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ORIGINAL ARTICLE

Anti-tick Potential and Intra-Specific Chemical Variations in *Ageratum conyzoides* L. Collected from Indus Plain and Central India

Bhanu Kumar[1,2], Pushpendra Kumar Shukla[1], Anil Kumar Sharma[3], Ajay Kumar Singh Rawat[1], Yashwant Singh Rawat[2], Srikant Ghosh[3], Sharad Srivastava[1,*]

[1]Division of Pharmacognosy, CSIR- National Botanical Research Institute, Lucknow, [2]Department of Botany, Kumaun University, Nainital, Uttarakhand, [3]Division of Parasitology, ICAR- Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

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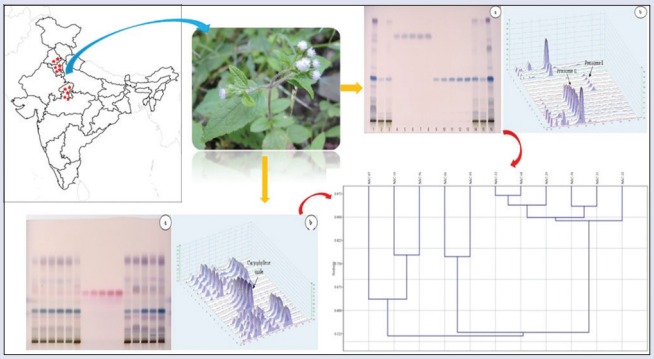
ABSTRACT

Background: Tick and tick-borne diseases significantly affect the animal husbandry sector worldwide. To overcome this menace, chemically synthesized acaricides are being used. However, these acaricides are toxic to the animals, pose environmental threat and also have led to the development of resistance in ticks. **Objectives:** Search for safe and efficacious plant-based alternatives to the chemical acaricides and to assess chemical variability among intra-specific germplasms. **Materials and Methods:** *Ageratum conyzoides* L. samples were collected from different locations and pharmacognostic analysis was performed. The plant extract was subjected to chromatographic profiling and quantification of three bioactive markers precocene I, precocene II, and caryophyllene oxide followed by *in vitro* anti-tick activity through the adult immersion test. **Results:** Precocene I content ranged from 0.001% to 0.019% while precocene II and caryophyllene oxide content varied from 0.003% to 0.11%. In the Indus plain samples, adult tick mortality (%) ranged from 15.0 ± 2.9 to 78.5 ± 4.1, whereas in the Central India, it ranged from 30.0 ± 12.9 to 81.4 ± 7.7 at 8% concentration of plant extract. Precocene II showed weak positive correlation (r = 0.11) with tick mortality. The results indicate that there is considerable variation in the marker compounds content and anti-tick potential of the plant extracts analyzed. There is significant ($P < 0.05$) variation in marker compound content as well as anti-tick potential of *A. conyzoides*. **Conclusion:** The results indicate there apart from the analyzed marker compounds there might be some other phytomolecules playing a role in the tick mortality which needs further investigation.

Key words: Acaricide, *Ageratum conyzoides* L., high-performance thin-layer chromatography, precocene, ticks

SUMMARY

- High-performance thin-layer chromatography analysis exhibited that precocene II content is higher than precocene I content in the samples of Indus plain and central India zones
- There is a significant variation in marker compound content as well as anti-tick potential of *Ageratum conyzoides*
- Anti-tick activity signifies that the anti-tick potential of precocene II was more than precocene I
- A strong positive correlation (r = 0.703) was found between precocene II content and tick mortality in Central India.



Abbreviations used: TTBDs: Tick and tick borne diseases; AIT: Adult immersion test; NMPB: National medicinal plants board; CPCSEA: Committee for the purpose of control and supervision on experimentation on Animals; API: Ayurvedic pharmacopoeia of India; HPTLC: High-performance thin-layer chromatography; BOD: Biological oxygen demand; ANOVA: Analysis of variance; GC-MS: Gas chromatography-mass spectrometry

Correspondence:

Dr. Sharad Srivastava,
Division of Pharmacognosy, CSIR- National Botanical Research Institute, Rana Pratap Marg, Lucknow - 226 001, Uttar Pradesh, India.
E-mail: sharad_ks2003@yahoo.com

