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Toxicity assessment and phytochemical analysis of *Broussonetia papyrifera* and *Lantana camara*: Two notorious invasive plant species

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Abstract

The blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security. As concern regarding the health issues of synthetic medicinal, industrial and agricultural chemicals increases, attention is focused on finding some alternative management strategies. Plant derived toxic chemicals being comparatively safer hold great prospects in this regard. In the present investigation, polar and nonpolar fractions of *Brousssonetia papyrifera* and *Lantana camara* were assessed for brine shrimp cytotoxicity, sandwich method and radish seed phytotoxicity in search of potential bioactive botanicals. *L. camara* methanol extract (LCME) with LD_{50}<1000ppm revealed *in vivo* cytotoxicity and its potential for antitumor compound exploration. *B. papyrifera* methanol extract (BPME) and *L. camara* chloroform extract (LCCE) represented good phytotoxicity while LCME was found to have significant phytotoxic potential with 60.48±1.77%, 54.70±2.26% and 72.85±2.69% radish root inhibition respectively. Similarly, leaf litter leachates of *L. camara* at 50mg concentration rendered 49.26±5.40% inhibition in lettuce root. Species-extract-concentration dependent toxic effects were observed for all the described assays. Our study suggests dose adjustments, isolation and structure elucidation of bioactive compounds for direct use or as lead compounds in the pharmaceutical or agrochemical industry.

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Introduction

The capability of plants to serve human beings in a range of aspects has been well documented since antiquity. Nature has produced wonderfully complex molecules in the form of secondary metabolites in plants that no synthetic chemist could ever dream up (Kumar *et al.* 2011) that have long been and will continue to be important sources and models for medicinal, agricultural and other industrial raw materials (Morris, 1999). Nowadays, people are more interested to utilize eco-friendly and bio-friendly plant based products (Bibi *et al.*, 2011). The search for such bioactive constituents has been quite productive in toxic plants (Rates, 2001). If explored fully and planned wisely, toxic compounds of plant origin elicit quite effective applications in managing health and improving the productivity of agricultural systems (Khanh *et al.*, 2005; Albuquerque *et al.*, 2011; Farooq *et al*, 2013).

Phytotoxins are considered a potential source of pharmaceutical drugs. Pharmacology is simply toxicology at a higher concentration and toxicology is simply pharmacology at a lower concentration, showing that dose adjustment differentiates a poison and a remedy (Parasuraman, 2011). Phytoxins are also used as agricultural chemicals such as bio-herbicides, bio-insecticides, bio-fungicides and bio-rodenticides the necessity of which is due to the emergence of resistance to older synthetic molecules and their hazardous effects on environment (Awan *et al.*, 2012).

Novel weapons hypothesis for plant invasion states that an invader adds toxic chemical(s) to the environment that exert strong toxic effects against native residents of the exotic range (Chengxu *et al.*, 2011; Qureshi *et al.*, 2014) but most invasive species have been neglected and much less surveyed for biologically active chemicals. For this study, two invasive species (*Lantana camara* and *Broussonetia papyrifera*) were selected being declared invasive in Pakistan.

L. camara is evergreen aromatic shrub in family Verbenaceae. Its toxic chemicals are reported to be present in all parts of the shrub which on release in surrounding interfere with many species (Choyal and Sharma, 2011). The toxicity of *L. camara* shrub is well known that has long been investigated for nematicidal, termiticidal, insecticidal and repellent activity (Verma and Verma, 2006; Mohamed and Abdelgaleil, 2008; Kalita and Bhola, 2013). On the contrary, *L. camara* has been reported to possess a number of medicinal properties to treat various human ailments such as malaria, dermatological and gastrointestinal diseases, tetanus, tumors and cancer (Kalita *et al.*, 2012).

B. papyrifera is a deciduous tree in Moraceae family. Its invaded areas are reported to be with changed vegetation patterns considerably having a lower diversity of herbaceous as well as woody species (Malik and Husain, 2007). Pollens from its flowers are allergens causing rhinitis and asthma (Hsu *et al.*, 2008). *B. papyrifera* has been used for the treatment of dysentery, hernias, oedema, tinea and in traditional Chinese medicine (Xu *et al.*, 2010). Its leaf extract is reported to be antifungal, antioxidant and antihepatotoxic (Yang *et al.*, 2014).

