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ALLELOPATHIC ACTIVITY OF *LEONURUS SIRIBICUS* L. ON SEED GERMINATION AND SEEDLING GROWTH OF WHEAT AND IDENTIFICATION OF 4- HYDROXY BENZOIC ACID AS AN ALLELOCHEMICAL BY CHROMATOGRAPHY

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Abstract

The aim of this study was to investigate the allelopathic effects of *L. siribicus* extract on seed germination and seedlings growth of wheat as well as to identify potential allelochemical. The different concentration (5, 10 and 15%) of aqueous extract were applied during the time of sowing and at 5 days after sowing of wheat seed. *L. siribicus* extract showed concentration and time - depending activity. Different concentration of aqueous extract inhibited seed germination, seedlings growth, when extracts were applied during the time of seed sowing. The stimulatory effect of seedlings growth were found for 5 % aqueous extract, in contrast 10 and 15% extract inhibited seedlings growth, when extracts were applied at 5 days after sowing. Apart from, 4-hydroxy benzoic acids affected seedlings growth irrespective of application time. The weight of dry matter of wheat seedlings were increased for 5% than 10 and 15% extracts. Thin layer chromatography suggested that the presence of 4-hydroxy benzoic acid including other allelopathic and growth regulatory compounds inhibited germination and seedlings growth. Mineral composition was determined and its might have some stimulatory effect on seedlings growth. It was interesting that 5% extract inhibited germination and seedlings growth, when it was applied during the time of seed sowing, but stimulated seedling growth, when it was applied at 5 days after sowing. The extract of this plants can be used either for bioherbicide as well as growth stimulatory agents for the organic farming system. To find out molecular mechanism behind it, further research is to be done.

Key words: Allelopathic, *Leonurus siribicus*, Germination, Wheat, Chromatography.

Introduction

The term allelopathy was first defined as the influence of one plant to another plant by releasing chemicals (Molish, 1937). The chemicals releases from one plant and which has direct or indirect beneficial or harmful effect on other plant is called allelochemicals (Rice, 1984). A substantial number of allelochemicals have been identified but most of these are secondary plant metabolites which belonging to the groups of terpenoids, phenolic compounds, long chain fatty acids, organic cyanides, organic acids, alakaloids and others (Macias *et al*., 2001; Oleszek & Stochmal, 2002). Organic acids are major water soluble allochemicals that can be inhibited seedlings growth, by altering nutrient uptake and transport, by reducing chlorophyll content (Baziramakenga *et al*., 1994). These organic acids, especially phenolic acids, such as cinnamic, vanillic, coumaric, and benzoic acid have been isolated and identified as allelochemicals (Ohno *et al*., 2001; Yu *et al*., 2003; Asao *et al*., 2003; Hao *et al*., 2006; Lee *et al*., 2006; Wu *et al*., 2008a,b). 4-hydroxy benzoic acid (HBA) one of the secondary plant metabolites inside the plant which is potential phytotoxic allelochemicals (Bolwell *et al*., 1986; Wu *et al*., 2001; Yu *et al*., 2003). This compound has also been identified and isolated in *Leonurus siribicus* L. (Sheng-Ming *et al*., 2006).They also isolated various phenolic compounds like 4-hydroxythiophenol, syringic acid, apigenin, genkwanin, isoquercitrin, rutin. In another studied showed that syringic acid and 4-hydroxybenzoic acid are closely related to allelopathic effect on some plants (Sasikumar *et al.*, 2001). *L. sibiricus* L. (Lamiaceae), plant native of India and now naturalized in South America, contains terpenoids and phenolic substances with demonstrated allelopathic effects. It is the common weeds grown in crop field, locally called "Honeyweed" or Siberian motherwort, is an annual or biennial herbaceous plant with upright stems that grown in central and Southwest Asia (Sheng-Ming *et al.*, 2006). *L. siribicus* is also commonly grown in crop filed in Bangladesh. *L. sibiricus* L. leaves have potential allelopathic effect on germination and seedlings growth of *Raphanus sativus*, *Lactuca sativa*, and *Lepidium sativum* (Almeida *et al*., 2008) and on *Solanum melongena*, *Abelmoschus esculentus*, *Amaranthus tricolor* and *Cucumis sativus In vitro* (Sayed *et al.*, 2012). It has been studied that leaves of *L. siribicus* have four major flavonoids (quercetin-3- O-a-L-rhamnopyranosyl- (1>6)-b-D-galacto pyranoside; rutin; hyperin, and isoquercetrin) and of three minor flavonoidic compounds (genkwanin, 3-hydroxy genkwanin, and quercetin) which might be inhibited the seed germination and seedling growth *Raphanus sativus*, *Lactuca sativa*, and *Lepidium sativum* (Almeida *et al*., 2008). It is important thing that HBA has been found in *L. sibiricus*. On the other hand, it has been reported that HBA has allelopathic effect on some plant, but no one did identify HBA as allelochemicals from *L. sibiricus* L. Although, various research works have been conducted with its medicinal value but research data are not available on its allelopathic effect on cereal crops like wheat. Thus, the present study was conducted in order to investigate the allelopathic effect of *L. siribicus* on seed germination and seedling growth of wheat as well as identification of potential allelochemicals.