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INTRODUCTION

Ticks are very common ectoparasites, infesting cattle, goats, sheep, dogs, etc., *Rhipicephalus microplus* is one of the most important ticks parasitizing on cattle. It is found worldwide mainly in tropical and sub-tropical countries and endemic to the Indian region.[1] The tick infestations are generally associated with decrease in milk production, anemia, and overall animal health.[2] Tick control is mainly done by using synthetic chemical acaricides such as pyrethroids, organophosphates, organochlorines, carbamates, avermectins, and fipronil.[3,4] However, these acaricides are toxic to the animals and harmful in many ways; nonetheless, large scale resistance to these chemicals has also been reported.[5-18] Apart from the chemical acaricides, several plants have been screened for their anti-tick potential. *Ageratum conyzoides* L. is one of such plant and is widely used as an ethnomedicine by the traditional

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healers and rural communities globally. It is used for treating cattle from infestation of ecto-papasites,[19] wound healing,[20] to stop bleeding,[21,22] to treat skin diseases,[23] treating kidney stones,[24] and as an antimicrobial agent.[25,26]

Current scientific investigations have revealed several other therapeutic properties of *A. conyzoides* L. namely, in the management of pediculosis with 96.7% efficacy after 60 min. of exposure,[27] as lipid lowering, hypoglycemic, and antioxidant properties.[28]

A number of metabolites, for example, pyrrolizidine alkaloids was reported in the samples collected from Kenya,[29] the essential oil containing ageratochromenes (42.5%), demethoxyageratochromene (16.7%), β-caryophyllene (20.7%)[30] were reported from this plant. Precocenes, also known as ageratochromenes are reported to cause precocious metamorphosis in insects.[31,32] Two bioactive compounds of *A. conyzoides* L. precocene I (7-methoxy-2,2-dimethyl-2-chromene) and precocene II (6,7-dimethoxy-2,2-dimethyl-2-chromene) have been reported to act as juvenile deficiency hormone in insects[20] and larvicidal activity against *R. microplus*.[33] The role of precocene I on the development and reproduction of pests has been studied and found that it has no acute toxicity to the adults and second instar nymphs; however, it was toxic to third instars in a dose-dependent manner.[34] Precocene II is also reported to have strong antifungal activity.[35] The insecticidal and anti-juvenile hormone activity of precocene II was also reported in the grasshopper.[36]

Humans, from ancient times have been dependent on the natural resources available nearby for their livelihood and healthcare. The practice of treatment through plants continued from one generation to next generation and this has developed into different systems of traditional medicine, also known as ethnomedicine. However, since the drugs or formulations used as ethnomedicine are not standardized for its chemical constituents and doses, it is less acceptable to the modern health-care system. The modern day drug discovery is largely based on the leads obtained from the plants mentioned in the ancient scriptures or used in traditional systems of medicine. The scientific validation of the traditional claims of different properties of plants is required to explore newer possibilities in drug discovery. The present study is conducted to analyze the chemical variations in germplasms of *A. conyzoides* L. collected from two phytogeographical zones and their potential as an anti-tick agent. Correlation between the quantitative value of the marker compounds and anti-tick activity has been analyzed.

MATERIALS AND METHODS

Plant germplasm collection

The plant collection was done as per the guidelines of National Medicinal Plants Board (Ministry of AYUSH; Government of India) in collaboration with the World Health Organization. All the necessary precautions were practiced in due course of plant collection. The plant samples were collected from natural habitats in the wild during the flowering stage. The aerial part of the plants was collected and herbarium sheets prepared. The plant material was authenticated, a unique voucher specimen number (e.g., LWG-305929) was allocated to each collection and the herbarium specimens were submitted to the herbarium of CSIR-National Botanical Research Institute, Lucknow, India. Complete passport data was recorded for each and every collection.

Reference biological materials for evaluation of anti-tick activity.