In the present study the toxicity levels of polar and nonpolar extracts of both species were investigated in laboratory with the objectives to explore their potential as sources of chemicals for medicinal (particularly anticancer) and/or agricultural (bio-herbicide, bio-pesticide) use in the future.

Materials and methods

Collection and extraction of plant material

The plant material was collected from PMAS-Arid Agriculture University, Rawalpindi, Pakistan. The fresh, healthy aerial parts of both species were collected and washed with clean water. Specimens were then dried in laboratory at room temperature. The dried specimens were ground to a fine texture and were separately macerated in methanol and chloroform in round bottom flasks for seven days

followed by filtration. The extracts were concentrated using laboratory vacuum rotary evaporator at 40°C. The extracts were weighed, labeled and stored at 4°C till further analysis.

Cytotoxic potential of two invasive species

Brine shrimp lethality assay. Cytotoxic potential was investigated by brine shrimp lethality assay according to the protocol of Rehman *et al.* (2005). Brine shrimp eggs were hatched into a small partitioned tank containing artificial sea water (38g/L, pH=8.5). Brine shrimp nauplii with second instar stage were used to perform the assay.

For this experiment, each extract in three concentrations (1000, 100 and 10 ppm) was taken into small sterile vials in triplicate (9-vials/extract). Ten shrimps were added to each vial using Pasteur's pipette. The vials were maintained under illumination at room temperature and survivors were counted after 24 h. The resulting data were analyzed by using formula;

$$\%\ Mortality = (pc - pt/pc)100$$

Data were evaluated by probit analysis (LdP Line Software) to determine the 'Lethality Dose 50' (LC_{50}) at 95% confidence intervals.

Phytotoxic potential of two invasive species

Radish seed germination assay. The assay was performed as described by Turker and Camper (2002). Two types of determinations were carried out:

(a) Detremination of Root length inhibition Radish seeds were sterilized with 1% mercuric chloride. The Whatman No.1 filter paper was placed in Petri plates and 5 ml for each extract concentration (10, 100, 500, 1000 and 10000 ppm) was added separately. 5 ml distilled water was added after solvent evaporation and then ten radish seeds were placed in each petri plate followed by tight sealing and incubation at 23±2°C. Root length was measured after 1, 3 and 5 days. Percentage growth inhibition by extracts was estimated using relation

% Growth Inhibition=100 (P_C-P_T) /P_C

Where P_T and P_C represent root length of the treatment and control respectively.

(b) Determination of germination index This part of the determination was similar to that of earlier determination except for the extract concentrations and the number of seeds. Here, three different concentrations (100, 1000 and 10000 ppm) and 100 radish seeds were used. Germinated seeds were counted daily up to 5 days. Germination index was calculated as

$$GI = (N1) + \frac{N2 - N1}{2} + \frac{N3 - N2}{3} + \cdots + \frac{Nn - Nn - 1}{n}$$

Where N1, N2, N3-----Nn=Proportion of seeds which germinated on day 1-----n. Each experiment was carried out three times. Results were expressed as the means of three replicates ± the standard deviation of triplicate analysis.

Sandwich method. Phytotoxicity of plant leachates was determined by Sandwich method following the protocol of Fujii *et al.* (2004). Agar solution (0.5% w/v) was prepared and autoclaved at 121°C for 15 min. Plant material was carefully weighed (10, 30, 50mg) and gently tipped into the wells of a six well multi-well plate. By using a pipette, first layer of agar (5 ml) was applied, dried plant material rose up that was allowed to gelatinize, on top of which a second layer of agar was applied. In each dish, five seeds of lettuce were placed above agar. Multi dishes were covered with aluminum foil to protect them from light and kept in incubator at room temperature. Length of radicle and hypocotyl was noted after 72 h for each plant. Percentage growth inhibition in root and hypocotyl in each treatment was estimated using the equation

% Growth Inhibition=100 (P_C-P_T) /P_C

Where P_T and P_C represent root/hypocotyl length of the treatment and control respectively.

Qualitative phytochemical analysis of plant extracts

Test for alkaloids. Mayer's reagent: Mercuric chloride (0.356 g) was dissolved in 60 ml of water and potassium iodide (5g) was dissolved in 20 ml water. Both solutions were mixed and volume was made up to 1000 ml with distilled water.

