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Assessment of intra-specific variability at morphological, molecular and biochemical level of *Andrographis paniculata* (*Kalmegh*)

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In the present investigation, 15 *Andrographis paniculata* genotypes collected from Chhattisgarh and adjoining states were grown in the field for the variability studies. Three different variability parameters namely morphological, molecular and chemotyping of active ingredient content were employed. Wide variations were recorded with regard to quantitative characters and RAPD profile. Ten polymorphic RAPD primers produced a total of 37 amplicons, which generated 70.27% polymorphism. The number of amplified products ranged from 2 to 7 for different primers, whereas the percentage of genetic similarity for the studied primers ranged from 51.4 to 97.0. There were two major clusters formed in the genotypes studied. The andrographolide content ranged from 0.69 to 1.85% and the genotype KI-2 had the highest estimated content. The study demonstrated that simultaneously morphological, molecular and biochemical analysis are useful for characterizing genetic diversity and defining relationships between *kalmegh* germplasm. It also gave possible indications to the phytochemical variation of different genotypes which were due to the genetic differences.

Keywords: *Andrographis paniculata*, andrographolide content, genetic diversity, germplasm, RAPD.

ANDROGRAPHIS PANICULATA (*Kalmegh*) is an erect growing annual medicinal herb and well known for its multiple health-promoting properties. It is found throughout India and other Asian countries namely China, Java, Thailand, etc. It was credited a wonder drug in 1919 for arresting the spread of the contagious disease 'global flu' epidemic. *Kalmegh* is also reported to possess antihepatotoxic[1], antibiotic[2], antimalaria[3], antihepatitic[4], anti-inflammatory[5] and anti-snakevenom[6] properties to mention a few, besides its general use as an immune stimulant agent[7]. It is used as a wonder drug in traditional Siddha and Ayurvedic systems of medicine as well as in tribal medicine in India and several Asian countries for multiple clinical applications.

A recent study conducted at Bastyr University, USA confirms anti-HIV activity of andrographolide[8]. It is widely distributed and exploited as medicinal plant in almost all regions of India (Himachal Pradesh, Andhra Pradesh, Chhattisgarh, Madhya Pradesh, etc.). It is placed at 17th position among the 32 prioritized medicinal plants of India with a demand of 2,197.3 tonnes (2005–06) and annual growth of 3.1%[9]. Several active components have been identified; two of them are andrographolide and neoandrographolide. Andrographolide (the diterpenoid lactones) is the main bitter principle found in high concentrations in the leaves of *kalmegh*[10]. Andrographolides are extracted for their use as drug. The aim of the present study was to find out whether phenotypic variations in the genotypes grown in different locations are merely epigenetic or genetic. Furthermore, if the same are genetically different, then their medicinally active principles have to be estimated to find out possible quantitative variation, which accord for the variation in medicinal activity of the plants collected from different locations. In general, genetic diversity can be measured at any functional level from blueprint (DNA) to phenotype[11]. In this study, possible genetic variation among *A. paniculata* genotypes collected from different locations was analysed using RAPD (random amplified polymorphic DNA) to

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Table 1. Collection of *Andrographis paniculata* genotypes taken for the study

Genotype no.	Collection site	Source
AP1	Kondagaon, Bastar, Chhasttigarh (CG)	Forest area
AP2	Kanker (CG)	Forest area
AP3	Kumharpara, Narayanpur (CG)	Government nursery
AP4	Baster (CG)	Forest areas
AP5	Jagdalpur (CG)	College of Agriculture, IGAU
AP6	Jora, Raipur (CG)	Central nursery
AP7	Maharashtra	Mr Rajaram Tripathi's farm
AP8	Neemach (MP)	Dr Geda collections
AP9	Chitrakoot (MP)	Dean Dayal Research Institute
KI1	College of Agriculture, Indore	Provided by Dr D. K. Shrivastava
KI2	College of Agriculture, Indore	Provided by Dr D. K. Shrivastava
KI3	College of Agriculture, Indore	Provided by Dr D. K. Shrivastava
KI4	College of Agriculture, Indore	Provided by Dr D. K. Shrivastava
KI5	College of Agriculture, Indore	Provided by Dr D. K. Shrivastava
KI6	College of Agriculture, Indore	Provided by Dr D. K. Shrivastava

Table 2. RAPD markers used for amplification

Primer	Primer
OP A01	OP ACO6
OP A02	OP AC10
OP A03	OP AC13
OP A06	OP AG01
OP A08	OP AG03
OP A10	OP AG08
OP A11	OP AG09
OP A13	OP AG19
OP A17	OP AH19
OP AA09	OP AH20
OP AA12	OP AI05
OP AC01	OP AI09
OP AC04	OP AI14
OP AC05	

discriminate molecular variability. Biochemical variations were estimated through quantifying andrographolide contents using HPLC (high performance liquid chromatography).