The reference colony of IVRI-I strain of *R. microplus* (National registration no. NBAII/IVRI/BM/1/1998) maintained in the entomology laboratory, division of Parasitology, ICAR-Indian Veterinary Research Institute was used as a standard to evaluate the acaricidal activity of the extracts prepared from the collected plant accessions. The tick strain was maintained by feeding on cross-bred male calves maintained in the tick proof animal shed following regulatory guidelines provided by "Committee for the purpose of control and supervision of experimentation on animals," a regulatory Indian body monitoring animal experimentation. The genetic homogeneity of the tick strain was established by analysis of the 16 srDNA gene sequences (accession nos. GU222462, GU323287, GU323288, HM176656, HM176657, HM176658).

Preparation of plant extract

The collected germplasms (aerial part) were manually screened and made free of any impurities. The samples were dried in shade and further dried in hot air oven at 40° C to remove any moisture content before grinding in an electric grinder. The coarse powder was subjected to methanolic extraction by continually shaking for 6 h and standing at the room temperature for up to 18 h. The extraction procedure was repeated thrice. The plant extract thus obtained was pooled, filtered, and concentrated under vacuum in a rotatory evaporator (Buchi Rotavpour, Switzerland) at 40°C. The extract obtained finally was freeze-dried and stored at 4°C for further use.

Physicochemical studies

The major physicochemical parameters to standardize herbal extract including water soluble extractive, alcohol soluble extractive, total Ash value, and the acid insoluble ash value were studied as per the standard protocols of Ayurvedic Pharmacopoeia of India.

Chromatographic analysis

High-performance thin-layer chromatography (HPTLC) was performed on Merck precoated silica gel $60GF_{254}$ (20 cm × 10 cm) plates. The stock solution of marker compounds and plant samples were freshly diluted with methanol and filtered to prepare working solution of 0.1 mg/ml and 10 mg/ml, respectively, before HPTLC profiling. Working dilutions of plant samples (15 µl) and precocene I, II, and caryophyllene oxide (3 µl) were applied on chromatographic plate as 6 mm wide bands positioned 10 mm above the bottom and 15 mm from the side of the plate, using CAMAG Linomat V automated TLC applicator with nitrogen flow providing a delivery speed of 150 nl s^{-1} (nanoliter per second). These conditions were kept constant throughout the study. Following sample application, the bands were developed in a CAMAG twin trough glass chamber, saturated with a binary mobile phase of Toluene-Ethyl Acetate (9.8:0.2, v/v) till proper separation of bands up to a height of 8 cm is achieved. For Caryophyllene oxide, the solvent system used was Petroleum ether: Ethyl acetate (10:1 v/v) for resolving the extract. The marker compound was visible after derivatization with anisaldehyde sulphuric acid reagent. The densitometric scanning was done at 520 nm and slit width 6 mm × 0.45 mm. The chromatogram was air dried with an air dryer, the marker compounds were quantified using TLC scanner model 3 equipped with CAMAG visionCATS V software at a wavelength of 300 nm in absorption-reflection mode. Quantification (% dry weight basis) was done on the basis of regression analysis of area versus concentration of standard dilutions.

Adult immersion test for anti-tick activity

For the evaluation of anti-tick activity of different accessions, previously standardized adult immersion test was adopted.[9] All the samples were dissolved in distilled water containing 2% tween-20 to prepare different concentrations. A dose-dependent response study was conducted by preparing 2%–10% concentrations of plant extract. All the Petri dishes containing ticks treated with plant extract were kept at 28°C and 85% ± 5% relative humidity in Biological Oxygen Demand incubator. The death of ticks was confirmed by observing loss of motility and pedal reflex

after exposing to light. The ticks surviving after exposure were reared subsequently for 14 days to generate the data on the efficacy of the plant accession. The posttreatment mortality was monitored starting from 72 h of treatment to 14 days when control ticks complete egg laying. The mortality data of different accessions were statistically analyzed.

Statistical analysis

Cluster analysis and Karl Pearson correlation were performed using PAST version 2.15 (University of Oslo, Norway).[37] The posttreatment mortality data were statistically analyzed using Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA) software. The significance of mean difference among the accessions was analyzed using the one-way analysis of variance ($P < 0.05$). The results were expressed as mean ± standard deviation.

RESULTS

Collection of plant samples

The germplasms were collected from 11 locations of the Indus plain and Central India region. Passsport data sheets were prepared for each collection [Table 1].

