Original Article

Evaluation of *Vrikshayurveda* treatments on physiological attributes and production of diterpenoids in *Andrographis paniculata* (Burm.f.) Nees.

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Abstract

Background: An ancient text on plant life *Vrikshayurveda* mentions the use of horticulture treatments, *Kunapa Jala* (KJ) and *Panchagavya* (PG) (cow milk, cow ghee, cow curd, cow dung, and cow urine) to enhance the efficiency of plants. **Aim:** An experiment was conducted to evaluate the effect of KJ and PG application on total leaf area, leaf area index (LAI) leaf area duration (LAD), crop growth rate (CGR), relative growth rate (RGR), and net assimilation rate (NAR); production of andrographolide (A1), neoandrographolide (A2), and 14-deoxy-11,12-didehydroandrographolide (A3) of the *Andrographis paniculata* (Burm.f.) Nees. **Materials and methods:** The experiment was conducted in randomized block design with six treatments, namely control, KJ, PG, farmyard manure, inorganic fertilizer, and humic acid. Simultaneous detection of contents was carried out using reversed-phase-ultra-flow liquid chromatography (RP-UFLC). The parameters were studied at 30, 60, 90, and 120 days after sowing (DAS). **Results:** KJ produced higher leaf area and LAI at 90 DAS, LAD between 90 and 120 DAS. PG produced higher CGR, RGR, and NAR between 60 and 90 DAS. RP-UFLC analysis revealed the maximum amount of ingredients at 120 DAS. At this stage, PG treatment recorded the maximum amount of A1, A2, and A3. **Conclusion:** *Kunapa Jala* and *Panchagavya* treatments were better or at par on physiological parameters and production of diterpenoids of *A. paniculata*.

Keywords: *Kunapa Jala*, *Panchagavya*, physiological attributes, reversed phase-ultra flow liquid chromatography, traditional horticulture, *Vrikshayurveda*

Introduction

Rapid population growth and industrialization have impacted agricultural practices and production. The utilization of scientific knowledge has helped agriculture tremendously and has helped in the green revolution in developing countries like India. Reduction in coverage area along with economic factors has adversely affected the ecology, which in turn has impacted health and environment.[1] Adoption of eco-friendly practices for sustainable agriculture is therefore the way ahead. Realization of the deterioration of soil health and damage caused to the beneficial soil microbial communities by decades of inorganic farming which has eventually led to a demand for organic practices in recent years. Organically farmed produce is now not only considered safer and healathier but also available at premium price in most of the markets the world over.[2] However, the use of such organic practices in agriculture had been in vogue in ancient Indian civilizations and these practices are even found mentioned in authentic texts as old as the *Vedas*. The *Vrikshayurveda* is one such text, which systematizes the use of biofertilizers, such as *Kunapa jala* (KJ) and *Pancha gavya* (PG) to enhance the biological efficiency of plants.

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The Sanskrit meaning of *Kunapa* is "stinking" as KJ is prepared by the decomposition of animal remains. PG is the blend of five products obtained from the cow, namely dung, urine, milk, curd, and ghee. The preparation of KJ and PG and their benefits have been well discussed, which have great relevance even today in the agriculture and horticulture sectors.[3,4,5] Although there are reports suggesting that fertilizers influence the growth attributes and production of secondary metabolites,[6,7] evaluation of the use of these age-old organic matters on growth and secondary metabolite production in medicinal plants are limited.

Andrographis paniculata (Burm.f.) Nees, belonging to the family Acanthaceae, is an erect, annual herb and 30–90 cm tall with the upper part of stem quadrangular while the lower part nearly rounded stem. Leaves are opposite sessile or subsessile, linear-lanceolateor lanceolate, 3–8 cm long, acute, glabrous, or minutely puberulous beneath and base cuneate, margin slightly undulate.[8] It is known as "*Kalmegha*," is one among the 32 prioritized medicinal plants of India by National Medicinal Plants Board.[9] Its estimated consumption in India is over 250 tons per year.[10] It is one of the most widely used medicinal plants in Ayurvedic formulations,[11] conventionally used as blood purifier, tonic, febrifuge, etc., In addition, it is also used in noncodified traditional practices to treat jaundice, malaria, tuberculosis, etc.[12] It is reported that most of the activities of *A. paniculata* are attributed to its diterpenoid contents namely andrographolide (A1), neoandrographolide (A2), and 14-deoxy-11,12-didehydroandrographolide (A3).[13-16] A1 has been shown to possess anti-inflammatory,[17] anticancer,[18] and cardioprotective properties.[19,20] A2 has been reported for anti-malarial and hepatoprotective activities.[21,22] The A3 has been reported as a hypotensive agent and for antiplatelet activity.[23,24]

Though plant *A. paniculata* is under cultivation in many parts of the country, it needs to go a long way to meet the ever-increasing market demand. It has necessitated to find feasible cultivation practices, which enhances the quality and quantity of the crop. In view of this, the present study was aimed to evaluate the effect of two age-old traditional preparations used for plant nutrition, **(**KJ and PG) through physiological attributes and quality through estimation of A1, A2, and A3 in *A. paniculata*.

