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Phytotoxicity of *Parthenium hysterophorus* L. and *Xanthium strumarium* L. Extracts on Wheat Seed Germination and Cytotoxicity on Brine Shrimp Nauplii

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Abstract

Parthenium hysterophorus L. and *Xanthium strumarium* L. are invasive alien species found around the world. The purpose of this study is to determine the phytotoxicity of *P. hysterophorus* and *X. stumarium* leaf and root extracts on wheat seedlings and their cytotoxicity on brine shrimp [*Artemia salina* (Linnaeus, 1758)] nauplii. The phytotoxicity of various extracts to seedlings was evaluated using petri dishes lined with moistened filter paper, while the cytotoxicity was determined using the brine shrimp lethality assay. It was found that 85-90% of wheat seeds germinated in dichloromethane (DCM) and methanol extracts of leaf and root of *P. hysterophorus and X. strumarium* at lower concentrations (0.15 to 0.62 mg/mL), 50-60% at medium concentration (1.25 and 2.5 mg/mL), and 10-20% at higher concentration (5 mg/mL) after three days of incubation. Root methanol extracts were more phytotoxic to root and shoot growth in seedlings (at 0.62-5.0 mg/mL) than other *P. hysterophorus* root and leaf extracts. Similarly, root DCM extracts were more phytotoxic to root and shoot growth in seedlings (at 0.31-5.0 mg/mL) than other *X. strumarium* root and leaf extracts. Moreover, the root DCM extract was more cytotoxic (LC_{50}: 302.93 μg/mL) to brine shrimp nauplii than the other extracts of root and leaf of *P. hysterophorus.* Similarly, root DCM extract was more cytotoxic (LC_{50}: 129.16 μg/mL) to brine shrimp nauplii than the other extracts of root and leaf of *X. strumarium.* This study revealed that the allelochemicals/phytotoxins present in the methanol and DCM extracts of the roots and leaves of *P. hysterophorus* and *X. strumarium* were phytotoxic to wheat seeds and seedlings, as well as cytotoxic to brine shrimp nauplii. This work will help future research into the effects of allelochemicals on natural vegetation and other crop plants, as well as the carcinogenic activity of allelochemicals identified in *P. hysterophorus* and *X. strumarium* on different human cell lines.

Keywords: Allelochemicals, Allelopathy, DCM, Extract, Seedling

Introduction

Parthenium hysterophorus L. (Asteraceae; wild carrot weed) and *Xanthium strumarium* L. (Asteraceae; rough cocklebur) are invasive alien species (Invasive Species Specialist Group [ISSG], n.d.). *P. hysterophorus* is distributed in many tropical and subtropical regions of the world, including Asia, Africa, Australia, and the Pacific region (Adkin & Shabbir, 2014), causing threats to natural environments, the agricultural sector, and conservation activities. *P. hysteroporus* contains certain allelochemicals, such as parthenin, hysterin, hymenin, and ambrosin that exert significant allelopathic impacts on different crop plants (Dukpa et al., 2020). In many dicotyledons and monocotyledon plants, the parthenin suppresses seed germination and radicle growth. Similarly, *X. strumarium* is originally native to North and South America; however, it has since become extensively naturalized and may be found primarily in temperate, subtropical, and Mediterranean regions (Waheed et al., 2024). *X. stramurium* contains allelochemicals like 1, 5-Dimethyltetralin, Eudesmol, l-Borneol, Ledene alcohol, -(-) Caryophyllene oxide, Isolongifolene, 7,8-dehydro-8a-hydroxy, L-Bornyl acetate, and Aristolene epoxide. According to Duke and Lydon (1987) *X. strumarium* consists of a variety of secondary metabolites and allelochemicals, such as sesquiterpene lactones (xanthatin and xanthinosin), tannins, flavonoids, and alkaloids. These chemicals, especially the sesquiterpenoids, and monoterpenes, were demonstrated to hinder the growth and germination of other types of plants, including

Bidens pilosa L., in a concentration-dependent way (El-Gawad et al., 2019). Both xanthatin and xanthinosin have potent phytotoxic effects by interfering with target plant cellular functions such as mitochondrial function and membrane stability.

Phytotoxicity refers to the detrimental effects of particular compounds (phytotoxins) or growing circumstances on plants, such as delayed seed germination or inhibited growth, root development, and overall health of plants (REAL CCS, 2014). Phytotoxinsare the toxic substances produced by plants, such as juglone (produced by *Juglans regia L.*), cyanogenic glycosides (produced by *Manihot esculenta* Crantz*, Sorghum bicolor* (L.) Moench*, Prunus avium* (L.) L.), coniine (produced by *Conium maculatum* L.), etc. that causes phytotoxicity. Allelopathy is a biological phenomenon in which organisms, particularly plants, emit biochemicals (allelochemicals) into the surroundings that affect the growth, existence, and reproduction of other living things (Rice, 1984). These compounds can have either inhibitory or stimulatory impacts on plant competitiveness, soil microbial populations and ecosystem dynamics (Cheng & Cheng, 2015). However, allelopathy may be used as natural herbicides for weed management (Weston & Duke, 2003), sustainable agriculture to reduce chemical pesticide dependency (Cheng & Cheng, 2015), and ecosystem dynamics to influence plant community structure and invasive species competition (Inderjit & Duke, 2003). Allelopathic interactions take place through a variety of mechanisms, including root exudation (Weston & Duke, 2003), leaf leachates (Cheng & Cheng, 2015), gas volatilization (He et al., 2012), and plant residue decomposition (Inderjit & Duke, 2003) in nature. Allelopathic compounds frequently impede root and shoot growth in targeted plants by interfering with cell division, enzyme activity, and hormone control (Rice, 1984).

Cytotoxicity is the capacity of a substance to harm or kill live cells. It is an important measure for determining the hazardous potential of natural or synthesized substances, particularly in drug development, environmental toxicology, and research on agriculture (Kroemer et al., 2009). Although cytotoxic compounds are also capable of killing normal human cells, their primary function is to selectively destroy rapidly dividing cells, including cancer cells. Therefore, cytotoxic compounds can be used to make drugs by making them specifically designed to target tumor cells and not normal cells. However, it may not function precisely, and that's why people experience side effects of drugs. Cytotoxicity studies investigate cell viability, integrity of the membrane, metabolism, and induction of apoptosis (Wang et al., 2012). The brine shrimp lethality assay (BSLA) is a popular cytotoxicity testing bioassay that utilizes *Artemia salina* nauplii (larvae) as an experimental organism. This assay is inexpensive, straightforward, and offers preliminary information about the hazardous or bioactive qualities of plant extracts (Meyer et al., 1982).