Dragendorff's reagent: Solution A: Basic Bismuth nitrate (1.7 g) and tartaric acid (20 g) was dissolved in 80 ml of distilled water.

Solution B: Potassium iodide (16 g) was dissolved in 40 ml of distilled water.

Solution A and B was mixed in 1:1ratio. Plant extract (0.5 g) was mixed with 8 ml of 1% HCl, warmed and filtered. Filtrate was treated separately with Mayer's reagent and Dragendorff's reagent. Turbidity or precipitation was observed to indicate the presence of alkaloids.

Test for flavonoids. Plant extract (0.5 g) was shaken with pet. ether to remove the fatty materials. The defatted residue was dissolved in 20 ml of 80% ethanol and filtered. Filtrate was mixed with 4 ml of 1% KOH. A dark yellow color was observed to indicate the presence of flavonoids.

Test for coumarins. Plant extract (0.5 g) was taken in a small test tube and covered with filter paper moistened with 1 N NaOH. The test tube was placed in boiling water for few minutes. The filter paper was removed and examined in UV light for yellow fluorescence to indicate the presence of coumarins.

Test for phenols. Plant extracts were treated with 3-4 drops of freshly prepared $FeCl_3$ solution. Appearance of bluish black color indicated the presence of phenols.

Test for saponins. Plant extract (0.5 g) was dissolved in boiling water in a test tube, allowed to cool and shaken thoroughly. Froth formation was observed to indicate the presence of saponins. ***Test for tannins.*** Plant extract (0.5g) was boiled in 20 ml of distilled water in a test tube and filtered. 0.1% $FeCl_3$ was added to the filtrate. Appearance of brownish green or blue black coloration showed the presence of tannins.

Test for glycosides. Extract (0.5 g) was dissolved in 2.0 ml of glacial acetic acid containing one drop of 0.1% $FeCl_3$ solution and was then underlaid with 1.0 ml of concentrated H_2SO_4. A brown ring at the interface indicated the presence of glycosides.

Results

LCMA showed cytotoxicity with median lethality dose of 354.27 ppm and 23.33% lethality at a concentration of 10 ppm. LCCE and BPME indicated weak cytotoxic activity with 50% lethality at 1000 ppm while BPCE was found not toxic with 40% lethality at 1000 ppm (Table 2). So, it is evident that LCME has cytotoxic activity which can further be exploited for pharmacological activity because brine shrimp is considered as a suitable probe for screening the pharmacological activities in plant extracts.

Table 1. Summary of plants, parts used, solvent used and extraction method.

Plant Species	Family	Vernacular name	Part used	Solvent used	Extraction method	Extract obtained
Broussonetia papyrifera (L.) L'Her. ex Vent. (Syn. *Papyrius papyrifera*, *Morus papyrifera*)	Moraceae	Jangli Shahtoot	Aerial parts (350g)	Methanol	Cold maceration	24g
			Aerial parts (420g)	Chloroform	Cold maceration	25.5g
Lantana camara L. (Syn. *Camara vulgaris*, *Lantana scabrida*)	Verbenaceae	Panj Phuli	Aerial parts (290g)	Methanol	Cold maceration	21g
			Aerial parts (350g)	Chloroform	Cold maceration	20.5

Table 2. *In vivo* cytotoxicity of *L. camara* and *B. papyrifera* extracts on Brine shrimp nauplii.

Plant Extract	Concentration (ppm)	Dead nauplii after 24 hrs.			Surviving nauplii after 24 hrs.			Total Survivors	Mortality (%)	LD_{50}
		R1	R2	R3	R1	R2	R3			
***B. papyrifera* (Met.)**	10	1	1	1	9	9	9	27	10.00	1000
	100	3	2	3	7	8	7	22	26.67	
	1000	5	5	5	5	5	5	15	50.00	
***B. papyrifera* (Chl.)**	10	2	3	3	8	7	7	22	26.67	>1000
	100	3	3	5	7	7	5	19	36.67	
	1000	4	4	4	6	6	6	18	40.00	
***L. camara* (Met.)**	10	1	4	2	9	6	8	23	23.33	354.27
	100	4	4	3	6	6	7	19	36.67	
	1000	6	7	5	4	3	5	12	60.00	
***L. camara* (Chl.)**	10	1	1	2	9	9	8	26	13.33	1000
	100	2	3	3	8	7	7	22	26.67	
	1000	4	6	5	6	4	5	15	50.00	