Physicochemical study and extraction

The water soluble extractive value ranged from 11% to 17% and alcohol soluble extractive value was varied from 4% to 6%. Total ash and acid insoluble ash value was in the range from 14% to 23% and 9%–12%, respectively [Table 2]. The Indus plain germplasms showed variations in the extractive values ranging from 93 mg to 158.5 mg. Maximum extractive value was found in the germplasms collected from Digoh, Haryana (NAC-68) while the minimum value was found in Kurukshetra, Haryana (NAC-32). The Central India germplasms showed variations in the extractive values ranging from 64.4 mg to 169.5 mg. The maximum extractive value was observed in the germplasms collected from Jabalpur, Madhya Pradesh (NAC-06).

Quantification of marker compounds

A sensitive and reproducible HPTLC method was developed for clear separation of precocene I, precocene II, and caryophyllene oxide [Figures 1a, b, 2a, b and 3a, b]. Precocene I content ranged from 0.001% to 0.019%, precocene II and caryophyllene oxide content ranged from 0.003% to 0.11%. Minimum precocene I content (0.001%) was detected in collected from Digoh germplasm (NAC-68) while maximum (0.019%) content was in NAC-95 germplasm. Similarly, minimum precocene II content (0.003%) was detected in NAC-31 and NAC-30 collected from Haryana and Punjab, respectively, while maximum content (0.11%) was detected in germplasm collected from Chitrakoot, Madhya Pradesh (NAC-07). The 3rd compound, caryophyllene oxide was at minimum quantity (0.003%) in germplasm, NAC-30 collected from Jalandhar, Punjab while maximum content in NAC-07 germplasm collected from Chitrakoot, Madhya Pradesh [Table 3].

Hierarchical cluster analysis

The data obtained from the quantification of marker compounds (precocene I, precocene II, and caryophyllene oxide) were used for the cluster analysis by generating UPGMA dendrogram. The results indicate that samples segregated into two distinct clusters [Figure 4]. Branch I contain three germplasms (NAC-07, NAC-10, and NAC-96), out of which NAC-07 stands alone having maximum content of Precocene II and Caryophyllene oxide. Branch II contains rest of the samples arranged into two branches. One of the branch contain only two samples NAC-06 and NAC-95 which are clustered together due to similarity in their precocene I and precocene II content. The second branch contains all other remaining samples.

Anti-tick activity

Amongst of germplasm collected from Indus plain region, maximum activity of 78.5 ± 4.1 was observed in germplasm collected from Karnal, Haryana (NAC-33) while minimum activity (15.0 ± 2.9) was reported in NAC-68 germplasm collected from Digoh, Haryana [Table 4]. The correlation of precocene I and precocene II with % tick mortality was negative, i.e., −0.32 and −0.27, respectively. Of the germplasms collected from central India, maximum anti-tick activity of 81.4 ± 7.7 was detected in the extracts prepared from the germplasm collected from Gwalior, Madhya Pradesh (NAC-10) while minimum activity (30.0 ± 12.9) was found in the extracts prepared from the germplasm of Bagraji, Madhya Pradesh (NAC-95) [Table 4]. A positive Karl Pearson correlation coefficient of 0.11 between tick mortality (%) with the concentration of precocene II and caryophyllene oxide was detected.

DISCUSSION

The problem of tick infestation in cattle is a matter of global concern not only because they directly harm cattle but also spread the pathogenic

Table 1: Brief passport data sheet of *Ageratum conyzoides* germplasms collected from Indus plain and central India