The allelopathic effect of invasive alien species on natural vegetation has been the subject of several studies; however, the allelovpathic effect of these invasive species on crop yield has received less attention. According to Naderi et al. (2024), *P. hysterophorus* decreased maize yields by as much as 46% at a density of 16 plants/m² according to a two year field research conducted in Pakistan. Even at low densities, losses in yield showed a nonlinear pattern, with notable decreases (e.g., 14% loss at 1 plant/m²). Hussain et al. (2014) studied the effect of *X. strumarium* on maize production. A few studies have been conducted on the effect of growth and productivity of wheat (*Triticum aestivum* L.) caused by *X strumarium* and *P hysterophorus* because of their potential phytotoxic compounds, competition for nutrients, light, and space, and allelopathic effects. Similarly, limited research has been conducted to study the effect of dichloromethane (DCM) and methanol extracts of leaf and root extracts of *P. hysterophorus* and *X. stramurium* on wheat seed germination and cytotoxicity on brine shrimp nauplii in lab condition. In a petri dish experiment, Shakya et al. (2021) studied the effect of *P. hysterophorus* leachates on wheat seed germination and growth metrics. The effect of *P. hysterophorus* residues on specific soil characteristics and the growth of *Cicer arietinum* and *Raphanus sativus* in soil and a lab setting were investigated by Batish et al. (2002).

Zahid et al. (2014) studied the effect of *Xanthium strumarium* on maize yield and yield components under natural soil conditions and found a reduction in the percentage yield of grain.

The majority of the research focused on the influence of methanol and aqueous extracts of leaves of *P. hysterophorus* (Amare, 2018; Bashar et al., 2021; Bashar et al., 2023) and leaves and fruits of *X. stramurium* (Benyas et al., 2010; Jalali et al., 2013; Mirzaee & Saeedipour, 2021; Seifu et al., 2024) on seed germination. A comparison of the allelopathic effect of leaf and root extracts on plants (seed germination) and their cytotoxic effect on animals (brine shrimp nauplii) could bring insight into the physiological link between plants and animals. Therefore, this research aims to assess the effect of allelochemical present in dichloromethane and methanol extracts of *P. hysterophorus* and *X. strumarium* leaf and roots on wheat seed germination, radicle and plumule growth in seedlings and cytotoxic potential in brine shrimp nauplii.

Materials and Methods

Plant collection and identification

P. hysterophorus and X. strumarium were collected in December 2023 at a height of 150 meters above sea level from Butwal, Western Nepal (Figure 1). The collected plants (Voucher no. PH05, XS07) were identified by using standard literature (Banik & Yomso, 2021; Chinnuswamy et al., 2018; Rajbhandari et al., 2016) and deposited in the Department of Botany, Butwal Multiple Campus, Tribhuvan University, Butwal, Nepal.

Shade drying and pulverization

The leaves and roots were cleaned under running tap water, dried in the shade, and then laid on newspaper at room temperature until completely dry. An electric grinder was used to grind the fully dried leaves and roots into a fine powder.

Extract preparation

Leaf and root powders (50 gm each) were macerated in 100 mL methanol and dichloromethane (DCM) separately at room temperature for 48 hours. They were filtered with the use of Whatman No. 1 filter paper. The residue was again macerated with 100 mL methanol and DCM for 24 hours twice more to complete the extraction, and the solution mixture was filtered using filter paper. The filtrate was evaporated and concentrated using a rotary evaporator at 37°C. The samples were then kept at 4°C in refrigerator for further testing.

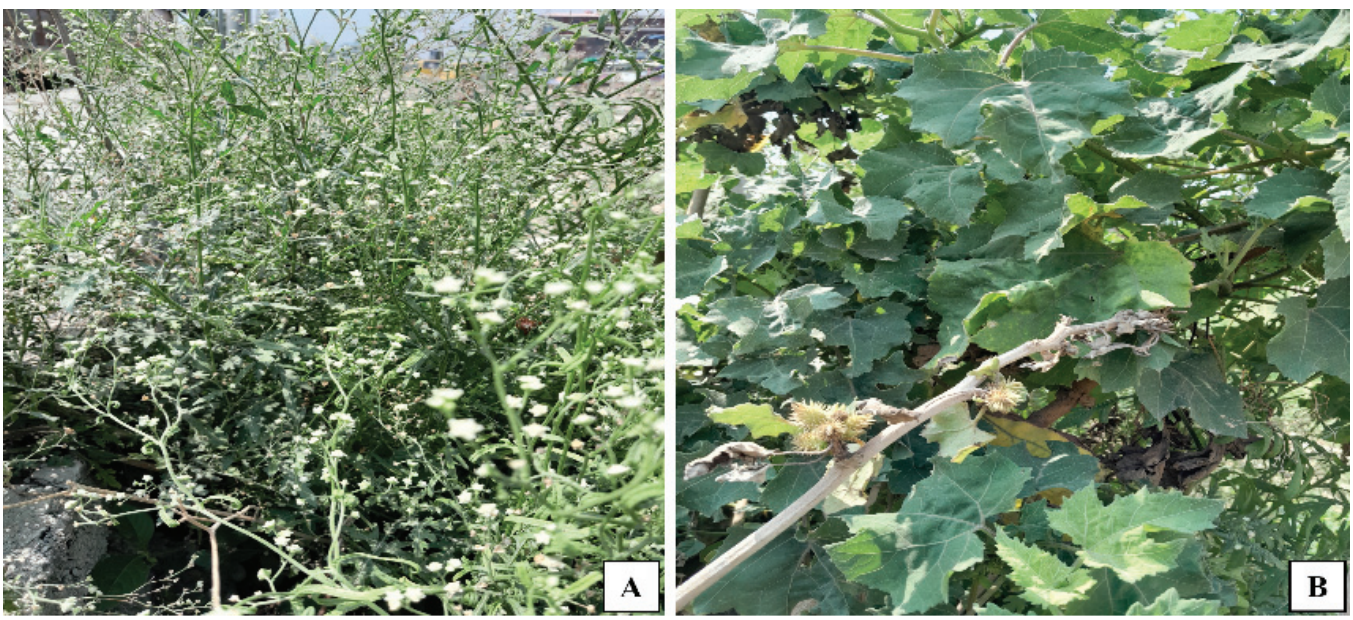


Figure 1: Collected plants, **(A)** *Parthenium hysterophorus,* **(B)** *Xanthium strumarium*

Evaluation of wheat seed germination and growth of seedlings using various extracts of P. hysterophorus and X. strumarium

Wheat seed collection and sterilization: Wheat (*Triticum aestivum*) seeds were obtained from the National Wheat Research Program, Bhairahawa, Rupandehi. The seeds were thoroughly rinsed with tap water and soaked in water for 24 hours. The seeds were then sterilized for 10 minutes with 1% sodium hypochlorite before being washed three times with sterilized distilled water. Filter papers, petri dishes, forceps, needles, and distilled water were sterilized in an autoclave at 121°C at 15 lb pressure for 15 min.

