

Potent phenolic allelochemicals from *Celosia argentea* var. *cristata* L. leaf extract based on bioactive fractions

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(Received in revised form: May 23, 2019)

ABSTRACT

We studied the phytotoxic allelopathic potential of *Celosia argentea* var. *cristata* L. against *Lepidium sativum* L. and identified the allelochemicals in its methanolic leaf extract using bioassay guided fractionation. The results revealed that cockscomb leaf extract fractions were phytotoxic at very low concentration (≥ 50 ppm). The most bioactive fractions were purified for isolation and identification of allelochemicals. Seven potent phenolic allelochemicals (p-coumaric acid, m-coumaric acid, vanillic acid, 4-hydroxybenzoic acid, m-hydroxybenzaldehyde, dihydroxybenzaldehyde and 4-hydroxybenzaldehyde) were identified by LCMS. The results suggested that allelochemicals play critical role in phytotoxic allelopathic effects of *Celosia argentea* in floriculture, natural and in agroecosystems.

Key words: Allelochemicals, allelopathy, bioassay, *Celosia argentea*, Cockscomb, ecology, *Lepidium sativum*, methanolic extract, ornamental, phenolic, phytotoxic, weed.

INTRODUCTION

Higher plants produce various secondary metabolites, which may be released into the environment. When secondary metabolites are released into the environment, they affect (negatively or positively) the growth of other plants and are known as allelochemicals and this phenomenon is called allelopathy (6). The allelochemicals mediate the plant-plant (plant-weed, weed-weed) plant-microbe, plant-insect and plant-disease interactions in natural as well as in agroecosystems (1,6,15,21,22,24). With the introduction of "Novel Weapons Hypothesis" which links allelopathy with invasive biology and ecology of plants, the allelopathy research has increased in recent years. The allelopathy phenomenon has been attributed to success of invasive plant species by outcompeting native plants in ecosystems (4,5).

The allelopathy of trees and crops has been well documented and potential allelochemicals have been identified (6). Though large number of allelochemicals have been identified from plants; but allelochemicals are present as mixture in donor plants (12). The most potent allelochemicals (bioactive at very low concentrations), have been identified from few plant species (28). For example, juglone from walnut (*Juglans nigra* L.), sorgoleone from sorghum (*Sorghum bicolor* L., Moench), catechin from spotted knapweed (*Centaurea stoebe* L.), momilactone A and B from rice (*Oryza sativa* L.), and L-DOPA from velvet bean (*Mucuna pruriens* L.) have been reported (5,10,14,27,28). Limited information is available regarding the potent phytotoxic allelochemicals from horticultural plants species, especially flowers.

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Cockscomb (*Celosia argentea* var. *cristata* L.) (family Amaranthaceae) is annual ornamental allelopathic plant and weed worldwide. Its inflorescence, young leaves and seedlings are consumed as vegetable in many countries. Besides, it is also noxious annual broadleaf weed in semi-arid regions in summer crops [cowpea (*Vigna unguiculata* L.), peanut (*Arachis hypogaea* L.), hyacinth bean (*Dolichos lablab* L.), pigeon pea (*Cajanus cajan* L.), maize (*Zea mays* L.), finger millet (*Eleusine coracana* L., Gaertn.), radish (*Raphanus sativus* L.) and sorghum (*Sorghum bicolor* L. Moench) (3,19).

It suppressed the growth of adjacent plant species (authors' personnel observation). Likewise, when its plant biomass was buried in soil, it inhibited the germination and growth of subsequent crops [millet (*Pennisetum typhoideum* Rich.) and sesame (*Sesamum indicum* L.) (2)]. Furthermore, *Celosia argentea* allelopathic extract inhibited the germination and seedling growth of many crops [*Arachis hypogaea*, *Vigna unguiculata*, *Dolichos lablab*, *Sorghum bicolor* and *Phaseolous aureus* Roxb.] (25). Its allelochemicals have already been isolated and identified (17). However, most potent allelochemical(s) responsible for its phytotoxic potential are not known.

This study aimed to identify the most potent allelochemical(s) in its leaves, responsible for growth inhibition of other plant species. We used *Celosia argentea* leaves having red colour inflorescence. The test specie was garden cress (*Lepidium sativum*) model plant species for allelopathic studies (13).

