by GC-MS: The total aqueous leachate (100 μ l) was mixed in 10 ml methanol. Gas chromatographic analysis was carried out on Fison GC 8000 Top coupled to Fison MD 800 MS under electron impact ionization (70 eV). The MS scan range was 30-200 atomic mass units (AMU). The chromatographic column for the analysis was a fused silica WCOT-Fused Silica capillary column (30 m x 0.25 mm i.d.; DB-35 MS 5CP, 0.25 μ m). The carrier gas used was helium at a flow rate of 1 mL/min. Samples were analyzed with the column held initially at 100°C for 3 min and then increased to 195°C with a 6°C/min heating ramp and then kept at 195°C for 3 min. Finally, temperature was increased to 250°C with a 10°C/min heating ramp and kept at 250°C for 10 min. The injection was performed in split mode (50:1) at 250°C. The identification of the components was based on the Wiley and Nist mass spectral library and interpretation was carried out to identify the possible compounds and their structure was determined using Chemspider Database and the possible activities of these structures were determined by PASS database.

Seed bioassay

To determine the allelopathic potentials of *Gmelina arborea* aqueous leachates on black gram and green gram rapid bioassay was done (10). 50 g clean sand of neutral pH was weighed into 4 inch dia pots. A Whatman no.1 filter paper was then placed on the sand surface in each dish. Ten mL extract of 5, 10 and 20% concentrations were added to the petri dishes as per treatments and deionized water was used as control. Twenty viable seeds of green gram and black gram were placed separately on the surface of petri dishes and maintained at Room Temperature for 5 days. The bioassay was conducted in completely randomized design with four replications. The number of germinating seeds was recorded each day. After 5 days, the seedlings were removed from Petri dishes and root and shoot lengths were measured. The fresh weight was recorded for 10 randomly selected seedlings. The seedlings were dried in hot air oven at 60°C and the dry matter was recorded.

Soluble protein Content: One g complete seedling of green gram and black gram were ground in mortar and pestle in phosphate buffer (pH 7.0). The contents were filtered using Whatman no.1 filter paper and centrifuged at 10,000 rpm at 4°C for 10 min. The supernatant was collected and the total protein content was determined using Folin phenol reagent (13).

Statistical analysis: The data were analyzed by conventional analysis of variance procedures. The means were compared by least significant differences ($p=0.05$) using SPSS Software package.

RESULTS AND DISCUSSION

The leachates of *Gmelina arborea* showed the presence of phenolic compounds on a Thin Layer Chromatogram. Further the FTIR spectrum showed distinct –OH stretch at 3416 cm^{-1} C-O stretch and C-H stretch at 1127 cm^{-1} & 2924 cm^{-1} respectively. The GC-MS revealed distinct peaks which pertained to the following compounds: 3-(4-hydroxyphenyl)prop-2-enoic acid, 3,4,5-hydroxybenzoic acid, 4-hydroxy-3,5-dimethoxybenzoic acid, 4-Hydroxy-3-methoxybenzoic acid which are all phenolic compounds and Shiromodiol diacetate, 2 Butyl methacrylate, Brusatol and Menthofuran which are all terpenoid and non-terpenoid compounds (Figure 1). The allelopathic effects could be attributed to the presence of these phenolics and terpenoids. From red pine benzoic acid and eleven other phenolic acids have been characterized by GC (12). Rimando *et al.* (19) also reported that 1,2- benzenedicarboxylic acid, bis(2-ethylhexyl) ester was found in the rice (*Oryza sativa* L.) cv. Taichung Native 1 and it could inhibit the germination of lettuce (*Lactuca sativa* L.). Several other benzenedicarboxylic acids like 1,2-benzenedicarboxylic acid in *Cucumis sativus* (2) and 1,3-benzenedicarboxylic acid (22) showed allelopathic effects. Several benzoic acid derivatives are allelopathic to several plant species. Sasikumar *et al.* (20) reported the allelopathic effects of benzoic acid derivatives on Redgram. Similarly several reports are available on the inhibitory effects of terpenoids released from trees.