Seed germination on petri dishes and seedling growth: Petri dishes were lined with blotting papers at the base and moisturized with sterile distilled water. Sterilized 10 uniform seeds were placed in each petri dish using forceps. Seeds were treated with 5.0, 2.5, 1.25, 0.62, 0.31, and 0.15 mg/mL concentration of extracts by the serial dilution (using sterile distilled water) of 10 mg/mL stock solution of DCM and methanol root and leaf extracts of *P. hysterophorus and X. strumarium*. Seeds treated with sterile distilled water were considered as the control treatment. The entire process was carried out in an air laminar flow cabinet. Now, the petri dishes with seeds were closed with a lid, labeled, and kept inside the incubator at 26±2°C for seed germination. The germination of seeds under different treatments was observed after every three days. The number of germinated seeds was recorded, and the radicle (root) and plumule (shoot) lengths in seedlings treated with concentration of extracts were measured with the help of a scale for 12 days.

Assessment of brine shrimp lethality assay using various extracts of P. hysterophorus and X. strumarium

The cytotoxicity of methanol and DCM crude extracts was assessed using a brine shrimp lethality assay (Fatope et al., 1993; Meyer et al., 1982) with a slightly modified protocol. First, 3.5 gm of NaCl was added and dissolved in 100 mL of distilled water to prepare an artificial seawater solution. Eggs weighing approximately 10 mg of brine shrimp (*Artemia salina*) were incubated in seawater for 48 hours at 23°C (using an 80-watt light bulb) to hatch the eggs into larvae or nauplii. Stock solution (10 mg/mL) of each plant extract (*P. hysterophorus* and *X. strumarium*) was prepared in a 2 mL Eppendorf tube separately. Then, various concentrations (5, 2.5, 1.25, 0.62, 0.31, and 0.15 mg/mL) of each extract were prepared using the serial dilution method with salt water. Potassium dichromate (concentrations of 10, 20, 40, 80, and 160 µg/mL) was employed as the positive control, and seawater (saltwater) as the negative control. In a well plate, a total of 150 µL of different concentrations of extracts and saltwater with 15 brine shrimp nauplii were treated with the help of a micropipette. It was incubated in light for 24 hours, and the number of living nauplii was determined. For every sample, triplicate experiments were conducted. Each plant extract's lethal concentration that causes 50% death (LC_{50}) was calculated using the regression line that was created by graphing the concentration against the percentage of mortality on a probit scale.

$$\% \text{ Mortality} = \frac{\text{No. of dead larvae (Nauplii)}}{\text{Initial No. of live larvae (Nauplii)}} \times 100$$

Data analysis

After three days of culture in petri dishes, the percentage of wheat seed germination with the control and extract treatments was determined using Microsoft Excel 2010.The mean length and standard error of the length of the roots and shoots of wheat seedlings with the control and various extract treatments in seeds were calculated after 3, 6, 9, and 12 days using Microsoft Excel 2010.Similarly, the LC_{50} of brine shrimp nauplii in various extract concentrations was computed using a simple linear regression line, and the percentage mortality of brine shrimp nauplii was determined using Microsoft Excel 2010. The correlation between extracts of *P. hysterophorus* and *X. strumarium* and the growth of wheat seedlings was determined using Principal Component Analysis (PCA) using R-software version 4.3.

Results and Discussion

Evaluation of wheat seed germination using various extracts of P. hysterophorus and X. strumarium

This study showed that wheat seeds were germinated initially into seedlings in various concentrations of leaf and root extracts of *P. hysterophorus and X. strumarium* in petri dish moistened with filter paper; however, the growth and development of radicle (root) and plumule (shoot) in seedlings differed depending on the concentration of extracts and time. Seed germination and growth and development of roots and shoots in wheat seedlings were evaluated for 12 days. It was found that 85-90% of wheat seeds germinated in DCM and methanol extracts of leaf and root of *P. hysterophorus and X. strumarium* at lower concentrations (0.15, 0.31, and 0.62 mg/mL), 50-60% at medium concentration (1.25 and 2.5 mg/mL), and 10-20% at higher concentration (5 mg/mL) after three days of incubation (Tables 1 and 2). This study also showed that DCM and methanol extracts from both plants had more or less similar effects on seed germination after three days, however, compared to *P. hysterophorus*, a somewhat lower percentage of seeds germinated in the DCM and methanol extracts of *X. strumarium* (Tables 1 and 2). Several studies showed that *X. strumarium* contains xanthatin and xanthinin, which prevent various kinds of crops and weeds from germinating and growing, indicating a potential allelopathic function. Shajie and Saffari (2007) found that *X. strumarium* leaf and stem extracts significantly lowered the germination, root, and shoot lengths of all tested crops, including corn, canola, sesame, lentils, and chickpea. Though seeds may not have had permanent physiological alterations within three days due to the application of extracts, the germination effect appeared in seeds. Slow penetration and bioaccumulation of lipophilic extracts (like DCM) and polar metabolites (like methanol-extracted phenolics) may be the cause of the delayed physiological response in seeds. These extracts may also take longer to pass through seed coats or cellular membranes. Plant extracts containing phenolic acids, such as ferulic acid, impair mitochondrial function and membrane integrity; however, because of their slow absorption, their effects require time to become visible (Einhellig, 1994). However, unlike soil, where factors such as microbial interactions, availability of nutrients, and retention of water change, a filter paper setup ensures consistent moisture delivery

Table 1: Percentage of seed germination in DCM and Methanol extracts of Leaf and root of *P. hysterophorus* after the three days

Extract concentration (mg/mL)	Total no. of seeds	Average no. of germinated seeds	Percentage of seed germination
0.15	120	108.00	90.00
0.31	120	106.85	89.04
0.62	120	104.57	87.14
1.25	120	72.00	60.00
2.5	120	66.57	55.47
5.0	120	24.28	20.23

Table 2: Percentage of seed germination in DCM and Methanol extracts of Leaf and root of *X. strumarium* after the three days

Extract concentration (mg/mL)	Total no. of seeds	Average no. of germinated seeds	Percentage of seed germination
0.15	120	105.42	87.85
0.31	120	103.42	86.18
0.62	120	102.28	85.23
1.25	120	68.28	56.90
2.5	120	59.71	49.75
5.0	120	12.00	10.00

and similar exposure to plant extracts, minimizing external variation (Khan et al., 2011). Some allelopathic chemical substances can affect seed coat permeability, reducing water absorption and slowing or entirely suppressing germination (Chou, 1999). Similarly, several allelochemicals found in plant extracts suppress amylase and protease enzymes, depriving the embryo of vital nutrients (Li et al., 2010). This technique enables a direct assessment of germination responses to various extract concentrations, including possible inhibiting or stimulating effects (Bewley et al., 2013).