The performance of *A. paniculata* genotypes was evaluated through field experimentation at the Department of Biotechnology, Indira Gandhi Agricultural University, Raipur. Seeds of 15 genotypes were collected from different farmer's fields, government nurseries and forests of Chhattisgarh and adjoining regions (Table 1). The seeds were sown in small plots in randomized complete block design (RBD) in the field. Observations were recorded on five randomly selected plants in each genotype. Morphological parameters included seven quantitative (plant height, number of branches, number of leaves, leaf length, leaf width, dry weight of whole plant and seed yield per plant) and three qualitative (leaf colour, flower colour and days required for flowering) characters for exploring the phenotypic diversity. RAPD analysis was used for detecting genetic variation and relatedness among different genotypes.

Total genomic DNA from the young leaves of the plants was isolated by using the method described earlier[12]. The quality and quantity of DNA was analysed by nanodrop and agarose (0.8%) gel electrophoresis. Extracted DNA was used in subsequent PCR amplifications, which were performed in a programmable thermocycler (PTC 100, MJ Research Pvt Ltd, USA). Each sample (20 µl) was amplified in a reaction mixture containing 4 µl (80 ng) of genomic DNA, 1 µl of 1.0 unit of *Taq* polymerase (Promega Co, USA), 2 µl of 15 pmoles of 10-mer RAPD primer (Operon Technologies Inc, USA), 2 µl of PCR buffer (500 mM KCl, 100 mM Tris-HCl, 1.0% Triton X-100 and 15 mM $MgCl_2$) and 2 µl (100 µm) dNTP mixture (Promega, USA). The cycling parameters were 3 min at 94°C for pre-denaturation, 40 cycles each of 1 min for denaturation, 1 min at 30°C for annealing, 2 min at 72°C for extension and a final extension at 72°C for 7 min. The mixture was cooled to 4°C and stored at –20°C until electrophoresis. The 10 µl amplified products was separated on 1.5% agarose gel (Pronadisa) in 1×-TAE buffer. The gels were run for 4 h at 90 V and stained with ethidium bromide and photographed under UV illumination. Specific amplified products were scored as present (1)/absent (0) depending on decreasing order of their molecular weights for each DNA sample. Genotypes were grouped by the Dice correlation coefficient and cluster analysis by the unweighted pair group method with arithmetic averages (UPGMA) and phylogenetic tree that summarizes the evolutionary relationship constructed by TREECON software package[13]. Reproducibility of RAPD banding profiles was determined from duplicate loadings of independent, RAPD reaction mixtures and cluster analysis then performed. To test the discriminatory ability of the primers, 27 random primers (Table 2) were initially checked, of which 10 primers were polymorphic and were then used for the dominant RAPD grouping and cluster analysis.

Fully grown plants of each genotype were screened for the active principle (andrographolide) contents. The methanolic extract of the shade-dried leaves was analysed by HPLC at 223 nm to estimate its concentration following