Materials and Methods

Experimental site, Collection of *Leonurus siribicus* and wheat seeds: The experiment was conducted at the Laboratory, Department of Biochemistry and Molecular Biology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Department of Biochemistry and Molecular Biology, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur and Department of Agricultural Chemistry, Bangladesh Agricultural University, Myemensingh, Bangladesh from November 2012-June 2014. Aerial parts of *L. siribicus* were collected from different agricultural fields of Ataikula (24° 2' 0" North, 89° 24' 0" East) and Atghoria (24.1333°N 89.2500°E) under the district of Pabna, Bangladesh in October, 2012. The collected plants were washed gently with tap water to remove the contaminants like soil and other hazard and kept it oven for drying in a constant 60^{o}C temperature until complete drying was ensured. Wheat seeds (Shatabdi cultivar) were collected from Wheat Research Institute, Dinajpur, Bangladesh. The purity and germination percentages of these seeds were 90% and 85-95% respectively.

Preparation of plant extracts and 4-hydroxy benzoic acid solutions: Dried aerial parts of *L. siribicus* were cut into small pieces and ground by mini grinder machine. The ground powder were kept in polythene bag with its mouth properly closed at 4^{o}C until extraction. Aqueous extract of *L. siribicus* was prepared following the previously stated protocol (Sayed *et al*., 2012; Aktar *et al*., 2012; Roy *et al*., 2012). In brief, the ground powder of *L. siribicus* species was weighted as 5, 10 and 15 g using electronic digital balance and powder were soaked overnight in 100 ml distilled water in a clean beaker. The leachate were filtered through a muslin cloth and squeezed by pressing with hands, thereafter, the filtrate was again filtered through Whatman No.1 filter paper to separate the suspended particles. The filtrate obtained after filtering was 5, 10 and 15% extract used for the study. Different concentration of 4-hydroxy benzoic acid (HBA) solution was prepared following previously stated by Hussain *et al.*, 2010. In brief, HBA was obtained from Sigma Chemical Company (Saint Louis, Missouri 63103, USA). The stock solutions of HBA was made by water: methanol (80:20) and the methanol was evaporated by rotary evaporator, finally concentration was adjusted to 5, 10 and 15 mM. The pH of these solutions was adjusted at 6. The control treatment was prepared by water and methanol.

Experimental design and treatment details: The experiment was designed following by Complete Randomized Design (CRD) with three replications where different concentration of *L. siribicus* extracts and HBA were applied during the time and at 5 days after sowing (DAS) of wheat seeds. The treatments were control (only distilled water), 5%, 10%, &15% *L. siribicus* extract for one set of experiment and control (distilled water and methanol), 5, 10 &15 mM of HBA for the second set of experiment.

Seed sowing: Seeds of wheat (Shatabdi) cultivars were surface sterilized by dipping the seeds in 1% mercuric chloride solution for 2 min and rinsed thoroughly with sterilized water. Wheat seeds (50 numbers) from each treatment were subjected to germination test in Petri dishes lined with doubled layered filter paper at laboratory maintaining 12 hours day and night, 15-20^{o}C temperature and 60-80% relative humidity until 15 days. About 20 ml of extracts and HBA of each were poured into the Petri dishes during the time of sowing and at 5 days after sowing of wheat seed, respectively. Also a control Petri dish in which only water was applied. Thereafter, filter papers were regularly moistened with distilled water.