The effects of standard phenolic compounds on germination of black gram and green gram were observed at 1% & 5% concentrations (Table 1). The allelopathic compounds significantly affected the seed germination and seedling growth (Table 2). Seedling length was drastically reduced in the order of 18, 25 and 31% of inhibition in black gram and 14, 21 and 31% inhibition of green gram at 5, 10 and 20% concentrations of the leachate respectively (Figure 2). The dry matter production and total soluble protein content were drastically reduced. More than 95% soluble proteins in germinating seeds comprises of enzymes, which play important role in germination; hence, the reduction in concentration of these soluble proteins has a direct effect on the seed vigour, which is evident from the corresponding inhibition in germination. There was a significant inhibition in the order of 22%, 40% & 59% in black gram and 18%, 26% & 38% in green gram at 5%, 10% & 20% concentrations of leachate respectively (Figure 2). Intervention of allelochemicals like phenolics and terpenoids in the metabolic process and growth properties of various plant species were reported (15). The allelopathic potential of sunflower on wild barley germination and seedling growth was reported by Ashrafi *et al.* 2008 (3). Terzi *et al.* has reported the allelopathic effects of Juglone and decomposed walnut leaf juice on muskmelon and cucumber seed germination and seedling growth (24). Wang *et al.* has reported that phenolics were involved in the phytotoxicity caused by sugarcane straw. Derivatives of benzoic acids can alter transpiration and ion uptake as well as induce oxidative cell damage (16).

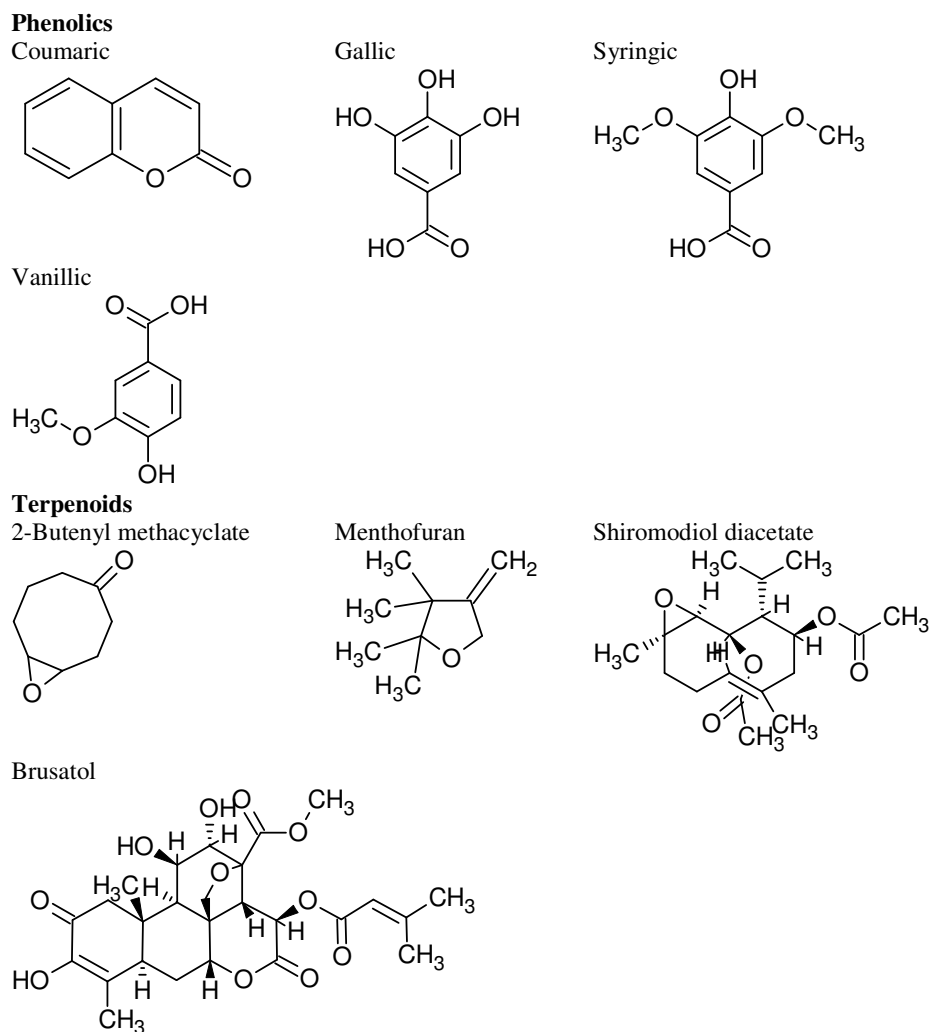


Figure 1. Chemical structures of the compounds identified by GC-MS

The activity of predicted compounds was determined by PASS software (Table 3). Both the phenolics and terpenoids seem to have inhibitory effects on two major enzymes namely Phosphatase and Prolyl aminopeptidase. The inhibition of phosphatase directly effects the energy metabolism and ATP production and also contributes to the seed vigour and retardation is visible in germination. Senna *et al*, 2005 (21) has reported the favorable role of acid phosphatase in Maize seeds germination where the activity of the

Table 1. Effects of standard phenolic compounds on germination of black gram and green gram.