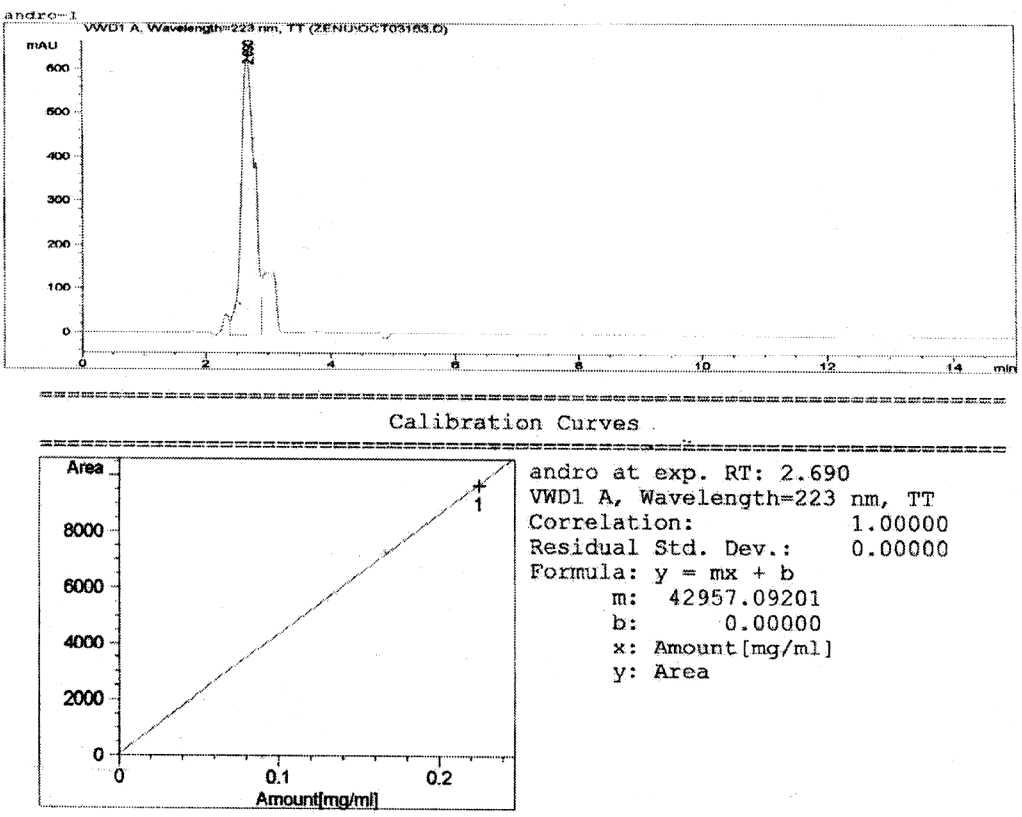

Figure 1. Retention time and calibration curve of standard andrographolid (Sigma grade).

the standard protocol[14]. Leaves at just before flowering of the plants were collected and dried in hot air oven (45°C) for 4 days followed by grinding. The fine leaf powder samples were then used for extracting the andrographolide. HPLC condition was optimized using a ODS C-18 column, mobile phase acetonitril : methanol : water (65 : 20 : 15), flow rate 1 ml per min, column pressure 88.7 bar and total run time of 10 min at 223 nm. The standard andrographolide (Sigma, USA) (22.3 mg) was dissolved in 100 ml methanol in which 20 µl of standard solution was injected into the HPLC column for making calibration curve (Figure 1). Standard andrographolide took 2.690 min as retention time and peak area corresponded to an amount of 0.222 mg/ml, i.e. 22.2 mg in 100 ml (Figure 1). Two gram test sample of dried powder was diluted 1000 times in HPLC solvent and 20 µl of it was injected in three replicates. The retention time and peak area were recorded for calculating the total andrographolide content in each genotype to compute an average content.

Out of 15 genotypes collected from Chhattisgarh and adjoining states, only 10 (AP-1, AP-2, AP-3, AP-4, AP-5, AP-6, AP-7, AP-8, AP-9 and KI-2) could be established in field for further studies. In the later stages, KI-2 superseded all the genotypes. A spectrum of morphological variations was noticed among the genotypes (Figure 2) though raised under uniform environmental and cultural conditions. The plant height ranged from 21.44 (AP-5) to 58.67 cm (KI-2) with a mean height of 39.89 cm; number of branches ranged from 18.44 (AP-2) to 33.44 (AP-6) with a mean value of 22.72, number of leaves ranged from 83.11 (AP-5) to 149.78 (AP-6) with an average of 111.68, leaf breath ranged from 1.07 (AP-6) to 2.96 cm (KI-2) with a mean value of 2.18, leaf length ranged from 5.88 (AP-9) to 7.28 cm (AP-5) with a mean of 6.54, dry weight of plant ranged from 30 (AP-5) to 50 g (KI-2) with a mean value 40.53, seed yield ranged from 2.67 (AP-2) to 4.27 (KI-2) with an average of 3.63. All the quantitative characters were significant at 5% level of probability except leaf length. The quantitative data indicated that genotype KI-2 gave the best performance. It had the maximum plant height (58.67 cm), leaf width (2.96 cm), dry weight of whole plant (50.0 g) and seed weight (4.27 g). The qualitative data revealed not much variation in flower colour among the genotypes, but leaf colour in KI-2 was comparatively distinct (light green) compared to others (dark green). The days required for flowering range from 82 (AP-6) to 158 (KI-2) with mean of 118 days. The genotype KI-2 took maximum number of days (158) for flowering (Figure 3) but showed the relatively best performance when compared for different quantitative characters and growth pattern. The data of