Germination and growth bioassay: Percent germination was calculated on 15th days as prescribed by ISTA (17) (Anonymous, 1993). The percent germination of each treatment was compared with the control using the equation stated by Islam & Kato-Noguchi (2013):

$$\text{Germination (\% of control)} = \frac{G_T}{G_O} \times 100$$

where, G_T= average number of germinated seed with treatment at the same time of measurements, G_0= average number of germinated seed with control in each time of measurements.

Shoot and root length were measured on 15th days from three randomly selected normal seedlings. Average of three root length was calculated and expressed in centimetre. The growth promotion or inhibition was calculated using the following equation previously described by Islam & Kato-Noguchi (2012).

The randomly selected three normal seedlings that were used for measuring root and shoot length used for recording dry weight of seedlings and expressed in grams. Dry weight of three seedlings was recorded after drying in oven maintained at 65^{o}C temperature for 72 hours. The dried seedlings were weighed and expressed in grams.

Preparation of *Leonurus siribicus* extract and Thin Layer Chromatography: For preparation of extract, 2 g dried and powdered was taken in a thimble and 50 mL distilled water was added in soxhlet apparatus. The extracton was continued for about 24 hours in a water bath keeping the temperature not more than 65°C ± 2°C.The extract was collected from soxhlet apparatus and the thimble was washed. The leaf extract was preserved in a conical flask for further use. Thin layer chromatography of this extract was done and it was then developed in a TLC tank containing (ethyl acetate: benzene=4:1) selected mixed solvent and R_f values of colored spots under iodine vapor was determined.

$$\text{Inhibition or promotion} = \left(1 - \frac{\text{Length of shoot or root with treatment}}{\text{Length of shoot or root with control}}\right) \times 100$$

Mineral composition of *Leonurus siribicus*: The study was concerned with the analysis of essential minerals i.e., Ca, Mg, P, S, K, Cu and Mn, in *L. siribicus* were determined by Hunter Method (1984) and Cu and Mn by wet oxidation methods as described by Jackson (1973).

Statistical analysis: The data were subjected to two-way analysis of variance (ANOVA) by GraphPad Prism 6.0 (GraphPad Software, Inc., LaJolla, California, USA).

Results

The aqueous extract of *L. siribicus* significantly inhibited seed germination when they were applied during the day of seed sowing. At 5, 10 and 15% extract showed the germination 31.68, 30.19 and 5.78%, respectively. In contrast, germination inhibitory activity was not observed at 5 days after sowing and showed 100% germination of wheat seeds (Fig. 1). On the other hands, 5 mM HBA was not inhibited germination while 10, 15 mM HBA inhibited seed germination when it was applied during seed sowing. At 5, 10 and 15 mM HBA had no any significant effect on seed germination, when it was applied at 5 DAS (Fig. 2). The two way ANOVA analysis showed that the main effects were sowing time and the interaction of sowing time and concentration on germination significant ($p<0.001$; $p<0.05$) for extract (Table 1) while concentration and the interaction between concentration and sowing time were significant ($p<005$) for HBA (Table 2). It is interesting that seedlings growth were stimulated by 5% and reduced by 10, and 15% aqueous extract when it was applied at 5 days after sowing (DAS)(Figs. 3&4). The two way analysis of variance (ANOVA) showed that the effects of extract concentration, seed sowing time and their interactions were significant at $p<0.001$, $p<0.01$ and $p<0.05$, respectively (Tables 3 and 4). HBA retarded the seedlings growth (shoot and root) irrespective of application time (Figs. 5 and 6). Although 5 mM HBA had little effects while 10 &15 mM HBA showed more or less similar significant ($p<0.05$) effect on seedlings growth (Table 4). But it was shown inhibitory effect of seedlings growth only 10 and 15% extracts when it was applied at 5 DAS. Surprisingly, 5% extracts showed stimulatory activity of seedlings growth.

The different concentration of aqueous extracts reduced the dry matter of wheat seedlings than control when it was applied during the seed sowing. In contrast, control treatment and 5% extract were not reduced the dry matter while 10 and 15% reduced the dry matter when it was applied 5 DAS (Fig. 7). The two way analysis of variance (ANOVA) showed that the effects of concentration, sowing time and their interactions were significant at $p<0.01$ and $p<0.05$ (Table 5). Thin layer chromatography (TLC) was performed to identify allelochemical from methanol extract of *L. siribicus.* For conducting the thin layer chromatography, HBA was used as standard with methanol extract of *L. siribicus.* Methanol extract showed similar spot with the standard of HBA (Fig. 8), indicated HBA was present in methanol extract, because R_f value was 0.96 in both cases. Although another two additional spots were found, but it was not identified. It might be phenolic or flavonoids compounds. The aqueous extract of *L. siribicus* were applied to investigate the effect of dry weight of wheat seedlings. According to our data, it was found that different concentration of extract significantly reduced dry weight of seedlings when extract were applied during seed sowing. It is notably that dry weight of wheat seedlings were increased significantly for 5 % of aqueous extract and reduced for 10 and 15% extract where it was applied at 5 DAS. Mineral composition was determined using ground powder of *L. siribicus* plant to investigate stimulatory activity on plant growth (Table 6). According to our data, P and Ca were found in higher than other minerals.