Sample code	Location/district of plant collection	Voucher number	Altitude (meter)	Latitude	Longitude	Soil type
Indus plain						
Punjab						
NAC-29	Chandigarh	305929	344	30°43′59.93″N	76°46′45.90″E	Loamy and clayey
NAC-30	Jalandhar	305930	240	31°19′33.65″N	75°34′34.26″E	Sandy and clayey
Haryana						
NAC-31	Panipat	305931	231	29°23′27.24″N	76°58′11.17″E	Sandy loamy
NAC-32	Kurukshetra	305932	258	29°58′11.59″N	76°52′41.47″E	Alluvial
NAC-33	Karnal	305933	251	29°41′11.50″N	76°59′26.27″E	Sandy loamy
NAC-68	Digoh	305968	220	29°37′06.67″N	75°40′18.19″E	Sandy loamy
Central India						
Madhya Pradesh						
NAC-06	Jabalpur	305906	417	23°13′11.44″N	79°50′00.55″E	Blackish clayey
NAC-07	Chitrakoot	305907	141	25°10′53.41″N	80°51′34.03″E	Sandy
NAC-10	Gwalior	305910	215	26°13′15.37″N	78°10′19.37″E	Alluvial
NAC-95	Bagraji	305995	435	23°18′50.78″N	80°15′46.07″E	Blackish clayey
NAC-96	Satna	305996	331	24°36′10.43″N	80°49′56.07″E	Red Gravel sandy

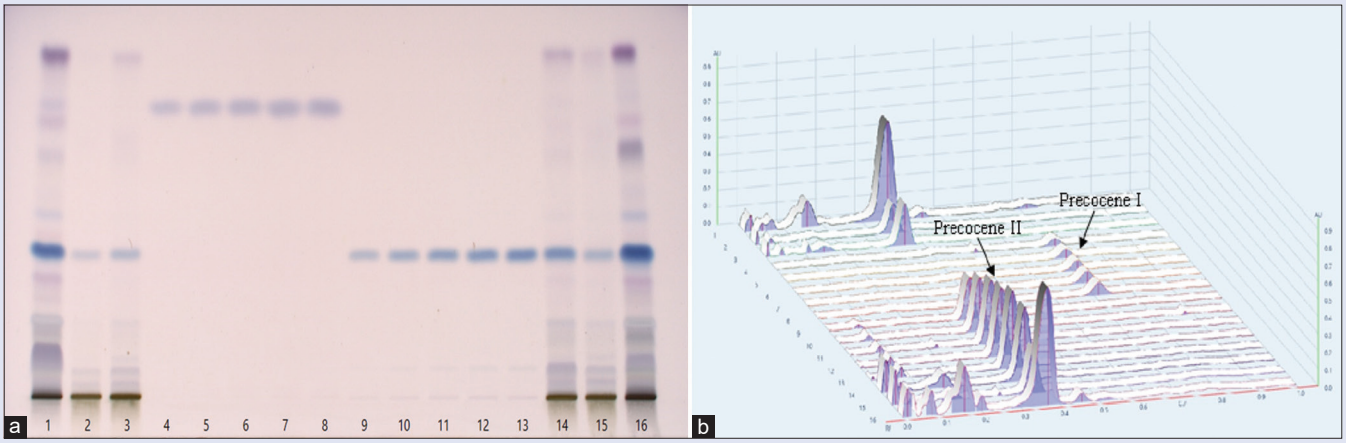

Figure 1: (a) High-performance thin-layer chromatography chromatogram of *Ageratum conyzoides* germplasms collected from Indus plain. Plate visualized after derivatization with Anisaldehyde sulfuric acid reagent. Track 1: NAC-29, Track 2: NAC-30, Track 3: NAC-31, Track 4, 5, 6, 7, 8: Precocene I, Track 9, 10, 11, 12, 13: Precocene II, Track 14: NAC-32, Track 15: NAC-33, Track 16: NAC-68. (b) Densitometric scan profile of *Ageratum conyzoides* germplasms collected from Indus plain