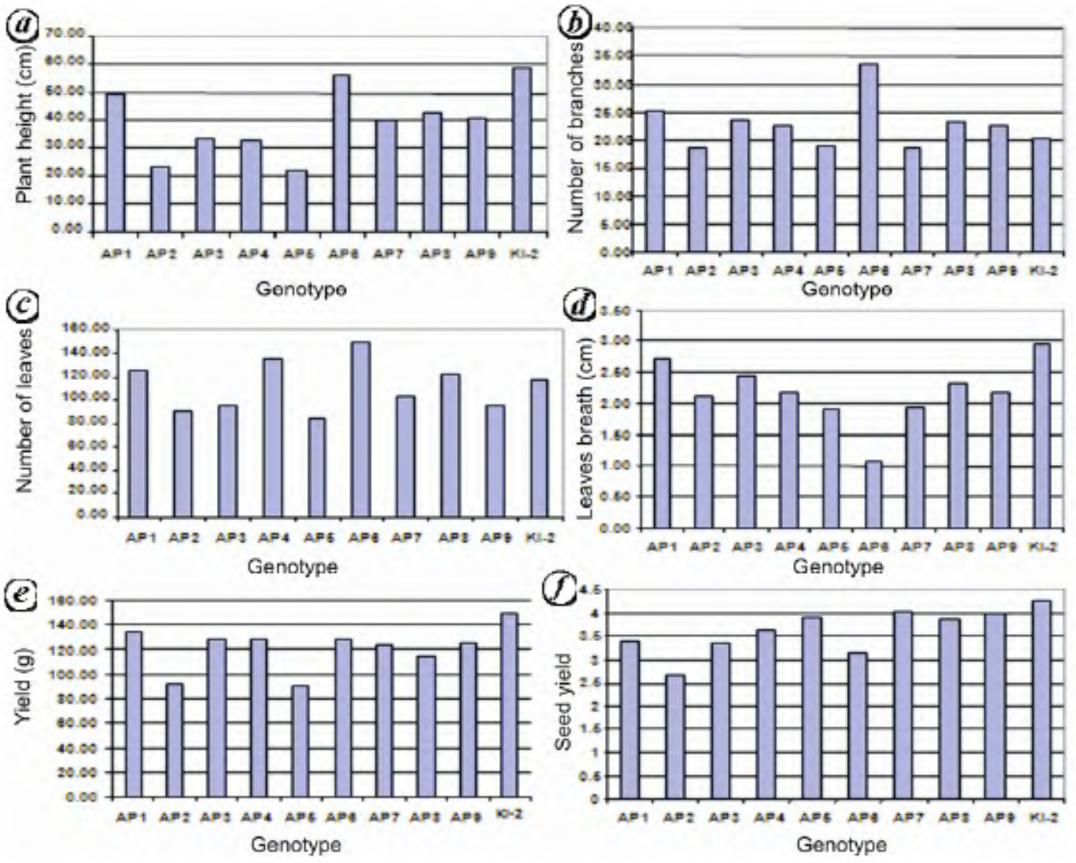

Figure 2. Morphological characterization (quantitative traits) of the *Kalmegh* genotypes. ***a***, Plant height; ***b***, Number of leaves; ***c***, Number of branches; ***d***, Leaf width; ***e***, Dry weight of whole plant; ***f***, Seed yield.

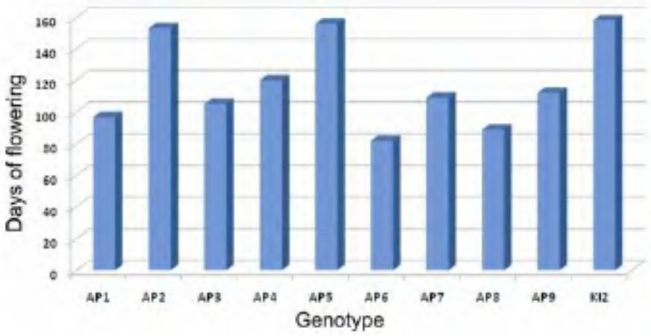

Figure 3. Variation in days of flowering (qualitative character).

morphological characters showed usefulness of selecting the genotypes for commercial cultivation. Further study is under way for at least 2 to 3 generations for validation of the performance.