MATERIALS AND METHODS

Collection of leaves and extraction of allelochemicals

The leaves of *Celosia argentea* were collected from fully mature plants in October during the flowering from the University of Agriculture Faisalabad and experiments were performed at Govt. College University, Faisalabad, Pakistan (31.25°N, 73.09°E, elevation 184 m and average annual rainfall 350 mm). The leaves were ground to powder after oven drying at 40 °C for 3 days. Ten g dried *Celosia argentea* leaf powder was added to 100 % methanol (100 ml), heated for 5 min at 50 °C and placed for 30 min in ultrasonic device (26). After filtration, the residue was rinsed with 80 % methanol (acidified with 0.1 % HCl). After evaporation of methanol, 100 ml distilled water was added and it was considered as 10 % methanolic extract. Acetone (2.5 L) was added to equal volume of 10 % methanolic extract and was placed for 24 h at 4 °C. After filtration of extract, rotary evaporator was used to evaporate the acetone. The 100 ml extract was partitioned thrice with 200 ml ethyl acetate. After removing ethyl acetate, the aqueous layer was used for acid hydrolysis. Extract was treated with 4N HCl at 90 °C for 1 h. After filtration, the 100 ml extract was re-extracted with 300 ml ethyl acetate. Anhydrous magnesium sulfate was added to extract and placed overnight at room temperature. Magnesium sulfate was separated by filtration and rotary evaporator was used to concentrate the ethyl acetate at 45 °C under vacuum and was dried using nitrogen gas. Thus, 7000 mg and 4020 mg residues of ethyl acetate and acid hydrolyzed fractions were obtained, respectively. The residues (10 mg) were dissolved in 10 ml of LCMS grade water to purify with solid phase extraction.

Preparative thin layer chromatography (TLC)

The above-mentioned 4 g residues were dissolved in methanol (286 ml) to make 14000 ppm solution. The silica gel 60GF254 slurry was prepared using water (6:10 w/v) to

make 280 TLC plates (0.75 mm thickness). For each fraction, 140 TLC plates were prepared using 2 ml extract per plate. The glass chambers were saturated with mobile phase solvents (methanol: n-hexane: dichloromethane, 1:4:5, v/v/v) 15 min before development of plates. The bands separated on TLC plates, were observed under UV light (254 nm). Bands of fractions of both plates (acid hydrolyzed and ethyl acetate) were divided into five fractions (F1, F2, F3, F4, and F5). The bands scratched from the plates were eluted with methanol, filtered and centrifuged to collect the supernatants. Methanol was evaporated to get 700 mg, 2970 mg, 460 mg, 400 mg and 3100 mg residues of F1- F5 fractions, respectively. Each residue (80 mg) was dissolved in methanol (20 ml) to prepare 4000 ppm solution. Further dilutions (50, 500, 1000 and 2000 ppm) were made with distilled water.

Based on the results of 1st bioassay using ethyl acetate and acid hydrolyzed fractions (F1, F2, F3, F4, and F5), the most bioactive fractions (F2 and F5) were further sub-fractionated using preparative TLC. The 2000 mg residues of most bioactive (F2 and F5) fractions were dissolved in 143 ml methanol to make 14000 ppm solution. The TLC procedure for subfractions was same as mentioned-above for fractions. Thus, from all TLC plates, 28 subfractions were collected viz., 129 (f1), 135 (f2), 112 (f3), 115 (f4), 120 (f5), 203 (f6), 160 (f7), 150 (f8), 120 (f9), 124 (f10), 112 (f11), 92 (f12), 70 (f13), 70 (f14), 78 (f15), and 75 mg (f16) residues from ethyl acetate subfractions, while 150 (f17), 110 (f18), 135 (f19), 113 (f20), 139 (f21), 165 (f22), 102 (f23), 350 (f24), 136 (f25), 120 (f26), 137 (f27) and 170 mg (f28) residues from acid hydrolyzed subfractions. Each residue (40 mg) was dissolved in 20 ml methanol to make 2000 ppm solutions. Further dilutions (50, 500 and 1000 ppm) were made from for bioassay.

Based on the results of 2nd bioassay using subfractions, the highly bioactive subfractions (f 7, f 8, f 24 and f 28) were purified further for LCMS analysis. For this, 30 mg residues of f 7, f 8, f 24 and f 28 subfractions were dissolved in 5 ml methanol to purify further with preparative TLC. The methanol was evaporated and 25 mg purified residues of highly bioactive subfractions were dissolved again in 12.5 ml methanol to make 500 ppm solutions in LCMS analysis.