The effect of five different concentrations (10000, 1000, 500, 100 and 10 ppm) of the extracts on root growth inhibition of radish seedling indicated highest percentage inhibition by LCME (72.85±2.69%) at 10000ppm. All extracts inhibited root growth at 10,000 ppm (Fig. 1). The effect of three different concentrations of each extract (100, 1000 and 10000 ppm) on seed germination was also observed and a gradual decrease in seed germination index for all extracts was observed until the fifth day of incubation with maximum inhibition at 10000 ppm (Fig. 2). BPCE showed most pronounced decrease in index of seed germination at 10000 ppm (63.13±5.86%). In all extracts, both the parameters were found to be directly correlated with concentration of extract used. However, seed germination velocity was less affected as compared to root length. This may be due to the reason that seeds are protected by their integuments, so they seem to be less sensitive to phytotoxins than seedlings (Dandelot *et al.*, 2008).

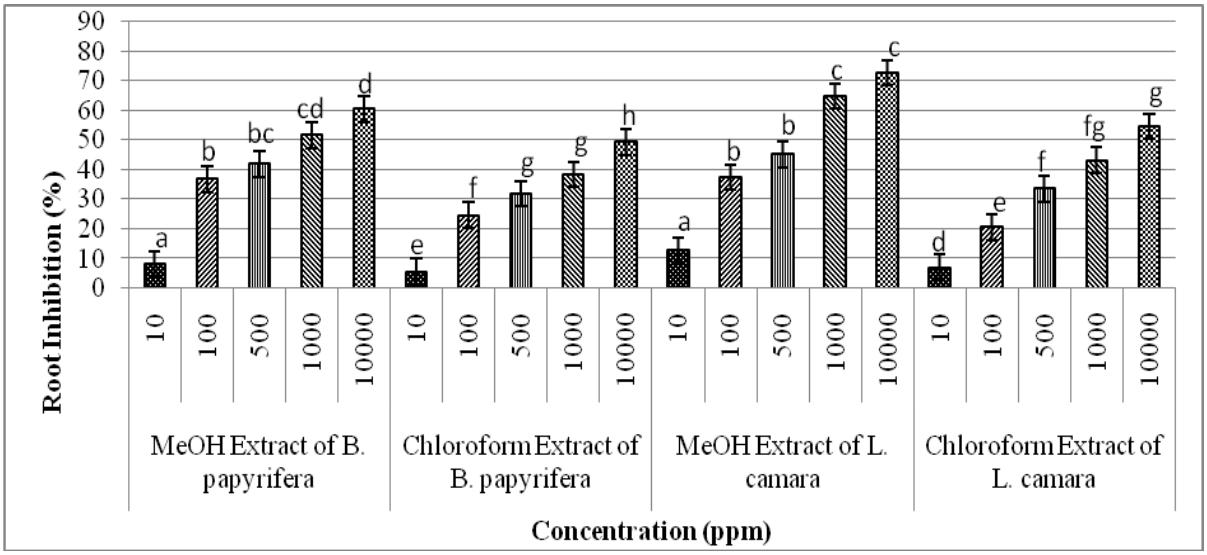

Fig. 1. Radish root inhibition (%) by *L. camara* and *B. papyrifera* extracts.