The genetic diversity and relationships among all 10 genotypes which were used for morphological analysis were also evaluated using RAPD[15,16] markers. Out of 27 primers (Table 2), 10 primers (37.03% of total primers) were found to generate clear and polymorphic bands (Figure 4). A total of 37 bands were generated from 10 primers, of which 26 were polymorphic. The percentage of polymorphism obtained by 10 primers was 70.27 (Table 3). Primers OPAI-5 and OPAA-9 generated 100% polymorphic bands and the lower polymorphism (33.3%) was observed in the case of primers OPAG-1, OPAG-3 and OPAI-9. The number of RAPD bands was in the range of 2–7 per primer. The percentage of polymorphism as revealed by individual primer varied from 33.3 to 100. The polymorphic patterns observed in different *A. paniculata* genotypes varied with the primer. The analysis of banding pattern revealed sufficient information to estimate the genetic variability among different genotypes. The generated dendrogram shows that the DNA analysis of genotypes grouped them broadly into two main clusters, i.e. A and B comprising 3 and 7 genotypes respectively (Figure 5). Similarity index values derived from the polymorphic data depicted the extent of genetic relatedness among genotypes (Table 4) and accordingly it ranged from 51.3 (AP-1 and AP-9) to 97.3% (AP-4, AP-6 and AP-8).

A tie was noted among the AP-4, AP-6 and AP-8 which shared the maximum similarity (97.3%). Therefore, based on the similarity index, it was concluded that the genotypes AP-1 and AP-9 (48.64%) were genetically most diverse followed by AP-1 and AP-4 (45.95%). The dendrogram showed a close relationship among geno-

Table 3. Primers and number of bands generated through PCR amplification and polymorphism (%)

Primer code	Total no. of bands	No. of amplified band(s)	Polymorphism (%)
OP AG-3	3	1	33.3
OP A-11	3	2	66.6
OP A-8	8	7	87.5
OP AC-10	5	4	80.0
OP AI-5	2	2	100.0
OP AA-9	3	3	100.0
OP AG-19	4	3	75.0
OP A-10	3	2	66.6
OP AG-1	3	1	33.3
OP AI-9	3	1	33.3
Total number of bands	37	26	70.2
Mean per primer	3.7	2.6	

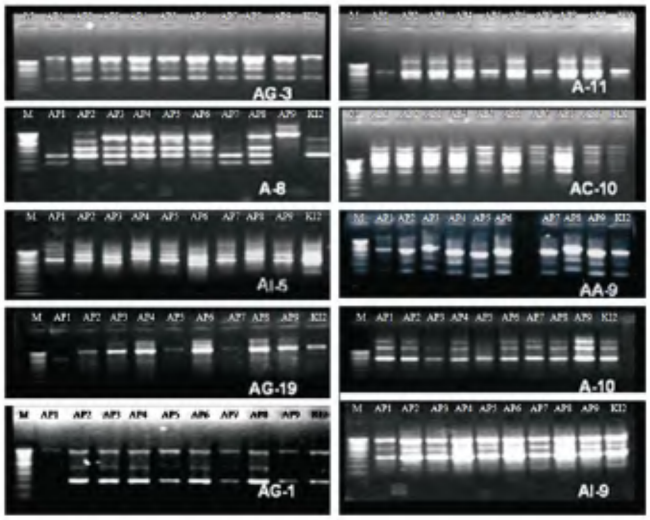

Figure 4. Polymorphic bands generated by different RAPD primers.