Materials and methods

Land preparation, sowing and treatments

The experimental field was brought to fine tilth by ploughing and harrowing. The field was divided into required number of plots of 2.2 m × 2.2 m size (gross size). After the layout of the plots, the treatments were allotted to different plots in each replication ($n = 3$). The treatment groups comprised of control, KJ, PG, farmyard manure (FYM), inorganic fertilizer *viz*. nitrogen: phosphorus: potassium (NPK) and humic acid (HA). Mature seeds of *A. paniculata* were procured from Agriculture University, Anand (Gujarat), India. Other raw materials required for the preparation of KJ, PG, FYM, branded HA and NPK were procured locally. The seeds were sown superficially; light irrigation was provided immediately after sowing. The seedlings were thinned at 30 days after sowing (DAS) to retain a seedling per hill with the spacing of 20 cm × 15 cm. A 10% of KJ and PG were dissolved in 20 L of water and applied to the respective plots at the time of land preparation and drenched at 45 DAS. The full dose of FYM was mixed well with the soil at the rate of 10 tons/hectare at the time of land preparation. Nitrogen (75 kg/ha), phosphorus (75 kg/ha) and potash (50 kg/ha) were applied in the form of urea, single super phosphate (P_2O_5) and muriate of potash (K_2O), respectively. Nitrogen was applied in two split doses. At the time of sowing, 50% nitrogen and full dose of phosphorus and potassium were applied to respective plots and the remaining 50% of nitrogen was top-dressed at 45 DAS. One percent of HA was dissolved in 20 L of water and applied to respective plots at the time of land preparation and drenched at 45 DAS.

Preparation of *Kunapa Jala* and *Panchagavya*

According to *Vrikshayurveda* texts, there are variants of KJ based on the types of ingredients involved. KJ was prepared according to the availability of ingredients, 1.5 kg each of sheep/goat meat, chicken meat and 1 kg of Indian mackerel fish (*Rastrelliger kanagurta*) were boiled in 16 L of water till properly cooked and transferred to an earthen pot. Each 500 g powders of black gram (*Vigna mungo* L.) and sesame (*Sesamum indicum* L.) were added along with milk (1 L), honey (500 g), and ghee (250 g). The pot was closed with lid and kept in warm place for 30 days with stirring at regular intervals. The content of the pot was filtered on the 31st day and the resultant filtrate was coaded as KJ. For application, 10% of KJ was used. All ingredients required for PG were collected from native Indian breed cow known as "Kilari." 20 kg of dung, 10 L each of urine and of tap water were added in an earthen pot and kept for 15 days with stirring every day for an hour in the clockwise and anticlockwise direction. On the 16th day, 5 kg of ghee was added, thoroughly mixed by stirring every day and kept for 5 more days. On 21st day, 10 L each of milk and curd were added and stirred every day till 30th day. On 31st day, the content of the pot was filtered. For application, 10% of PG was used.[25]

Physiological attributes

Plants from different treatment groups were harvested at 30, 60, 90. and 120 DAS. Plants were shade-dried to constant weight, dry weight of plant, and dry weight leaf. The fresh leaves were detached and total leaf area (cm^2) was measured using leaf area meter (Biovis). The leaf area index (LAI), leaf area duration (LAD), crop growth rate (CGR), relative growth rate (RGR), and net assimilation rate (NAR) were studied according to standard procedures.[22,23,25,26]

Plant material and extraction

The plants harvested from different groups were shade-dried to constant weight at room temperature and coarsely pulverized. The powders of different treatment groups were extracted by cold maceration using methanol overnight and sonicated using ultra sonicator (Revotek). The extracts were filtered and stored

at 4°C until further use. The extracts were diluted to 0.5% for reversed-phase ultra-flow liquid chromatography (RP-UFLC) analysis.

Simultaneous detection and quantification of diterpenoids using reversed-phase-ultra flow liquid chromatography analysis

Instrumentation

The RP-UFLC analysis was performed on the Shimadzu chromatographic system (LC-20AD) consisting of a quaternary pump, manual injector, degasser (DGU-20A5), and dual λ UV absorbance diode array detector (SPD-M20A). The built-in LC-Solution software system was used for data processing. Chromatographic separation was achieved on Waters Nova-Pak C18 column (5 µm, 4.6 mm × 250 mm) for standards A1, A2, and A3.

Chromatographic conditions

The mobile phase comprised acetonitrile: water (38:62) was used for separation with an injection volume of 20 µL. A chromatographic condition of 1.1 mL/min flow rate was maintained and retention times (RTs) were observed for A1, A2, and A3 and detection was observed at 205 nm.