Table 1. Analysis of variance (ANOVA) for the effects of concentration and sowing time on the germination percent of wheat by aqueous extract of *L. siribicus.*

Source of variation	df	% Germination
Concentration	2	547.8^{NS}
Sowing time	1	30524***
Concentration × Sowing time	2	729.5*
Error	4	160.5

Notes: NS = Not significant; *** Significant p< 0.001; * p<0.05

Table 2. Analysis of variance (ANOVA) for the effects of concentration and sowing time on the germination percent of wheat by aqueous extract of *L. siribicus.*

Source of variation	df	% Germination
Concentration	2	1493*
Sowing time	1	2735^{NS}
Concentration × Sowing time	2	2414*
Error	4	357.3

Notes: NS = Not significant; * Significant p<0.05

Table 3. Analysis of variance (ANOVA) for the effects of concentration and sowing time on the shoot and root growth of wheat by aqueous extract of *L. siribicus.*

Source of variation	df	% Inhibition	
		Shoot growth	Root growth
Concentration	2	7759**	5880***
Sowing time	1	15979**	13359**
Concentration × Sowing time	2	1113*	2135*
Error	4	170.2	454.4

Notes: ***Significant p<0.001; ** p<0.01; *p<0.05

Table 4. Analysis of variance (ANOVA) for the effects of concentration and sowing time on the shoot and root growth of wheat by 4-hydroxy benzoic acid.

Source of variation	df	% Inhibition	
		Shoot growth	Root growth
Concentration	2	982.0*	2166**
Sowing time	1	137.7^{NS}	7.044^{NS}
Concentration × Sowing time	2	29.18^{NS}	177.9^{NS}
Error	4	247.2	1292

Notes: NS = Not significant; * Significant p<0.05; ** Significant p<0.01

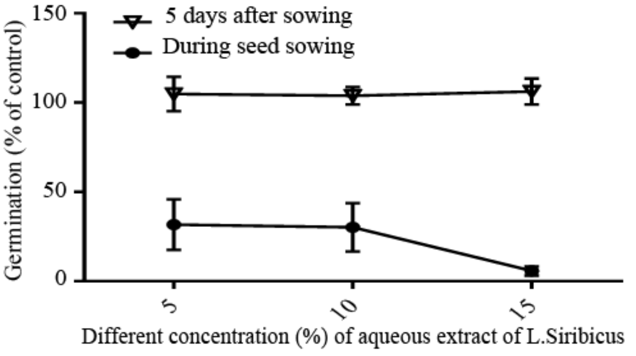

Fig. 1. Effect of aqueous extract of *L. siribicus* on seed germination of wheat during seed sowing and at 5 days after seed sowing. Each value is the average of 3 measurements; bar indicates mean ± SD.

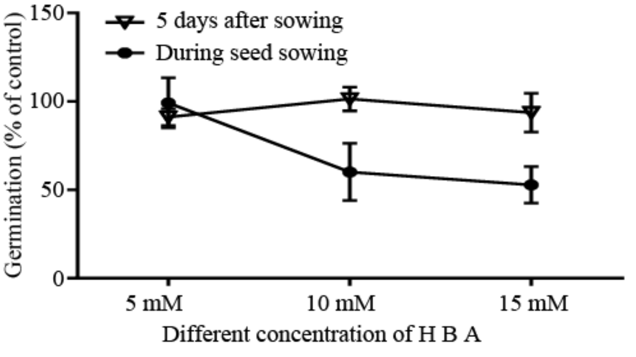

Fig. 2. Effect of different concentration of 4-hydroxy benzoic acid (HBA) on seed germination of wheat during seed sowing and at 5 days after seed sowing. Each value is the average of 3 measurements; bar indicates mean ± SD.