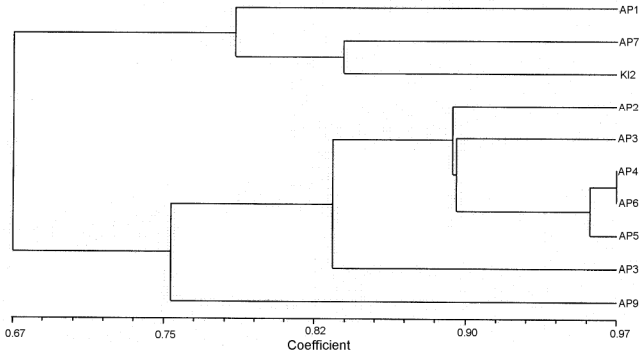

Figure 5. Dendrogram obtained from RAPD analysis using UPGMA.

types AP-4, AP-6 and AP-8 (97.3%) and followed by AP-4 and AP-6 (94.59%). Although the data presented are not conclusive to infer per cent phylogenetic relationships among the genotypes, they effectively reflect the utility of RAPD in the diversity analysis in this important medicinal herb. We are currently involved to improve this analysis by scoring additional loci using the available 10-mer primers and to find out the correlation between the observed chemical variations and RAPD profiles and also to increase the number of genotypes for wide and comprehensive information about the pattern of genetic variation in this species. Similar RAPD analysis was done to determine intra-specific variability in *A. paniculata*[17]. The RAPD-based marker also proved useful for diversity analysis in different plant species like *Azadirachta*[18], *Juniperus communis*[19], *Codonopsis pilsula*[20], *Allium schoenoprasum*[21], etc.

The HPLC estimation also showed the considerable phytochemical (andrographolide) variation in the studied *A. paniculata* genotypes. The phytochemical diversity measured as quantitative difference in the accumulated andrographolide ranged from 0.69 to 1.85% of dry weight with a mean value of 1.23%. The highest andrographolide content was detected in the genotype KI-2 (1.85%) followed by AP-1 (1.56%), AP-4 (1.5%), while the least amount was found in genotype AP-2 (0.69%) (Figure 6). Plant-specific marker compound (andrographolide) showed quantitative variations among the genotypes, which could not be correlated with allelic variation but the active principle content when measured under uniform growing conditions, the variations so observed presumably had a genetic basis. Earlier, Sabu *et al.*[22] carried out a similar study in *A. paniculata* and they correlated the andrographolide variation with genetic differences. The variation analysis was done by the following equation: VP = VG + VE, where VP = total phenotypic variance, VG = genetic variance and VE = environmental variance. Since all the plants were grown under the same growing conditions, influence of factors, if any, affecting the growth of the plants will be uniform to all accessions, hence it should ideally be VP = VG.

The present study revealed that the genotype KI-2 had not only the desirable phenotypic performance but was also the highest accumulator of andrographolide (1.85%). This genotype may potentially be multiplied and used on

Table 4. Similarity matrix of 10 genotypes generated by 10 primers

Genotypes	AP1	AP2	AP3	AP4	AP5	AP6	AP7	AP8	AP9	KI2
AP1	1.00									
AP2	0.62	1.00								
AP3	0.62	0.89	1.00							
AP4	0.54	0.92	0.86	1.00						
AP5	0.59	0.86	0.86	0.84	1.00					
AP6	0.56	0.89	0.90	0.97	0.81	1.00				
AP7	0.78	0.73	0.73	0.65	0.70	0.68	1.00			
AP8	0.59	0.86	0.92	0.95	0.78	0.97	0.70	1.00		
AP9	0.51	0.73	0.73	0.81	0.65	0.78	0.68	0.81	1.00	
KI2	0.78	0.84	0.73	0.76	0.81	0.73	0.84	0.70	0.68	1.00

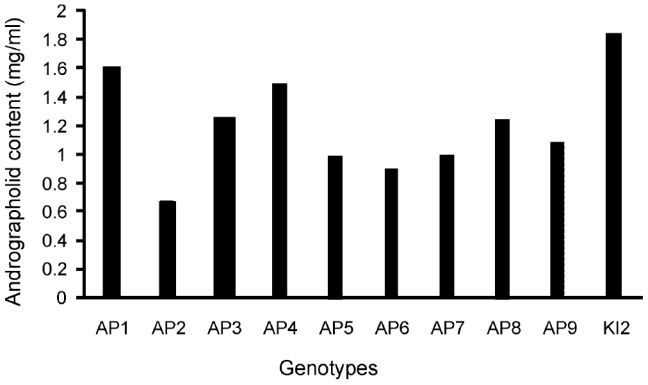

Figure 6. Andrographolid contents in different *Kalmegh* genotypes.

a large scale for commercial cultivation and also for future crop improvement. However, the stability in expression of the traits has to be revalidated under different geographical conditions.