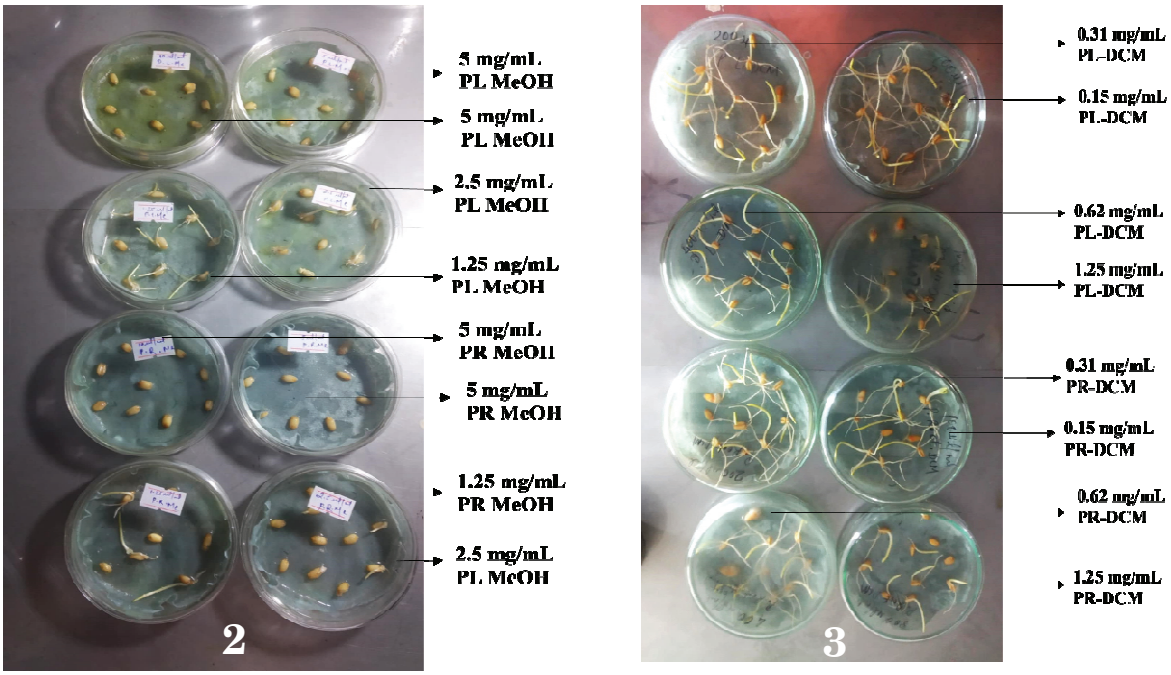
Effect of DCM and methanol extracts of P. hysterophorus on the growth and development of shoots and roots in wheat seedlings

This study showed that methanol extracts of leaf and roots were more effective for the growth inhibition of root and shoots in wheat seedlings than the DCM extracts of leaf and roots of *P. hysterophorus* (Tables 3 and 4; Figures 2 and 3). Methanol is a polar solvent that may extract a wider variety of allelochemicals and phytotoxins, such as phenolics, flavonoids, tannins, alkaloids, phenolic acids (e.g., ferulic acid, p-coumaric acid), and parthenin (Harborne, 1998; Singh et al., 2002). These compounds inhibit cell division, activity of enzymes, and uptake of nutrients, resulting in decreased root and shoot growth (Singh et al., 2003). According to studies, methanol extracts of *P. hysterophorus* have higher amounts of water-soluble allelochemicals that affect the seedling metabolism (Kanchan & Jayachandra, 1980). The growth inhibition of roots and shoots in seedlings was directly proportional to extract concentrations, while the growth and development of roots and shoots in seedlings were found to be directly proportional to time. During the initial growth stage of seedlings for three days, the length of roots and shoots was greater in lower concentrations (from 0.15 to 0.62 mg/L) of DCM and methanol extracts of leaf and root than in the control treatment; however, at higher concentrations, the length of roots and shoots were decreased than that of the control treatment (Tables 3 and 4). It may be due to hormesis, which is an alternating response in which low concentrations of hazardous chemicals stimulate growth while greater quantities inhibit it (Calabrese & Baldwin, 2003). At low concentrations (0.15 - 0.62 mg/L), some phenolic compounds and sesquiterpene lactones of *P. hysterophorus* might act similarly to plant growth regulators.

When compared to the extracts of plant parts, root extracts (both methanol and DCM) of *P. hysterophorus* showed a higher inhibition for the growth and development of roots and shoots in wheat seedlings than the leaf extracts (both methanol and DCM). It could be owing to the larger concentration of allelochemicals in roots than in leaves. In nature, plants discharge allelochemicals into the soil through their roots. This process can happen actively during root growth or passively via diffusion. The discharges may contain phenolics, flavonoids, terpenoids, and organic acids, all of which may interfere with seed germination or plant growth (Inderjit & Duke, 2003).

Table 3: Effect of DCM and methanol leaf extracts of *P. hysterophorus*on wheat seed germination