Bioassays and identification of allelochemicals

The bioassays were done using the previously described procedure (17).

Bioassay I: Methanolic extracts of acid hydrolyzed and ethyl acetate fractions (F1, F2, F3, F4, and F5) were used at concentrations 0-4000 ppm. Methanolic solution (2 ml) of each concentration was added to filter paper in petri dish (4.5 cm dia). In control treatment petri dishes, 2 ml of 100% methanol was added, and it was evaporated followed by addition of 2 ml distilled water. Five seeds of *Lepidium sativum*, surface sterilized (with 2% sodium hypochlorite for 2 min), were placed equidistant on filter paper in each petri dish. The petri dishes were sealed and placed in a growth chamber (24± 2°C and 14/10 h, dark/light). Germinated seeds were counted daily for 10-days. Data on root length, shoot length and seedling dry weight were recorded 6 days after sowing.

Bioassay II: Methanolic extracts of acid hydrolyzed and ethyl acetate subfractions (f1 to f28) were used at concentrations 0-4000 ppm. Methanolic solution (2 ml) of each concentration was added to filter paper in petri dish (4.5 cm dia). In control treatment petri dishes, 2 ml of 100% methanol was added, and it was evaporated followed by addition of 2 ml distilled water. Five seeds of *Lepidium sativum*, surface sterilized (with 2% sodium hypochlorite for 2 min), were placed equidistant on filter paper in each petri dish. The petri

dishes were sealed and placed in a growth chamber (24 ± 2 °C and 14/10 h, dark/light). Germinated seeds were counted daily for 10-days. Data on root length, shoot length and seedling dry weight were recorded 6 days after sowing. Based on results of bioassay II, highly bioactive subfractions were used for further LCMS analysis. The LCMS analysis was done using the same procedure as previously described (23).

Statistical analysis

Data were analyzed by ANOVA and post-hoc Duncan's Multiple Range Test was applied to compare treatment means at $P < 0.05$. Experiment was arranged in completely randomized design with 5 replications.

RESULTS AND DISCUSSION

Phytotoxic allelopathic potential

(i). **F1-F5 fractions** : Although all fractions (F1-F5) were inhibitory to seeds germination of *Lepidium sativum*, but F2 and F5 were inhibitory and at > 500 and > 50 ppm concentrations, completely inhibited the seeds germination of *Lepidium sativum* (Table 1). Furthermore, when means of concentrations of each fraction were compared, the F2 and F5 inhibited the final germination by 71.66 and 82.91 %, respectively than control. At highest concentration (4000 ppm), the F1 fraction completely inhibited the germination, while F3 and F4 were inhibitory to seeds germination of *Lepidium sativum* at all concentrations. Nevertheless, F1, F3 and F4 suppressed the seeds germination and seedling growth of *Lepidium sativum* at higher concentrations [1000-4000 ppm] (Table 1 and Fig. 1). The different methanolic leaf extract fractions had various inhibitory allelopathic effect on germination and seedling growth of target plant species (18).

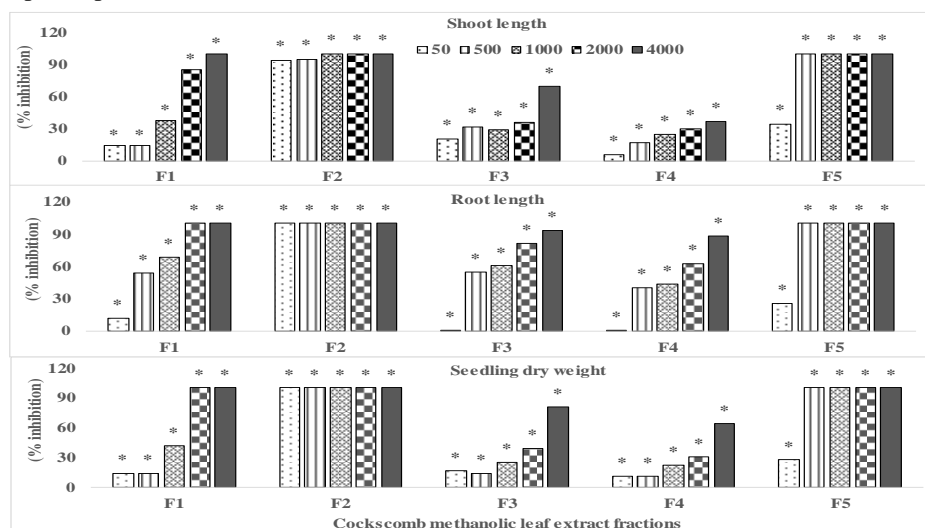


Figure 1. Phytotoxic effects of *Celosia argentea* methanolic leaf extract fractions (F1-F5) at 0-4000 ppm concentrations on shoot and root length and seedling dry weight of *Lepidium sativum*. The data on Y-axis are presented as % inhibition as compared with control; *Shows significantly different from respective control at $P < 0.05$; F=fraction.