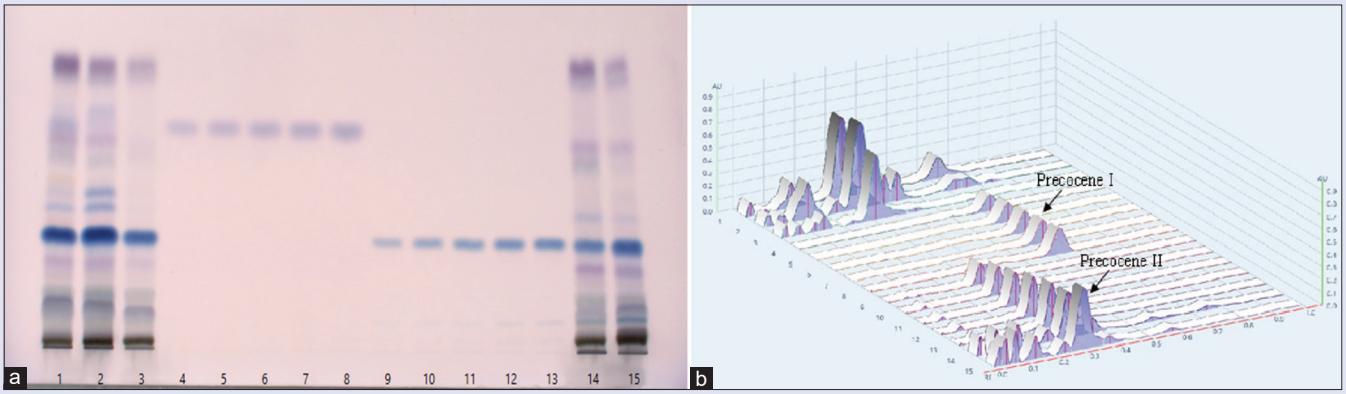

Figure 2: (a) High-performance thin-layer chromatography chromatogram of *Ageratum conyzoides* germplasms collected from Central India visualized after derivatization by Anisaldehyde sulphuric acid reagent. Track 1: NAC-06, Track 2: NAC-07, Track 3: NAC-10, Track 4, 5, 6, 7, 8: Precocene I, Track 9, 10, 11, 12, 13: Precocene II, Track 14: NAC-95, Track 15: NAC-15. (b) Densitometric scan profile of *Ageratum conyzoides* germplasms collected from Central India

Table 2: Physico-chemical parameters of *Ageratum conyzoides* L. from Indus plain and central India

Sample code	Location/district of plant collection	Water soluble extractive (%)*	Alcohol soluble extractive (%)*	Total ash (%)*	Acid insoluble ash (%)*
Indus plain					
Punjab					
NAC-29	Chandigarh	11.91±2.8	4.08±1.2	18.35±2.1	11.95±2.1
NAC-30	Jalandhar	12.33±2.2	5.08±1.1	18.9±1.8	10.2±2.3
Haryana					
NAC-31	Panipat	11.33±1.5	5.33±1.6	21.2±2.4	10.0±3.4
NAC-32	Kurukshetra	17.8±1.8	6.9±0.8	23.4±1.5	10.4±2.8
NAC-33	Karnal	17.58±2.4	5.12±1.2	17.7±2.9	11.05±1.8
NAC-68	Digoh	17.45±2.2	6.77±1.6	14.82±3.2	9.66±2.5
Central India					
Madhya Pradesh					
NAC-06	Jabalpur	15.2±2.1	5.61±1.5	15.11±2.6	11.62±1.3
NAC-07	Chitrakoot	14.4±1.8	5.66±0.7	15.25±2.5	12.0±2.1
NAC-10	Gwalior	16.58±1.5	5.45±1.6	15.02±1.3	11.22±1.8
NAC-95	Bagraji	14.8±2.5	5.12±2.2	16.45±1.7	12.8±2.2
NAC-96	Satna	14.9±3.1	5.32±2.3	15.33±2.5	11.9±2.6