Extracts	Concentration (mg/mL)	Mean length of roots ± S.E (cm)				Mean length of shoots ± S.E (cm)			
		3 days	6 days	9 days	12 days	3 days	6 days	9 days	12 days
DCM	5.0	0.3±0.51	0.6±0.35	0.8±0.67	0.8±0.56	0.5±0.65	0.8±0.49	1.2±0.56	2.5±0.45
	2.5	0.3±0.67	1.3±0.67	1.5±0.45	2.1±1.12	0.4±0.87	0.9±0.41	2.5±67	3.4±0.76
	1.25	0.4±0.54	2.3±0.98	2.4±0.98	2.4±1.54	0.6±0.45	4.6±1.64	8.9±2.42	9.6±1.54
	0.62	1.6±0.87	3.7±0.54	4.4±0.45	5.5±0.54	0.6±0.31	5.2±2.21	10.6±2.65	11.5±4.21
	0.31	1.6±0.39	3.95±0.71	4.6±1.21	4.6±0.43	0.7±0.12	4.5±3.11	9.8±3.21	12.5±2.54
	0.15	2.5±0.56	4.8±1.1	5.6±0.62	6.1±2.21	1.2±0.54	5.4±0.78	10.7±4.42	12.3±1.87
	Control	0.6±0.87	2.8±0.54	3.6±0.76	4.1±0.94	0.3±0.56	2.6±0.56	7.7±0.78	9.8±0.54
Methanol	5.0	0.1±0.43	0.1±0.56	0.1±0.78	0.4±0.34	0.4±0.54	0.7±0.54	0.8±0.64	1.2±0.76
	2.5	0.2±0.34	0.6±0.51	0.8±0.34	1±0.76	0.2±0.34	0.9±0.76	2.1±0.34	2.3±0.52
	1.25	0.4±0.87	1.1±0.86	1.3±0.87	1.5±0.34	0.5±0.52	1.4±1.23	1.6±0.44	1.9±0.76
	0.62	1.1±0.21	2.8±0.42	3.8±1.42	4.5±2.21	0.7±0.76	1.8±0.67	4.4±1.54	5.2±1.54
	0.31	1.5±0.67	3.3±0.47	4.2±2.41	5.2±1.65	0.7±0.34	3.1±2.41	7.8±2.54	9.9±3.45
	0.15	2.2±0.51	4.1±1.31	4.7±1.52	6.2±1.65	1.3±0.87	3.9±1.87	8.3±3.31	10.1±0.8
	Control	0.6±0.87	2.8±0.54	3.6±0.76	4.1±0.94	0.3±0.56	2.6±0.56	7.7±0.78	9.8±0.54



Figures 2 and 3: Effect of methanol and DCM extracts in wheat seedlings at various concentrations (PR MeOH=*Parthenium* root methanol extract, PL MeOH= *Parthenium* leaf methanol extract, PR-DCM= *Parthenium* root DCM extract, and PL-DCM= *Parthenium* leaf DCM extract)

Effect of DCM and methanol extracts of X. strumarium on the growth and development of shoots and roots in wheat seedlings

The growth inhibition, as well as the growth and development of roots and shoots in seedlings, was shown to be directly proportional to extract concentrations and time. This study showed that DCM extracts of leaf and roots were more effective for the growth inhibition of roots and shoots in seedlings than the methanol extracts of leaf and roots of *X. strumarium* (Tables 5 and 6; Figures 4 and 5). DCM is more effective at separating non-polar and semi-polar compounds, such as terpenoids, steroids, and specific flavonoids, that are frequently linked to allelopathic and phytotoxic actions (Gniazdowska & Bogatek, 2005). DCM-extracted non-polar compounds may permeate plant cell membranes more effectively, causing greater toxicity and impairment of cellular functions than polar methanol extracts (Macías et al., 2007). According to Singh et al. (2003) extracts of *X. strumarium* produced using non-polar solvents had more biological activity due

Table 4: Effect of DCM and Methanol root extracts of *P. hysterophorus* on wheat seed germination

Extracts	Concentrations (mg/mL)	Mean length of roots ±S.E (cm)				Mean length of shoots ±S.E (cm)			
		3 days	6 days	9 days	12 days	3 days	6 days	9 days	12 days
DCM	5.0	0.2±0.34	0.7±0.88	0.9±0.34	1.1±0.56	0.2±0.12	1.2±0.67	3.3±0.38	4.4±0.56
	2.5	0.4±0.56	0.8±0.56	1.2±0.78	1.8±0.53	0.3±0.34	1.2±0.51	4.5±0.81	5.1±0.34
	1.25	0.4±0.67	2.1±0.23	2.3±1.57	2.5±86	0.4±0.54	3.5±1.09	8.5±2.23	9.3±2.03
	0.62	1.8±0.83	4.1±1.04	4.4±2/09	4.6±1.85	0.7±0.32	4.8±2.65	10.1±0.4	11.5±0.6
	0.31	1.9±0.56	4.5±2.13	4.8±0.52	5.1±2.54	0.7±0.31	4.4±1.52	8.3±0.75	10.1±0.8
	0.15	1.9±0.76	4.9±0.89	5.1±0.62	5.2±1.37	0.9±0.46	5.5±2.75	10.2±0.8	11.2±0.4
	Control	0.6±0.87	2.8±0.54	3.6±0.76	4.1±0.94	0.3±0.56	2.6±0.56	7.7±0.78	9.8±0.54
Methanol	5.0	0.0±0.00	0.0±0.00	0.0 ±0.0	0.0±0.0	0.0±0.00	0.0±0.00	0.0±0.0	0.0±0.0
	2.5	0.1±0.44	0.2±0.34	0.2±0.43	0.3±0.34	0.1±0.45	0.4±0.23	1.4±0.21	2.0±0.34
	1.25	0.2±0.33	0.4±0.35	1.7±0.53	2.3±0.76	0.3±0.54	1.6±0.35	6.4±0.53	9.5±0.76
	0.62	0.3±0.56	0.8±0.56	2.3±0.76	3.4±0.65	0.4±0.32	2.1±0.76	6.8±0.61	9.5±1.23
	0.31	0.7±0.62	2.5±0.66	3.9±0.91	4.2±1.05	0.6±0.23	2.8±0.61	7.9±1.74	10.1±0.8
	0.15	0.8±0.23	2.9±1.05	4.1±1.03	4.5±0.54	0.6±0.34	3.3±0.67	8.3±2.34	10.5±0.6
	Control	0.6±0.87	2.8±0.54	3.6±0.76	4.1±0.94	0.3±0.56	2.6±0.56	7.7±0.78	9.8±0.54

Table 5: Effect of DCM and Methanol leaf extracts of *X. strumarium*on wheat seed germination