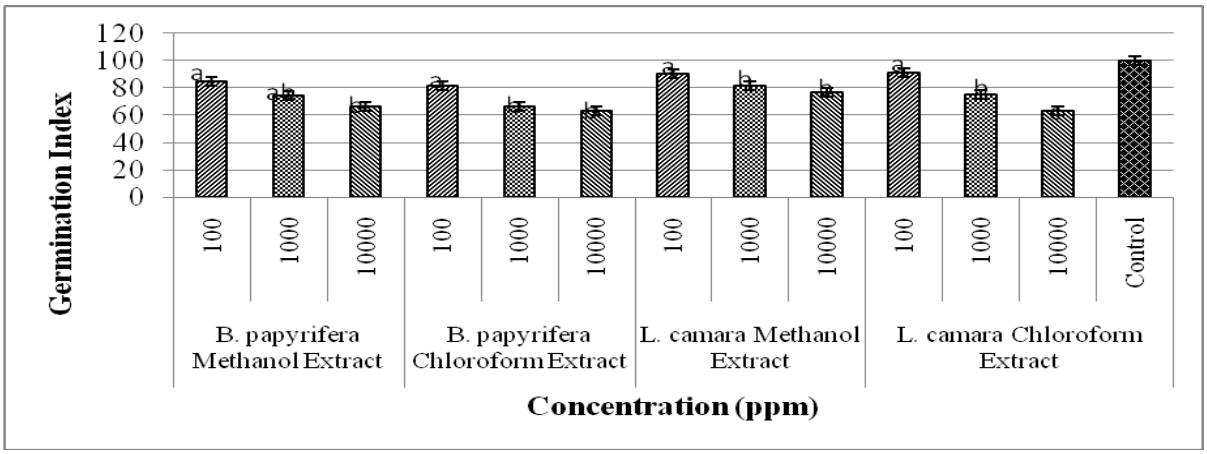

Fig. 2. Radish seed germination index by application of *L. camara* and *B. papyrifera* extracts.

Exposure of lettuce seeds to both invasive species has registered growth inhibition in roots and hypocotyles. Inhibition of root and hypocotyl length in lettuce seedlings was influenced by the concentration of plant material. Maximum toxic potential on root inhibition has shown by *L. camara* dried sample at 50mg concentration (49.26±5.40%). At the same concentration *B. papyrifera* showed inhibition of 42.94±1.32% (Fig. 3). In the same vein maximum toxic potential on hypocotyl inhibition in lettuce seedlings was predicted by *B. payrifera* 50mg sample (32.15±2.18 %) while *L. camara* showed 25.90±1.04% inhibition at the same concentration.

Preliminary phytochemical analysis indicated that alkaloids, saponins, tannins, glycosides, coumarins, flavonoids and phenols were present in LCME and BPME. Saponins were found to be absent in LCCE while tannins and saponins were not indicated in BPCE (Table 3).

Table 3. Preliminary phytochemical analysis of plant extracts.

Plant Extract	Alkaloides	Flavonoids	Saponins	Tannins	Coumarins	Glycosides	Phenols
LCME	+++	+++	++	++	+	++	+++
LCCE	++	+	-	+	+	+	++
BPME	+++	++	+++	+	+	++	+++
BPCE	++	+	-	-	++	+	+

'+' weak '++' moderate '+++' strong presence '-'absence

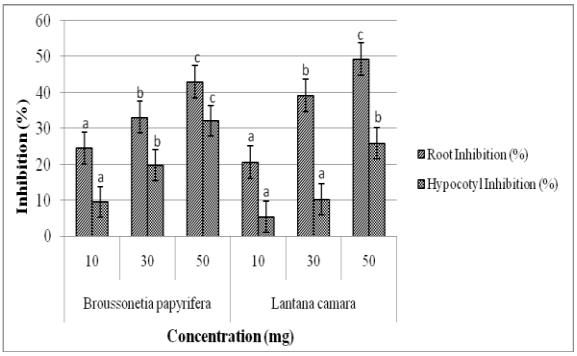

Fig. 3. Root and hypocotyl reduction (%) in lettuce seedlings by *L. camara* and *B. papyrifera*.

Discussion

With direct or indirect utilization capability in medicinal, agricultural and industrial raw materials, study of phytotoxins serve society to protect humans and the environment from the deleterious effects of synthetic toxicants. The Novel Weapons Hypothesis of plant invasion holds that invader plants release some toxic substances that may be synthesized in any plant part, but leaves are considered to be most consistent producers of these phytotoxins (Umer *et al.*, 2010). In this scenario, we investigated toxicity levels of polar and nonpolar extracts from aerial parts

of two species declared invasive in Pakistan through prescreening dose response bio-assays (brine shrimp cytotoxicity assay, sandwich method and radish seed phytotoxicity assay) and qualitative phytochemical analysis was performed to determine possible secondary metabolites accountable for toxicity.