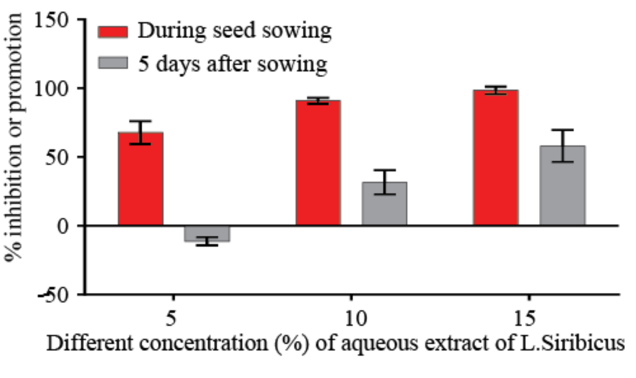

Fig. 3. Effect of aqueous extract of *L. siribicus* on shoot growth of wheat seedlings during seed sowing and at 5 days after sowing. Each value is the average of 3 measurements; bar indicates mean ± SD.

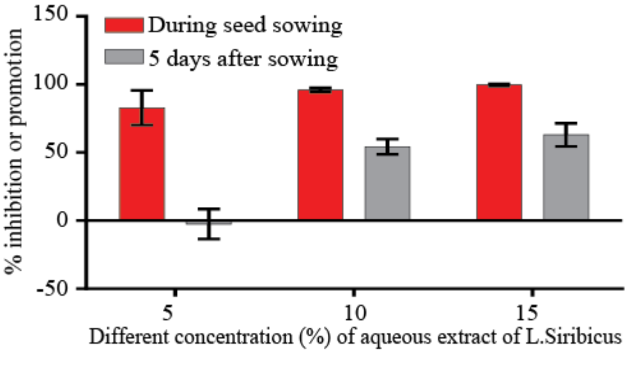

Fig. 4. Effect of aqueous extract of *L. siribicus* on root growth of wheat seedlings during seed sowing and at 5 days after sowing. Each value is the average of 3 measurements; bar indicates mean ± SD.

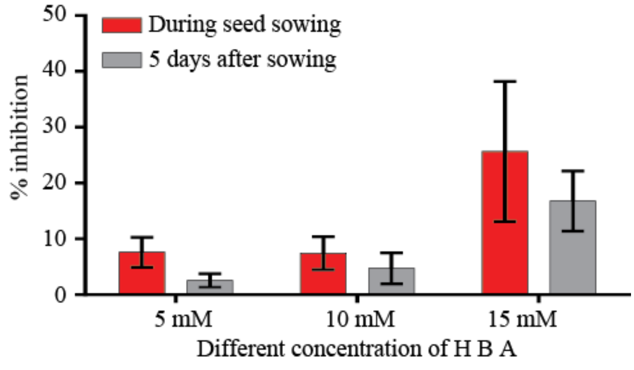

Fig. 5. Effect of different concentration of 4-hydroxy benzoic acid (HBA) on shoot growth of wheat seedlings during seed sowing and at 5 days after sowing. Each value is the average of 3 measurements; bar indicates mean ± SD.

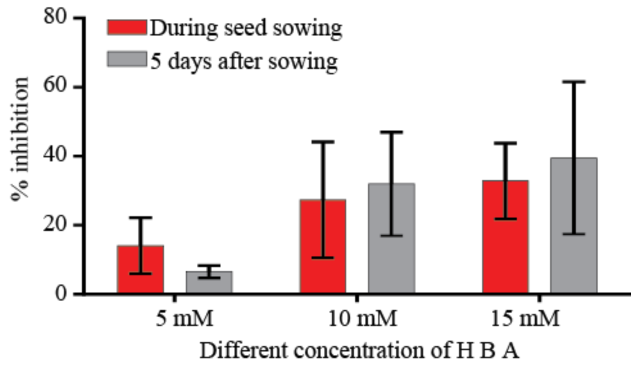

Fig. 6. Effect of different concentration of 4-hydroxy benzoic acid (HBA) on root growth of wheat seedlings during seed sowing and at 5 days after sowing. Each value is the average of 3 measurements; bar indicates mean ± SD.