Extracts	Concentrations (mg/mL)	Mean length of roots ±S.E (cm)				Mean length of shoots ±S.E (cm)			
		3 days	6 days	9 days	12 days	3 days	6 days	9 days	12 days
DCM	5.0	0.0±0.0	0.1±0.21	0.1±0.31	0.2±0.21	0.0±0.0	0.3±0.23	0.5±0.23	0.8±0.45
	2.5	0.1±0.23	0.1±0.34	0.3±0.21	0.4±0.23	0.1±0.23	0.4±0.34	0.9±0.45	2.3±0.54
	1.25	0.4±0.32	0.9±0.54	1.3±0.54	1.4±0.51	0.1±0.23	1.1±0.56	2.4±0.54	5.7±0.89
	0.62	0.5±0.32	1.0±0.23	1.5±0.76	1.9±0.48	0.2±0.32	1.4±0.54	3.6±0.78	6.3±1.29
	0.31	0.5±0.23	1.5±0.76	2.2±1.09	3.1±1.23	0.2±0.23	1.7±0.76	5.3±1.06	7.1±2.92
	0.15	0.8±0.54	3.1±0.32	3.9±0.89	4.5±2.14	0.3±0.21	2.3±97	8.2±1.38	10.2±0.5
	Control	0.6±0.87	2.8±0.54	3.6±0.76	4.1±0.94	0.3±0.56	2.6±0.56	7.7±0.78	9.8±0.54
Methanol	5.0	0.1±0.24	0.1±0.23	0.1±0.23	0.1±0.23	0.1±0.32	0.1±0.23	0.7±0.34	1.4±0.43
	2.5	0.2±0.26	0.3±0.32	0.4±0.41	0.5±0.23	0.1±0.23	0.5±0.34	1.9±0.76	3.5±0.49
	1.25	0.6±0.43	1.3±0.45	1.3±0.76	1.5±0.65	0.2±0.34	1.6v0.65	4.5±0.87	5.6±0.76
	0.62	0.8±0/49	1.5±0.57	1.8±0.87	3.5±0.76	0.3±0.24	1.8±0.45	5.1±0.56	6.5±1.04
	0.31	1.1±0.78	3.1±0.78	3.6±0.56	4.8±1.92	0.4±0.31	2.7v0.78	8.3±0.54	10.2±0.6
	0.15	1.3±0.36	3.8±0.87	4.2±0.67	5.3±0.34	0.5±0.42	3.8±0.34	9.7±0.87	12.5±0.8
	Control	0.6±0.87	2.8±0.54	3.6±0.76	4.1±0.94	0.3±0.56	2.6±0.56	7.7±0.78	9.8±0.54

to the presence of lipophilic secondary metabolites. During the initial growth stage of seedlings for three days, the length of roots and shoots was greater in lower concentrations (from 0.15 to 0.31 mg/L) of DCM and methanol extracts of leaf and root than in the control treatment; however, at higher concentrations, the length of roots and shoots were decreased than that of the control treatment as in *P. hysterophorus* (Tables 5 and 6).

When compared to the extracts of plant parts, root extracts (both methanol and DCM) of *X. strumarium* showed a higher inhibition for the growth and development of roots and shoots in wheat seedlings than the leaf extracts (both methanol and DCM). It might be because roots have a higher concentration of allelochemicals than leaves do. However, allelochemicals enter the soil via many mechanisms and affect plant competition by reducing seed germination, root elongation, and shoot elongation in nature. The longevity and impact of these compounds are determined by soil composition, microbial activity, and environmental conditions.

Correlation between extracts of P. hysterophorus and X. strumarium and growth of wheat seedlings using principal component analysis (PCA)

The effect of plant extracts treatment of *Parthenium hysterophorus* and *Xanthium strumarium* on the growth of shoot and roots in wheat seedling was analyzed by PCA (Appendix 1 & 2). The result of the individual showed that the proportion of variance was found in axes PCA1 to PCA%. However, more than 95% of the proportion of variance was explained by PCA1 and PCA2.

The biplot of PCA on DCM and Methanol leaf extract (Figure 6) and root extract (Figure 7) of *P. hysterophorus* showed negative relationships with shoot and root elongation of wheat seedlings. It means when the concentration of plant extract increased, the plant part growth decreased. On the other hand, the growth of root and shoot elongation showed a positive relationship with time, i.e., when the days increased, the plant part elongation (growth) was also found to increase in extract used of all concentrations.

The growth of roots and shoots in seedlings by the use of DCM and methanol of both plant parts was found higher than the control condition. At high concentrations (5 mg/mL), the methanol root extract of the *P. hysterophous* did not affect the elongation of shoots or roots.

A similar negative effect was found between the shoot and root elongation of the wheat seedling and the PCA biplot on the concentration of DCM and methanol leaf extract (Figure 8) and root extract (Figure 9) of the *Xanthium strumarium*. In other words, the growth of root and shoot elongation showed a positive relationship with time, meaning that as the number of days increased, the plant part elongation (growth) also increased in the extract used of all concentrations. This indicates that the stem and root elongation decreased as the concentration of plant extract increased.

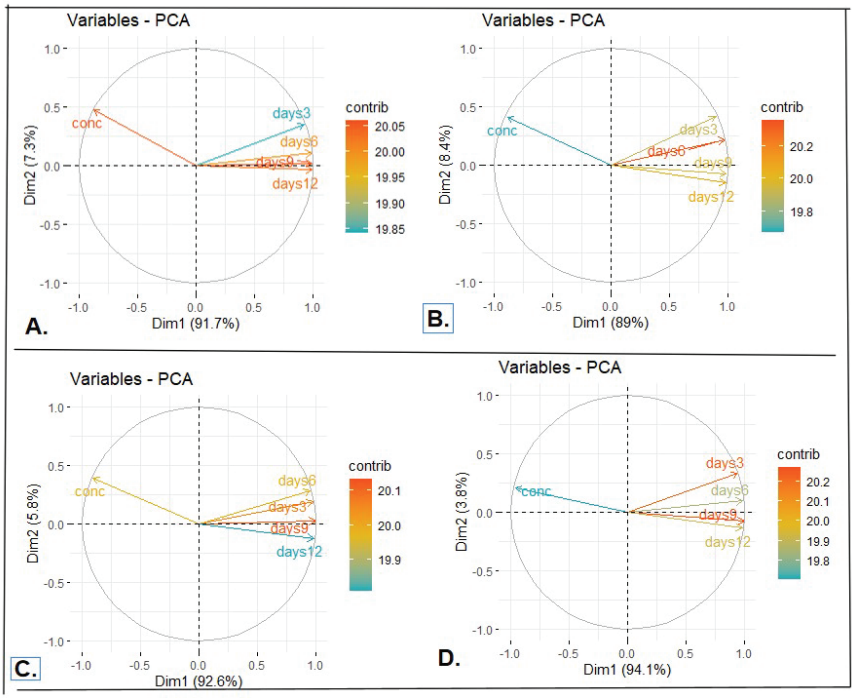


Figure 7: Bi-plot of individuals and variables on the effect of root extract on DCM (**A.** root growth & **B.** shoot growth) and on Methanol (**C.** root growth & **D.** shoot growth) of *P. hysterophorus* on wheat seed germination

At high concentrations (5 mg/mL), the DCM root extract of the *Xanthium strumarium* did not affect the elongation of shoots or roots. In most cases, the growth rate of the root and shoot of the treated plant was found higher than the high concentration and controlled condition. A similar result was found in the germination and growth of turnip, spinach, and ladies' finger by treatment of aqueous extract of *Terminalia belerica* in comparison with control (Talukder et al., 2015).