The brine shrimp lethality assay is used to evaluate a broad spectrum of pharmacological activities of natural substances taking into account the basic premise that toxicology is simply pharmacology at a lower dose. From pharmacological point of view, a good relationship has been found with the brine shrimp lethality and antitumor activity of plant extracts (Pour and Sasidharan, 2011). Out of four extracts, cytotoxicity was shown by LCME (LD_{50} = 354.27ppm) while the other extracts showed LD50≥1000ppm that is not significant. Cytotoxicity of this plant is also reported earlier in cell line experiments (Raghu *et al.*, 2004 and Pour *et al.*, 2011). The observed lethality of this extract to brine shrimps indicates the presence of cytotoxic and probably antitumor components in this plant.

Determination of phytotoxicity of a plant species helps in the formulation of natural plant growth regulators or biological herbicides (Khan *et al.*, 2011). The radish seed germination assay is a valuable tool that indicates general phytotoxicity because of their sensitivity to toxic compounds. All extracts exhibited phytotoxicity in radish seed bioassay at high dose maximum inhibition was shown by LCME (72.85±2.69%). Proportional inhibitory effects of concentration were also reported by Mahmood *et al.* (2010) while investigating the phytotoxic potential of *Sorghum bicolor* (sorghum), *Helianthus annuus* (sunflower), *Brassica napus* (brassica), *Zea mays* (maize), *Oryza sativa* (rice) and *Morus alba* (mulberry) water extracts suppressing germination and growth of horse purslane in the laboratory bioassay. Khan *et al.* (2011) also revealed dose dependent phytotoxic effects of methanol extracts of thirteen medicinal plants. Dose effect was also reported by Pukclai and Kato-Noguchi (2012) while investigating allelopathic effect of *Amomum krervanh* Pierre ex Gagnep against five test plant species; *Digitaria sanguinalis* L., *Lactuca sativa* L., *Lepidum sativum* L., *Medicago sativa* L. and *Phleum pratense* L.

Phytotoxicity assessment through sandwich method revealed maximum root inhibition by 50mg pulverized sample of *L. camara* (49.26±5.40%) while maximum hypocotyl inhibition by 50mg *B. papyrifera* (32.15±2.18%). These findings support more root sensitivity to phytotoxins in comparison to hypocotyl. Relative greater root sensivity may be explained by the fact that after the seed germination, roots are the first to come in direct contact with toxic chemicals (Khaliq *et al.*, 2013). Parallel results were forwarded by Fujii and Aziz (2005) while examining the phytotoxic effect of the extracts from 14 plant species of plain areas on the growth of lettuce seeds. Anjum *et al.* (2010) evaluated inhibitory effect of 14 medicinal plants through sandwich technique and suggested strong inhibitory effects of *Albizia lebbeck* and *Broussonetia papyrifera*.

Plants owe their bioactivities by the presence of certain biochemicals. Chemicals that impose toxic influence are called phytotoxins that are classified as secondary plant metabolites (Hadacek, 2002) belonging to diverse chemical groups. Alkaloids, cumarines, flavonoids, hydroxamic acids and phenolic acids are generally cited phtotoxins responsible for their toxic activities in ecosystem. The phenolic compounds (such as flavonoids, tannins and phenols) are reported as the most common and widely distributed toxic metabolites in plants (Arowosegbe *et al.*, 2012). Similarly, various studies indicated that different phytochemicals cause cytotoxicity/cell damage. Alkaloides, flavonoids, tannins and other phytochemicals produce cyototxic effects on tumors (Sharma *et al.*, 2011). Toxicity of plants may be induced by one of these biochemical or different biochemicals may act in synergism to induce toxicity (Hussain and Reigosa, 2011). Thus, the toxicity of extracts in this work could be ascribed to

the toxic compounds such as phenolic compounds, alkaloids and saponins that were found to be present in the extracts. Whatever may be the toxic principle, the promising result displayed by the polar plant extracts in the cytotoxicity and phytotoxicity assays justified the efficacy of these plants as a potential source of pharmaceutical, industrial and agro-chemical.

Conclusion

In conclusion, this study demonstrated that *L. camara* and *B. papyrifera* have bioactive toxic principles. LCME has cyotoxic activity towards brine shrimps and comparatively high phytotoxic ability. Thus, we consider that preferentially this plant might have some useful influences in two fields i.e. medicine and agriculture. However, further toxicity studies are needed for dose adjustment and for isolation and structure elucidation of bioactive compounds responsible for the observed toxicity.

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