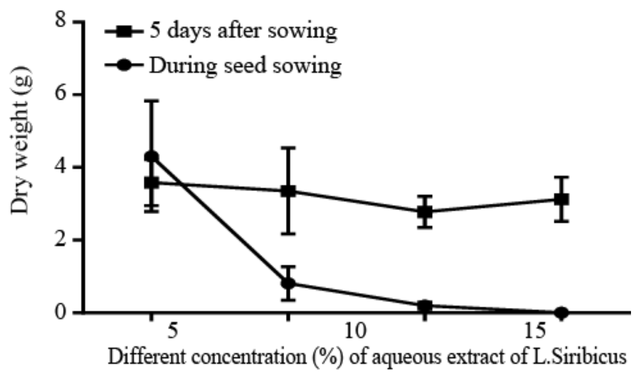

Fig. 7. Effect of aqueous extract of *L. siribicus* on dry matter of wheat seedlings. Each value is the average of 3 measurements; bar indicates mean ± SD.

Table 5. Analysis of variance (ANOVA) for the effects of concentration and sowing time on the dry matter of wheat seedlings by aqueous extract of *L. siribicus.*

Source of variation	df	Dry matter (g)
Concentration	2	23.74*
Sowing time	1	21.25*
Concentration × Sowing time	2	13.86**
Error	4	1.917

Notes: ** Significant $p < 0.01$; * $p < 0.05$

Table 6. Mineral composition of *L. siribicus* (mean ± SD).

Minerals (ppm)						
P	S	Cu	Mn	K	Ca	Mg
11.76 ± 2.3	1.576 ± 0.43	0.075 ± 0.02	0.054 ± 0.01	0.10 ± 0.04	14.59 ± 3.0	1.52 ± 0.35

Fig. 8. TLC showing a distinct spot of 4- hydroxy benzoic acid using methanol extract of *Leonurus siribicus* L.

Discussion

Our results were similar with previously studied by Almeida, *et al.,* 2008 and they found that both of aqueous and methanol extract of *L. siribicus* significantly inhibited seed germination of *Lactuca sativa*. Another study was stated that aqueous, ethanol and acetone extract of *L. siribicus* reduced the germination rate of some vegetables seed (Sayed *et al.*, 2012). Methanol extracts reduced or delayed the germination of some weed seeds (Islam & Kato-Noguchi, 2014). It was interesting that aqueous extract of *L. siribicus* did not show any inhibitory effect of seed germination irrespective of concentration when it was applied 5 DAS. The aqueous extract of *L. siribicus* adversely inhibited initial shoot and root growth of wheat seedlings when it was applied during time of seed sowing. In this regards there were supporting data that this species (*L. siribicus*) introduced into the environment radical exudates that increased the germination of rice, wheat and mustard depending on concentration (Mandal, 2001); the aqueous extract of its leaves inhibited the corn germination and the growth of tomato seedlings (Almeida *et al*., 2003). It was shown that the inhibitory effect on seed germination due to some flavonoids and phenolic compounds (Mandal, 2001 and Almeida *et al*., 2008). Recently, published report stated that flavonoids like 3´-OH-genkwanin and quercetin showed the stronger anti-germinative activity that was isolated from both of aqueous and methanol extract of *L. siribicus* (Almeida *et al*., 2008). A substantial number of allelochemicals have been identified but most of these are secondary plant metabolites which belonging to the groups of terpenoids, phenolic compounds, long chain fatty acids, organic cyanides, organic acids, alakaloids and others (Macias *et al*., 2001; Oleszek & Stochmal, 2002). Our results also strongly suggested that some toxic compounds or allelo-chemicals were present in aqueous extract that inhibited seed germination of wheat depending on application time. Intriguingly 5, 10 and 15 mM HBA reduced the germination when it was applied during the sowing time. These data strongly revealed that HBA is one of the allelochemicals that was reduced the germination (Shann & Blum, 1987).