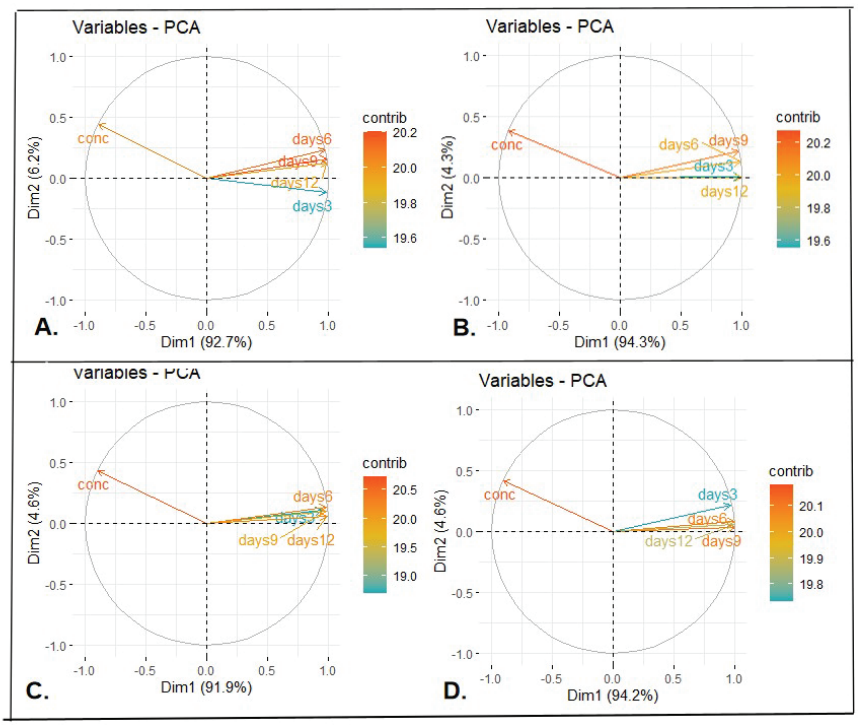


Figure 8: Bi-plot of individuals and variables on the effect of leaf extract on DCM (**A.** root growth & **B.** shoot growth) and on Methanol (**C.** root growth & **D.** shoot growth) of *Xanthium strumarium* on wheat seed germination

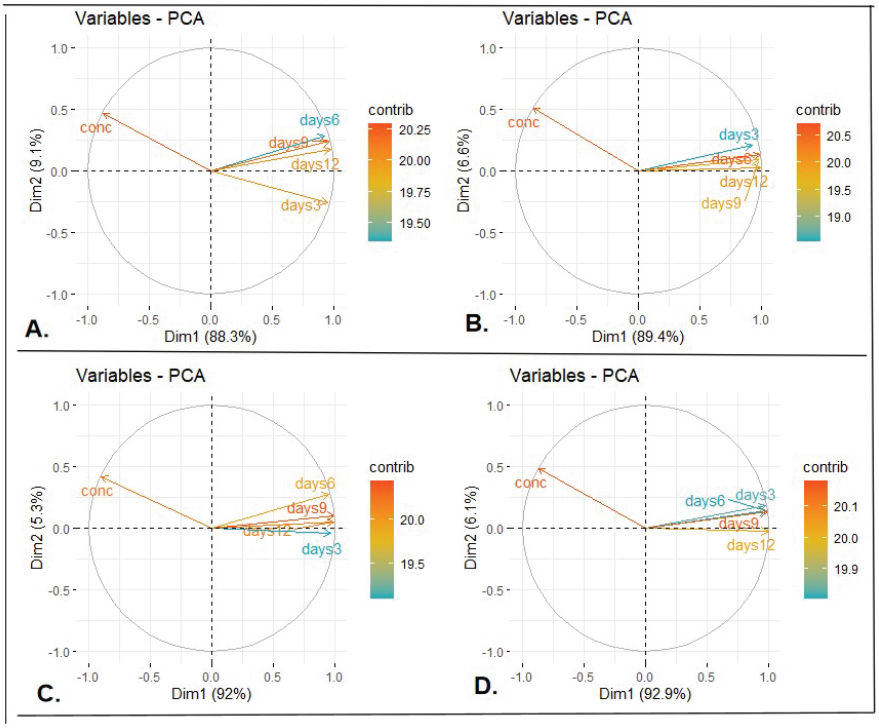


Figure 9: Bi-plot of individuals and variables on the effect of root extract on DCM (**A.** root growth & **B.** shoot growth) and on Methanol (**C.** root growth & **D.** shoot growth) of *Xanthium strumarium* on wheat seed germination

Assessment of brine shrimp lethality assay using various extracts of P. hysterophorus and X. strumarium

This study found that DCM and methanol extracts of *X. strumarium* leaf and root extracts showed higher cytotoxicity to brine shrimp nauplii than *P. hysterophorus* leaf and root extracts. The lethal concentration 50% (LC_{50}) is a widely utilized measurement to determine the cytotoxicity of substances or extracts, especially in toxicological and pharmaceutical studies. A lower LC_{50} value for extracts indicates higher cytotoxicity. The root DCM extract of *X. strumarium* had the highest cytotoxic activity (LC_{50}: 129.16 µg/mL), followed by the root methanol extract of *X. strumarium*, the root DCM extract of *P. hysterophorus*, and the leaf methanol extract of *P. hysterophorus* against brine shrimp nauplii (Table 7). The percentage mortality of brine shrimp nauplii was dosage-dependent after 24 hours of incubation.