Compound	Black gram germination (%)		Green gram germination (%)	
	Conc. (1%)	Conc. (5%)	Conc. (1%)	Conc. (5%)
Control	85	85	67.7	67.7
Coumaric acid	84	82	65	62
Gallic acid	79	78	60	58
Syringic acid	78	76	61	59
Vanillic acid	83	81	63	61
Siromodiol diacetate	84	83	66	64
T test	NS	NS	NS	#

Significantly different at P<0.05. NS: not significantly different (P> 0.05).

Table 2. Effects of concentrations of aqueous leaf leachates of *Gmelina arborea* on germination and seedlings growth of blackgram and greengram

Conc. (%)	Black Gram				Green Gram			
	Germination (%)	Seedling length (mm)	Dry Weight (g)	Protein Content (µg/g)	Germination (%)	Seedling length (mm)	Dry Weight (g)	Protein Content (µg/g)
0	85.0	175.5	0.56	643.3	66.7	161.0	0.22	486.6
5	68.33	143.9	0.52	502.1	51.66	139.0	0.19	398.6
10	56.66	131.3	0.48	386	43.33	127.9	0.18	357.3
20	40.0	120.75	0.45	263.3	35.0	110.8	0.16	303.3
SE	5.41	6.29	0.01	31.62	4.16	5.62	0.01	19.7
T-test	#	#	NS	NS	#	#	NS	NS

Significantly different at P<0.05. NS: Not significantly different (P> 0.05), SE: Standard Error.

Table 3. Activity of compounds determined by PASS Database

S.No	Compound	Pa	Pi	Activity
Phenolics				
1	Coumaric acid (3-(4-hydroxyphenyl)prop-2-enoic acid)	0.784	0.007	Oxidoreductase inhibitor
2	Gallic acid (3,4,5-hydroxybenzoic acid)	0.928	0.013	Prolyl aminopeptidase inhibitor
3	Syringic acid (4-hydroxy-3,5-dimethoxy-benzoic acid)	0.899	0.025	Prolyl aminopeptidase inhibitor
4	Vanillic acid (4-Hydroxy-3-methoxybenzoic acid)	0.896	0.027	Prolyl aminopeptidase inhibitor
Terpenoids				
5	6-Oxabicyclo nonan-7-one (2-Butenyl methacrylate)	0.973	0.002	Phosphatase inhibitor
6	3-Methylidene-4,4,5,5-tetramethyl tetrahydro furan-2-one (Menthofuran)	0.905	0.008	Phosphatase inhibitor
7	Shiromodiol diacetate	0.821	0.033	Phosphatase inhibitor
8	Brusatol	0.713	0.002	Chloride channel inhibitor