Seedlings growth of wheat were significantly retarded by 5, 10 and 15% of aqueous extract, when extract were applied during seed sowing. This results were agreed with previously studied by Mandal (2001); Almeida, *et al*. (2008); Sayed, *et al*. (2012) and Islam & Kato-Noguchi., (2014). Root exudates of *L. siribicus* showed inhibitory effect on initial growth of rice, wheat and mustard seedlings depending on concentration (Mandal, 2001). In addition that seedlings growth of some vegetables were significantly retarded by aqueous, ethanol and acetone extracts (Sayed *et al*., 2012). Besides that, both aqueous and methanol extract shown inhibitory effect of seedling growth of *Raphanus sativus, Lactuca sativa*, *Lepidium sativum* and some weed seeds (Almeida *et al*., 2008 and Islam & Kato-Noguchi, 2014). It has been studied that initial growth of seedlings were reduced by some flavonoids compound like rutin, isoquercetrin and 3´-OH-genkwanin that were identified in both aqueous and methanol extract of *L. siribicus* (Almeida *et al*., 2008). The inhibitory activity of different concentration of extract suggested that probably the active allelochemicals or flavonoids affected the cellular lengthening mechanisms (Hoagland & Williams, 2004) and possible detoxification mechanisms might have happened. According to our experimental results, it was suggested that lower (5%) and higher (10 or 15%) concentration of aqueous extract showed stimulatory and inhibitory effect on initial growth of wheat seedlings, respectively. This results were fully agreed with the findings that was previously studied by Mandal, (2001). It was found that root exudate of *L. siribicus* showed concentration dependent activity, that is, low concentration showed stimulatory activity of initial growth of rice, wheat and mustard. Mandal (2001) stated that caffeic acid was found in *L. siribicus* showed inhibitory effect at the concentration of 500, 250 and 125 ppm; after that (lower concentration) it showed stimulatory effect. Caffeic acid is produced from cinnamic acid which is produced from the shikimic acid pathway and widely distributed among the plant kingdom (Mandal, 2001). Our findings suggested that low concentration (5%) of aqueous extract can be applied for stimulating the initial growth of wheat seedlings to achieve final maximum yield. On the contrary, higher concentration (10, 15%) might be used for controlling weed in wheat field. For proper management of weeds and crops, the allelopathic compounds should be tested in various combinations for their activity. Our results were agreed with previous data studied by Sayed *et al*. (2012). These data were strongly correlated with our above data that 5% aqueous extract of honeyweed (*L. siribicus*) had stimulatory rather than inhibitory effect on seedling growth of wheat, when it was applied at 5 days after sowing. Shoot and root growth were adversely affected by HBA when it was applied irrespective of time. These findings were very much similar with previously stated that HBA reduced the growth of *Lactuca sativa* (Hussain *et al*., 2010). It is notably that HBA in addition to other phenolic compounds like 4-hydroxythiophenol, syringic acid, apigenin, genkwanin, isoquercitrin, rutin were identified by Sheng-Ming, *et al.* (2006) from methanol extract of *L. siribicus*. To the best of our knowledge, although, HBA was first identified by Sheng-Ming, *et al*. (2006) in methanol extract of *L. siribicus*, but no one mention allelopathic properties of its due to HBA. So far we know, it is the first and noble findings that HBA was identified in methanol extract of *L. sirbicus* as potential alleochemical. To support the TLC result, we applied different concentration (1-5%) of commercial HBA to see the effect of seed germination and seedlings growth of wheat and found inhibitory effect of germination and seedlings growth. It has been studied that HBA interfere with enzymatic system as well as the major physiological processes like phytohormone activity, mineral uptake, plant water balance and stomatal function, photosynthesis, respiration, organic synthesis of certain compounds and flow of carbon (Einhellig, 1995 &1986). However, they may not alter cell division or directly affect gene translation. The most important and first action site of HBA to interfere the plant growth is plasma membrane. They reduced the transmembrane electrochemical potential with the immediacy and extent of that action depending on the concentration and lipid solubility of the compound (Glass, 1973). All of these minerals may be contributed to stimulate the seedling growth, although no reports were found in favour of this argument. It has been well documented that phenolic acids suppress absorption of phosphate, potassium, nitrate, and magnesium ions, and overall changes in tissue content of mineral ions are one of the effects on plants grown with phenolic acids in the growth medium (Alsaadawi *et al*., 1986; Balke, 1985; Baziramakenga, *et al*., 1994; Bergmark *et al*., 1992; Booker, *et al*., 1992; Glass, 1973,1974; Kobza & Einhelling, 1987). So, it may be concluded that allelochemicals suppressed the mineral absorption of wheat seedlings in higher concentration of *L. siribicus* (10, 15%), but at lower concentration (5%), it's contributed all of these minerals to stimulate the growth.

Conclusions

Now it is very clear that, *L. siribicus* has both stimulatory and inhibitory effect on seedling growth of wheat. It may be applicable for other crops for stimulating initial growth of seedlings. On the contrary, 10 or 15% aqueous extract might be applied to control weed in crops field, if it can be applied at 5 DAS. In this case, further intensive study are to be needed to find out inhibitory and stimulatory effect of *L. siribicus* extract on weeds and crops, respectively.

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