Table 1. Phytotoxic effects of F1-F5 fractions of *Celosia argentea* leaf extract on germination of *Lepidium sativum*

Fraction	Conc (ppm)	Germination (%)	Time to start germination (days)	Germination time (days)	Germination Index
Control	0.0	96a	1ef	2.53ef	15.57a
F1	50	86abc (-10.41)	1ef (0)	2.86de (13.04)	12.03bc (-22.73)
	500	88abc (-8.33)	1ef (0)	2.09fg (-17.39)	10.80bc (-30.63)
	1000	64e (-33.33)	1.60de (60)	2.52ef (-0.39)	8.18def (-47.46)
	2000	18f (-81.25)	3.40b (240)	3.65bc (44.65)	0.53j (-96.59)
	4000	-	-	-	-
	Mean	51.2 (-46.66)			
F2	50	74d (-22.91)	3b (200)	4.14ab (63.63)	3.79hi (-75.65)
	500	62e (-35.41)	4a (300)	4.54a (79.44)	1.94ij (-87.54)
	1000	-	-	-	-
	2000	-	-	-	-
	4000	-	-	-	-
	Mean	27.2 (-71.66)			
F3	50	96a (0)	1ef (0)	1.55 (-38.73)	12.60b (-19.07)
	500	90abc (-6.25)	1ef (0)	1.53gh (-39.52)	12.50b (-19.71)
	1000	92ab (-4.16)	1ef (0)	1.57gh (-39.74)	11.60bc (-25.49)
	2000	92ab (-4.16)	2.00cd (100)	2.60ef (2.76)	6.27fg (-59.73)
	4000	80cd (-16.66)	2.40c (140)	3.50c (38.33)	5.24gh (-66.34)
	Mean	90 (-6.25)			
F4	50	96a (0)	1f (0)	1.22h (-51.77)	11bc (-29.35)
	500	94a (-2.08)	1ef (0)	2.39ef (-5.53)	15.83a (1.66)
	1000	88abc (-8.33)	1ef (0)	1.63gh (-35.57)	10.20cd (-34.48)
	2000	88abc (-8.33)	1ef (0)	2.85de (12.64)	10.67bc (-31.47)
	4000	64e (-33.33)	1ef (0)	2.64ef (4.34)	8.50de (-45.40)
	Mean	86 (-10.41)			
F5	50	82bcd (-14.58)	2cd (100)	3.32cd (31.22)	6.53efg (-58.06)
	500	-	-	-	-
	1000	-	-	-	-
	2000	-	-	-	-
	4000	-	-	-	-
	Mean	16.4 (-82.91)			

Data are presented as mean of 5 replications; Significant differences among the treatments are indicated by different letters in the same column ($P < 0.05$ by Duncan's Multiple Range test); The values in parentheses indicate % increase (+) or decrease (-) over control; F= Fraction

(ii). Subfractions f 1 to f 28

Based on results of 1st bioassay with fractions F1-F5 (Table 1 and Fig. 1), two most bioactive fractions (F2 and F5) were selected for further sub-fractionation. Twenty-eight subfractions were obtained from F1 and F5 fractions (Table 2). Keeping in view the bioactivity and phytotoxic effects of subfractions (f 1-f 28) on seeds germination and seedling growth of *Lepidium sativum*, the subfractions were divided in 4-Categories:

(A) Highly bioactive : Four subfractions f 7, f 8, f 24, and f 28, which completely inhibited germination of *Lepidium sativum* at lowest concentration of >50 ppm, were considered as the highly bioactive subfractions (Table 2).

(B) Moderately bioactive : The subfractions f 6, f 9, f 10 and f 27 suppressed root growth of *Lepidium sativum* (50-80 %) \geq 500 ppm were categorized as moderately bioactive.