The better understanding of genetic variation at the intraspecific level help in identifying superior genotype(s) for crop improvement as well as to evolve strategies for the effective *in situ* and *ex situ* conservation programmes, although such empirical determination of genetic diversity can be obtained by evaluating morphological, physiological and biochemical traits. The results of the diversity analysis have shown significant level of variation among the collected genotypes at phenotypic, genetic and phytochemical levels. The results obtained in the study are important in the exploration of *A. paniculata* genotypes for commercial production of andrographolide due to its high andrographolide content in KI-2 genotype.

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Pollination biology, breeding system and reproductive success of *Adhatoda vasica*, an important medicinal plant

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***Adhatoda vasica* Nees. (Acanthaceae) is an important and widely used medicinal plant. The flowers last for 3–5 days, they are protandrous and pass through three distinct phases: male, bisexual and female. Two species of carpenter bees, *Xylocopa verticalis* and *Xylocopa* sp. are the effective pollinators. Pollen grains are deposited on the dorsal surface of the thorax during *Xylocopa* visit to the flowers in the male phase, and the stigma rubs the pollen-coated thorax and is pollinated when the bees visit the flowers in bisexual and female phases. There is a high level of geitonogamy. Pollination efficiency under field conditions is high (95%). However, fruit set is poor (6%). The species is self-incompatible. None of the self-pollinated flowers sets fruits, but over 50% of the cross-pollinated flowers sets fruits. The results indicate that protandry does not prevent self-pollination, but reduces interference in export and import of pollen. Although the flowers have adapted well to achieve a high level of pollination, reproductive success in terms of fruit set is low, largely due to the limitation of compatible pollen.**

Keywords: *Adhatoda vasica*, breeding system, pollination biology, protandry, *Xylocopa* spp.

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ADHATODA VASICA Nees. (Acanthaceae) is a common perennial shrub that grows on wastelands and gardens in most parts of India, Nepal and parts of Pakistan. It is an important medicinal plant and its roots and leaves are used as anti-asthmatic, anti-spasmodic, bronchodilator and expectorant. Its bronchodialatory effects are due to the presence of alkaloids, vasicine and vasicinone. In the ayurvedic system of medicine, *A. vasica* is commonly known as vasa and has been in use for over 2000 years[1,2]. Many ayurvedic preparations containing leaf and root extracts of *A. vasica* are commercially available. In spite of its importance and wide distribution, no information is available on its reproductive biology, particularly pollination biology. This communication presents the results of studies on reproductive biology of *A. vasica*. The primary objectives were to study floral phenology, identify effective pollinators and to investigate pollination efficiency, breeding system and reproductive success.

Studies were carried out during two seasons (January–March 2007 and 2008) during the peak of flowering on a population growing on fallow land in Odekar Farm near Thovinakere, (13°32′.583N and 77°02′.945E), located about 30 km from Tumkur, Karnataka. Comparative studies were also carried out on another population growing about 4 km away from this population. To study floral phenology, flower buds that would open the next day were tagged ($N = 50 \times 2$) and were kept under observation (every hour on the first day and every morning, noon and evening on subsequent days until senescence) to record the time of anthesis, anther dehiscence and structural changes associated with ageing of flowers. The amount of nectar was measured with 5 µl calibrated microcapillaries (microcaps, Drummonds) and the concentration of sugar in the nectar was estimated with a portable refractometer.

Pollen viability was assessed on the basis of fluorescein diacetate test[3]. Pollen samples were collected in the morning from freshly opened flowers and maintained under laboratory conditions, and were tested for viability each day until they lost viability completely. Stigma receptivity was assessed on the basis of pollen germination following manual pollination. Flower buds that would open the next morning were tagged and bagged. The time and day of their opening were recorded and the samples were kept under observation for their sexual phase. Flowers ($N = 10$ for each phase) were pollinated during male, bisexual and female phases with fresh pollen (collected from another plant) and rebagged. They were excised 12 h after pollination, fixed in FAA and used to study pollen germination using aniline blue fluorescence[4]. The stigmas were observed under a fluorescence microscope and those that permitted pollen germination were considered receptive.

Initial observations revealed that several insects visit the flowers throughout the day from 0600 to 1800 h. The frequency of visits and foraging time were continuously recorded from 0600 to 1800 h for three days (36 h of total