Pa: Possible Activity, Pi: Possible Inactivity

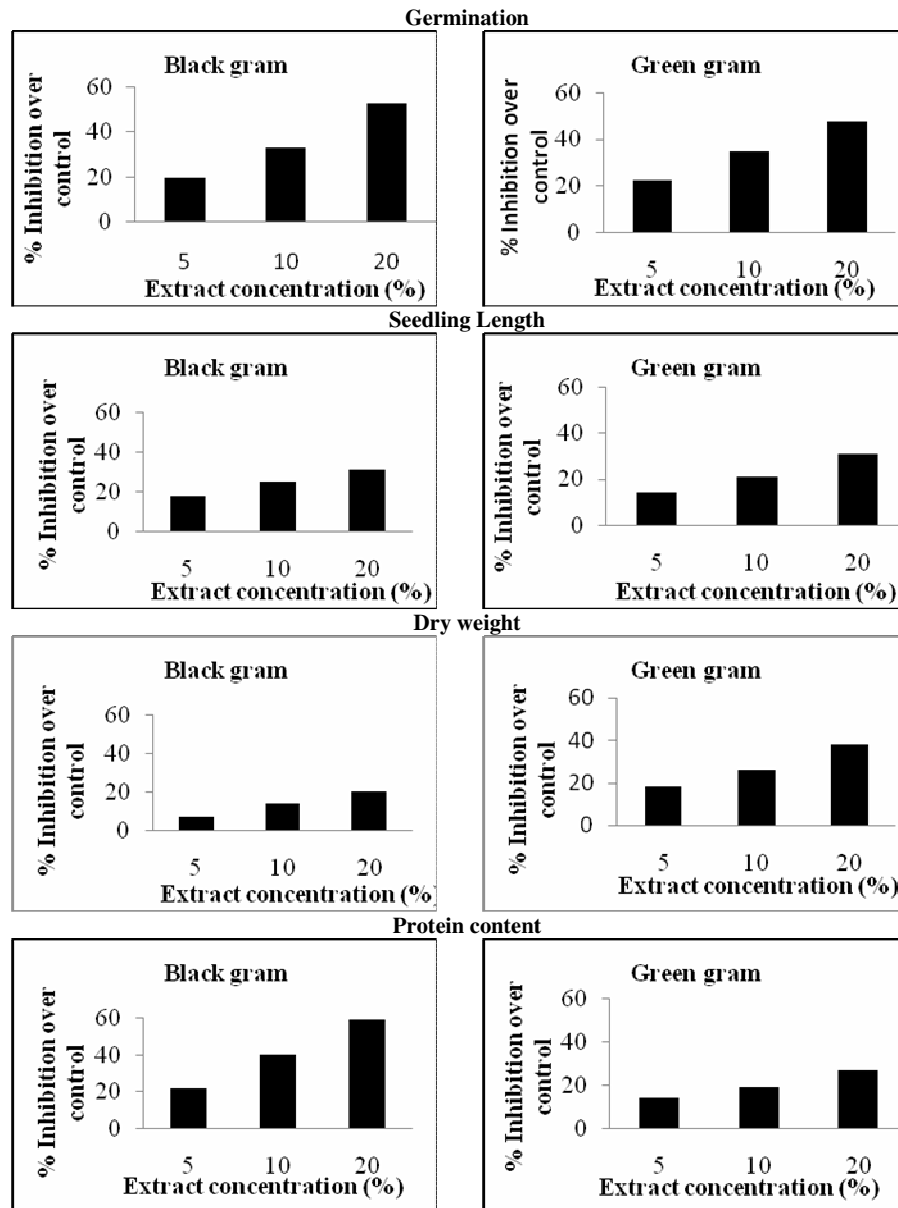


Figure 2. Comparison of inhibition percentage on black gram and green gram due to allelochemicals of *Gmelina arborea*.

directly effects the energy metabolism and ATP production and also contributes to the seed vigour and retardation is visible in germination. Senna *et al*, 2005 (21) has reported the favorable role of acid phosphatase in Maize seeds germination where the activity of the acid phosphatase increases in 24 hours of onset of germination. Similarly the levels of Prolyl aminopeptidase and acid phosphatase were higher in germinating seeds of *Lens esculenta* (5). These enzymes have the potential role in liberation of proline and other aminoacids that are released by the enzymatic hydrolysis of several seed storage protein. Similarly hydrobrusotol is a calcium channel inhibitor. Matveyeva *et al*, 2003(14) have reported several compounds like furosemide, bumetanide etc...Which are inhibitors of calcium cotransporters to suppress the germination rate to less than 50%. There is potential role of EMP enzymes particularly dehydrogenases in seed germination. David *et.al.* (7) has reported the decreased level of alcohol dehydrogenases during seed germination upon treatment with inhibitors. PASS prediction has revealed that coumaric acid inhibits the oxidoreductase (enzymes catalyzing the oxidation reduction reactions in several biochemical pathways). Hence the leachate of *Gmelina arborea* affects several such dehydrogenases, which cumulatively leads to loss of seed vigour.

The present study herein brings to limelight the inhibitory effects of allelochemicals of *Gmelina arborea* on the two important enzymes that are very essential during seed germination. The allelopathic effects can hence be attributed to the role of the compounds in inhibiting some of the potential enzymes in a seed, leading to the retarded are no germination and poor vigor index under the influence of the allelochemicals. Many reports are available which indicate the allelopathic effects of trees on crop plants. Though these phytochemicals may be useful in weed, insect and nematode control. Hence a critical evaluation of the allelopathic potential of the trees in the vicinity of the fields is essential and some measures have to be considered to prevent the clogging of the fields with the dried leaves from the neighboring trees.

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