(C) Less bioactive : Less bioactive subfractions (f 5, f 25 and f 26) reduced root length of *Lepidium sativum* by 20-50 % at \geq 500 ppm.

(D) Least bioactive : The least bioactive subfractions such as f 1, f 2, f 3, f 4, f 11, f 12, f 13, f 14, f 15, f 16, f 17, f 18, f 19, f 20, f 21, f 22 and f 23 suppressed the *Lepidium sativum* root growth up to 20 % at \geq 500 ppm (Table 2).

Table 2. Bioactivities of methanolic leaf extracts subfractions (f 1-f 28) and allelochemicals identified from highly bioactive subfractions

Bioactivity Category	Growth inhibition	Subfractions	Allelochemicals identified in Subfractions
A.Highly bioactive	Completely inhibited <i>Lepidium sativum</i> germination at >50 ppm	f 7, f 8, f 24, f 28	f 7 : <i>m</i> -Hydroxybenzaldehyde, <i>p</i> -coumaric acid and vanillic acid , f 8 : 4-Hydroxybenzoic acid and <i>m</i> -coumaric acid; f 24 : Dihydroxybenzaldehyde; f 28 : 4-Hydroxybenzaldehyde
B.Moderately bioactive	Inhibited <i>Lepidium sativum</i> root growth by 50-80% at \geq 500 ppm	f 6, f 9, f 10, f 27	-
C.Less bioactive	Inhibited <i>Lepidium sativum</i> root growth by 20-50% at \geq 500 ppm	f 5, f 25, f 26	-
D.Least bioactive	Inhibited <i>Lepidium sativum</i> root growth by <20% at \geq 500 ppm	f 1, f 2, f 3, f 4, f 11, f 12, f 13, f 14, f 15, f 16, f 17, f 18, f 19, f 20, f 21, f 22 and f 23	-

Previously, phytotoxicity of *Celosia argentea* allelopathic leaf extract has been reported (25). In the present study, differential phytotoxicity of different fractions and subfractions helped to isolate most bioactive fractions for further isolation and identification of allelochemicals. The highly bioactive subfractions were further analyzed using LCMS for identification of allelochemicals.

Identification of allelochemicals in highly bioactive subfractions by LCMS

In f 7 subfraction, three compounds (*m*-hydroxybenzaldehyde, vanillic acid and *p*-coumaric acid) were identified. Two compounds (4-hydroxybenzoic acid and *m*-coumaric acid) were identified in subfraction f 8, while dihydroxybenzaldehyde and 4-hydroxybenzaldehyde were identified in subfraction f 24 and f 28, respectively (Table 2). These seven secondary metabolites have previously been reported as allelochemicals

inhibitory to growth of plant species (7,20). It is notable that there was at least one allelochemical in each four highly bioactive subfractions, which belongs to benzaldehydes and benzoic acids which have recently been reported as a new class of bioactive and allelopathic compounds (7). Ninety-two benzene derivatives have been evaluated for allelopathic potential against germination and root growth of *Brassica campestris*. Benzaldehydes and benzoic acids including m-hydroxybenzaldehyde, 4-hydroxybenzoic acid, dihydroxybenzaldehyde and 4-hydroxybenzaldehyde were most phytotoxic against the test species with IC₅₀ (concentration inhibiting root growth of test species by 50%) of <100 mg/L (7).

Furthermore, benzoic acid derivatives have been used as commercial herbicides (8). Therefore, the phytotoxic allelopathic potential of *Celosia argentea* might be due to presence of seven allelochemicals especially benzaldehydes and benzoic acids. The efficacy of allelochemicals to inhibit the plant growth is often attributed to their combined action (11,15). Generally, there are more than one allelochemicals released by plants, which may work in synergistic way to suppress growth of nearby plants. Previous reports suggested that a mixture of allelochemicals had synergistic effect, thus resulting in higher plant growth inhibition by an allelochemical mixture than growth inhibition by respective individual allelochemicals (9,11,17).

CONCLUSIONS

Cockscomb is allelopathic plant and its phytotoxicity might be due to the presence of allelochemicals. Seven allelochemicals were found in *Celosia argentea* leaves, which may contribute to its allelopathy. It seemed that the allelochemicals in leaf extract of *Celosia argentea* may have joint phytotoxic action, which affected the growth of neighbouring plants. These allelochemicals may play significant role in ecology of *Celosia argentea* in floriculture and as weed in agroecosystems.

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