*n=3, values are mean±SD. SD: Standard deviation

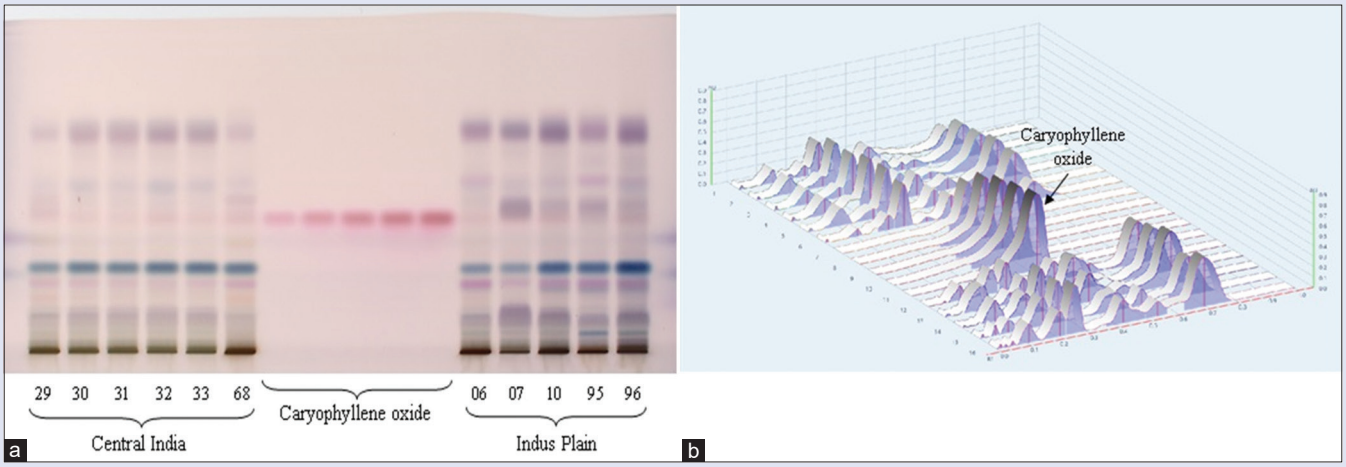

Figure 3: (a) High-performance thin-layer chromatography chromatogram of *Ageratum conyzoides* germplasms against caryophyllene oxide. Plate visualized after derivatization with Anisaldehyde sulphuric acid reagent. (b) High performance thin layer chromatography densitometric profile of *Ageratum conyzoides* germplasms. Scanning done at 520 nm

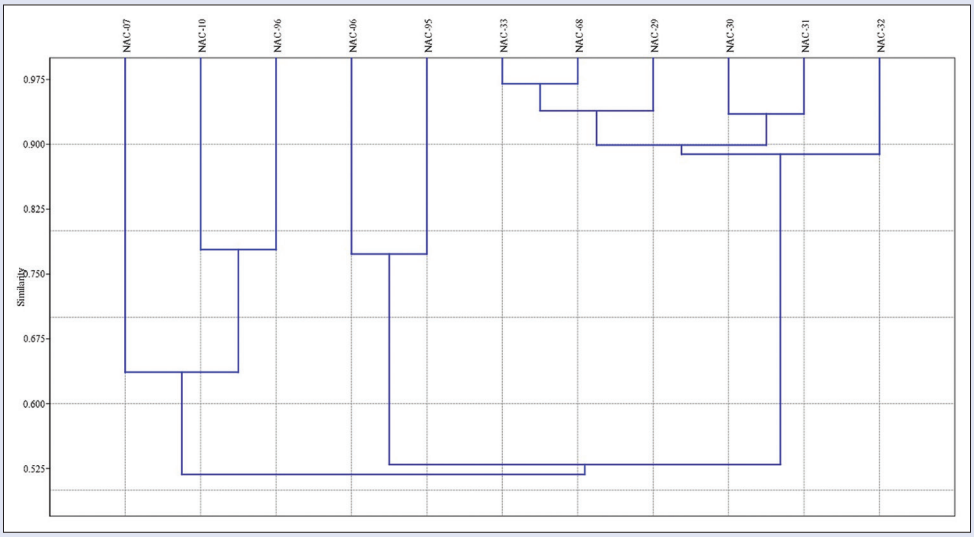

Figure 4: UPGMA dendrogram of germplasms on the basis of precocene I, precocene II, and caryophyllene oxide content

organisms such as protozoans and viruses.[4] Major synthetic chemical acaricides used worldwide for the control of ticks include pyrethroids, carbamates, organophosphates, ivermectin, etc., which are toxic and unsafe for animals.[6] The residues of these compounds, over a long period of continuous use also reach to the meat and the milk, making them unsafe for human consumption. Moreover, recent reports have recorded that there is the development of large scale resistance to these chemicals in the different parts of the country and also in the other parts of the world.[9,12,15] The loss caused by ticks on the productivity of animals, especially milk production in cattle goes down to approximately 50%. In other animals, the quality of hides and wool is also badly affected. Therefore, it is very much required to find out effective, safe, eco-friendly, and sustainable alternatives to the synthetic acaricides.