The cytotoxicity of extracts was compared with a positive control, potassium dichromate (LC_{50} = 65.5 µg/mL). Meyer et al. (1982) defined cytotoxicity as a chemical compound or extract with an LC_{50} value of less than 1000 µg/mL in the brine shrimp lethality test (BSLA). According to Oketch-Rabah

Table 7: LC_{50} values of various extracts of *P. hysterophorus* and *X. strumarium*

Plants	Plant parts	Extracts	IC_{50} (µg/mL)
Parthenium hysterophorus	Leaf	DCM	438.24
		Methanol	497.44
	Root	DCM	302.93
		Methanol	395.48
Xanthium strumarium	Leaf	DCM	293.03
		Methanol	392.31
	Root	DCM	129.16
		Methanol	216.38
Potassium dichromate (positive control)	IC_{50}: 65.5 µg/mL		

et al. (1999) the indicated cytotoxic activity is poor at LC_{50} of 500-1000 µg/mL, medium at 100-500 µg/mL, and strong at 0-100 µg/mL. Therefore, all the extracts of *P. hysterophorus* and *X. strumarium* were moderately cytotoxic to brine shrimp nauplii. However, the root extracts of both plants were more cytotoxic than the leaf extracts, and the DCM extracts of both plants were more cytotoxic than the methanol extracts on the brine shrimp nauplii. It shows that the concentration of allelochemicals depends on the plant parts. Earlier studies found that DCM extract was more cytotoxic to nauplii than other types of extracts from other plants, including *Lilium nepalense* D. Don (Thapa et al., 2023), *Tephrosia purpurea* (L.) Pers.*, Andrographis paniculata* (Burm.f.) Wall. ex Nees, and *Oldenlandia umbellata* L. (Suneka and Manoranjan, 2021).

Relationship between the phytotoxicity and cytotoxicity of various extracts of P. hysterophorus and X. strumariumto wheat seedlings and brine shrimp nauplii

According to this study, the specific extracts with allelochemicals of *P. hysterophorus* and *X. strumarium* that exhibited higher phytotoxicity to wheat seedlings (plant cells) also typically had higher cytotoxicity to brine shrimp nauplii (animal cells). It might be because allelochemicals in plants and animals work similarly. For instance, the DCM extracts of *X. strumarium* leaves and roots showed more cytotoxicity to brine shrimp nauplii and greater inhibition to the growth of roots and shoots in wheat seedlings than those of methanol extracts of *X. strumarium* leaves and roots. Similarly, in the case of plant parts, the roots of both plants showed higher cytotoxicity to brine shrimp nauplii and greater inhibition to the growth of roots and shoots in wheat seedlings than the leaves. This study can infer that the extract contains allelochemicals or phytotoxins that have growth-inhibitory or general cytotoxic effects. These results indicate that the extract might have a non-specific mechanism affecting both plants and animals, and broad-spectrum biological action, which could include antibacterial, anticancer, or herbicidal effects (Weston & Duke, 2003). According to Mousavi et al. (2021) allelopathic and cytotoxic properties are often linked with phenolic compounds (such as caffeic acid, rosmarinic acid) and terpenoids (such as carvacrol, thymol).These substances impair cellular processes, such as the integrity of membranes, mitochondrial activity, and oxidative balance, in aquatic organisms and plants (Pinheiro et al., 2015). *Juglans regia* releases allelochemicals that reduce nearby weeds and exhibit cytotoxicity to brine shrimp, indicating broader antibacterial or antipredator properties (Dorąevic et al., 2022). However, methanol extracts of leaf and roots of *P. hysterophorus* showed higher inhibition to the growth of roots and shoots in wheat seedlings, and the DCM extracts of leaf and roots showed higher cytotoxicity to brine shrimp nauplii.

Conclusion

This study showed that the allelochemicals or phytotoxins found in the methanol and DCM extracts of the roots and leaves of *P. hysterophorus* and *X. strumarium* were phytotoxic to wheat seeds and seedlings, and cytotoxic to brine shrimp nauplii. The concentration of allelochemicals may vary across various types of solvent extracts, and their distribution may vary among plant parts. The root methanol extract of *P. hysterophorus* was more effective at inhibiting seed germination and the growth of roots and shoots in wheat seedlings; however, the root DCM extract of *X. strumarium* was more effective in inhibiting the growth of roots and shoots in wheat seedlings, indicating that the nature (polar or non-polar) of phytotoxic chemicals differ based on plant species. Similarly, root DCM extracts of *X. strumarium* and *P. hysterophorus* were more toxic to brine shrimp nauplii than leaf methanol extracts. Moreover, the roots of both plants were more phytotoxic to seedling growth and cytotoxic to brine shrimp nauplii than the leaves, indicating that the roots contained a higher concentration of allelochemicals. This study also exhibits that allelochemicals present in both plant extracts have similar modes of action for phytotoxicity to seedling growth and cytotoxicity to brine shrimp nauplii. This study will be helpful for future research into the effect of allelochemicals on natural vegetation

and crop plants, as well as the anticancer activity of allelochemicals found in *P. hysterophorus* and *X. strumarium*on various human cell lines.

Author Contributions

C B Thapa did conceptualization, methodology, formal data analysis, writing draft, and final editing and U Thapa did plant collection, data curation, and lab work. B R Nepali did Data analysis, and editing.

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Appendix 1: Result of individuals based on the explained variance of PCA analysis on the effect of DCM and methanol extracts of *Parthenium hysterophorus* on the growth of wheat seedlings

Used plant parts	Particular	DCM				Methanol			
		Root length		Root length		Root length		Root length	
		PCA1	PCA1	PCA1	PCA1	PCA1	PCA2	PCA1	PCA2
Leaf extract	Standard deviation	2.14	2.14	2.14	2.14	2.14	0.48	2.17	0.48
	Proportion of variance	0.91	0.92	0.92	0.92	0.92	0.05	0.94	0.05
	Cumulative proportion	0.91	0.92	0.92	0.92	0.92	0.96	0.94	0.98
Root extract	Standard deviation	2.14	2.15	2.15	2.15	2.15	0.51	2.17	0.55
	Proportion of variance	0.92	0.93	0.93	0.93	0.93	0.05	0.93	0.06
	Cumulative proportion	0.92	0.93	0.93	0.93	0.93	0.97	0.34	0.96

Appendix 2: Result of individuals based on the explained variance of PCA analysis on the effect of DCM and methanol extracts of *Xanthium strumarium*on the growth of wheat seedlings

Used plant parts	Particular	DCM				Methanol			
		Root length		Shoot length		Root length		Shoot length	
		PCA1	PCA2	PCA1	PCA2	PCA1	PCA2	PCA1	PCA2
Leaf extract	Standard deviation	2.15	0.55	2.17	0.46	2.14	0.48	2.17	0.48
	Proportion of variance	0.93	0.06	0.93	0.04	0.92	0.05	0.94	0.05
	Cumulative proportion	0.93	0.98	0.93	0.98	0.92	0.96	0.94	0.98
Root extract	Standard deviation	2.10	0.67	2.11	0.57	2.14	0.51	2.17	0.55
	Proportion of variance	0.88	0.09	0.89	0.07	0.92	0.05	0.93	0.06
	Cumulative proportion	0.88	0.97	0.89	0.96	0.92	0.97	0.34	0.96