To tackle this problem, different methods have been recommended and phyto-acaricide has been considered as one of the most viable option.[38] Plants possess a number of natural compounds which can intervene on all biological processes of insects interrupting their life cycle and are considered as an important part of ethno-veterinary practices. Several medicinal plants have been screened for their anti-tick potential,[39] but although it is well documented that same plant accessions collected from different geographical locations have varied level of properties, very limited information are available regarding geographical variations among the germplasms of active plants collected from different geographical locations.

To establish the quality (identity, purity, and strength) of the raw material collected from different places, physicochemical analysis was done which was found to be in accordance with earlier studies.[40] Slight variations in extractive and ash values were found which may be due to the different soil characteristics at different locations as indicated by previous reports.[41] HPTLC analysis exhibited that precocene II content is higher than precocene I content in the samples of both Indus plain and Central India zones. There are reports that precocene I is the biosynthetic precursor of precocene II and that's why precocene II content is higher in mature plants.[42] This may be a possible reason for the higher content of pecocene II in the plant. A comparative gas chromatography-mass spectrometry study of *A. conyzoides* collected from Etawah, Uttar Pradesh has reported that peak area (%) of precocene II was 57.08% in methanol extract while precocene I was 1.10% in methanol extract[43]

Table 3: Precocene I, Precocene II and Caryophyllene oxide content of *Ageratum conyzoides* L. germplasms collected from Indus plain and Central India

Sample code	Location/ district of plant collection	Precocene I content (%)	Precocene II content (%)	Caryophyllene oxide content (%)
Indus plain				
Punjab				
NAC-29	Chandigarh	0.0017	0.016	0.023
NAC-30	Jalandhar	ND	0.003	0.003
Haryana				
NAC-31	Panipat	ND	0.003	0.024
NAC-32	Kurukshetra	ND	0.005	0.050
NAC-33	Karnal	ND	0.011	0.033
NAC-68	Digoh	0.001	0.015	0.033
Central India				
Madhya Pradesh				
NAC-06	Jabalpur	0.014	0.05	0.031
NAC-07	Chitrakoot	0.009	0.11	0.111
NAC-10	Gwalior	0.002	0.10	0.079
NAC-95	Bagraji	0.019	0.04	0.066
NAC-96	Satna	ND	0.05	0.069

ND: Not detected

Table 4: Anti-tick activity of *Ageratum conyzoides* L. germplasms collected from Indus plain and central India

Sample code	Location/district of plant collection	Percentage tick mortality at 8% concentrations
Indus plain		
Punjab		
NAC-29	Chandigarh	64.2±9.2
NAC-30	Jalandhar	71.4±8.2
Haryana		
NAC-31	Panipat	39.3±6.8
NAC-32	Kurukshetra	71.4±11.7
NAC-33	Karnal	78.5±4.1
NAC-68	Digoh	15.0±2.9
Central India		
Madhya Pradesh		
NAC-06	Jabalpur	51.7±9.1
NAC-07	Chitrakoot	62.7±4.3
NAC-10	Gwalior	81.4±7.7
NAC-95	Bagraji	30.0±12.9
NAC-96	Satna	56.5±6.1

which corresponds with the findings in this study. In the above study, peak area of precocene II was also found higher in Dichloromethane (DCM) and hexane extracts. Chromatographic analysis demonstrated considerable variation in marker compound content which resulted into the clustering of samples into groups.

Anti-tick activity signifies that the potency of precocene II was more than precocene I. A strong positive correlation (r = 0.703) was found between precocene II content and tick mortality in Central India; however, precocene I and caryophyllene oxide exhibited weak correlation. In a previous study on *Calea serrata* of *Asteraceae* family, the isolated precocene II produced 99.9% larval mortality in *R. microplus* at 4.25 mg/ml (0.425%) concentration.[44] which agrees with the high positive correlation of tick mortality with precocene II content observed in the present study.

CONCLUSION

The present study highlights the variations in the chemical profile and anti-tick potential of *A. conyzoides* germplasms collected from different locations. The study emphasizes on the role of collection of germplasms from the different regions for chemical profiling of variations to identify elite germplasms. The identification of other phytomolecules in *A. conyzoides* responsible for Anti-tick activity needs to be studied.

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Conflicts of interest

There are no conflicts of interest.

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