Calculations, calibration curves and linearity

Standards A1, A2, and A3 were accurately weighed separately and dissolved in methanol to prepare 1000 PPM solutions for each. The solution of three standards was mixed to prepare the stock solution for simultaneous detection and was serially diluted in methanol to obtain working concentrations for plotting calibration curves. Ten different concentration levels of mixed stock solution (0.05, 0.5, 1, 5, 10, 25, 50, 100, 250, and 500 µg/mL) were used during the study. All the solutions were stored at 4°C until further use.

Validation parameters

The accuracy was determined following the spike recovery method, in which the amount of a target compound was determined as a percentage of the theoretical amount present in the matrix. Spiked samples were prepared over the range of 50%–200% of the target concentration. Individually prepared replicates ($n = 3$) at each concentration were analyzed. It was expressed by calculating the percent recovery (% R) of the analyte recovered.[27]

The precision was checked by intra- and inter-day repeatability of responses after replicating injections and expressed as relative standard deviation percent (RSD%) among responses. The precision was determined by replicate analyses at the concentration of 5, 10, and 25 µg/mL of standard.[27]

The limit of detection (LOD) and limit of quantification (LOQ) were determined with the signal: Noise method. Signal: Noise ratios of 3.3 and 10 were used for estimating the LOD and LOQ, respectively.[27]

Chemicals and standards

The solvents, namely, acetonitrile and water were of HPLC grade (Fischer Scientific, India). andrographolide, neoandrographolide, and 14-deoxy-11,12-didehydroandrographolide (>95% pure) HPLC standards were procured from Natural Remedies, India.

Statistical analysis

Various physiological observations made during the study were subjected to statistical analysis using Fisher's method of analysis of variance (ANOVA).[28] RP-UFLC data was expressed as mean values and standard deviations for replicates ($n = 3$). Statistical analysis of data was performed by ANOVA and tested for significance by the Dunnett test using GraphPad Instat3 software,[29] which allowed comparison of test groups against control to individualize the significant differences. Differences were considered highly significant at $P < 0.01$.

Observations and Results

Physiological attributes

Table 1 shows the effect of treatments on physiological attributes namely leaf area and LAI at different growth stages. The maximum leaf area was observed at 90 DAS, at this stage, KJ (94.44 cm^2) treated group was highest which was on par with PG (92.45 cm^2) and NPK (89.36 cm^2) treated groups. The LAI is the ratio between photosynthetic surfaces (leaf laminar area) to the ground area occupied by the plant. The maximum LAI was observed at 90 DAS, at this stage, KJ (0.315) treated group was highest which was on par with PG (0.308) and NPK (0.298) treated groups.

The results of LAD, CGR, RGR, and NAR are presented in Table 2. The maximum LAD was observed during 90–120 DAS, at this stage, KJ (5.56) possessed higher LAD which was on par with PG (5.43) and NPK (5.18) treated groups. The highest CGR was observed during 60–90 DAS, at this stage, PG treated group (0.071 g/m^2/day) was highest which was on par with KJ (0.069 g/m^2/day). In the beginning stages, all treatment groups performed with higher RGR values and later on declined, at 60–90 DAS PG treated group (0.0110 g/g/day) was highest which was on par with KJ (0.0108 g/g/day). At 60–90 DAS PG treated group (0.211 g/m^2/day) produced higher NAR which was on par with KJ (0.199 g/m^2/day).

An RP-UFLC method for simultaneous detection and quantification of A1, A2 and A3 was validated. Figure 1 shows a typical chromatogram for simultaneous detection of three standards (100 µg/mL) showing the separation of A1, A2, and A3 at 3.82 ± 0.02, 5.47 ± 0.04 and 8.46 ± 0.04 min, respectively.

The linearity of the calibration curve for A1, A2, and A3 was calculated and constructed by plotting the peak area against concentration. [Figure 2] The RT, linear regression equation (y = mx + c) and correlation coefficient (r^2) are shown in Table 3.

Validation parameters

The accuracy was determined by recovery experiments. The recovery studies were carried out at three different concentration levels in triplicate (5, 10, and 25 µg/mL) using

Table 1: Effect of treatments on physiological attributes viz. leaf area and leaf area index

Treatment	Leaf area (cm^2)				LAI			
	30	60	90	120	30	60	90	120
Control	5.96	30.60	80.05	74.85	0.020	0.102	0.267	0.249
KJ	6.65	35.06	94.44	86.72	0.022	0.117	0.315	0.289
PG	6.44	34.07	92.45	83.73	0.021	0.114	0.308	0.279
FYM	5.88	33.76	83.01	79.57	0.020	0.113	0.277	0.265
NPK	6.63	34.83	89.36	80.47	0.022	0.116	0.298	0.268
HA	6.41	33.66	81.93	71.82	0.021	0.112	0.273	0.239
SEM±	0.33	1.92	2.71	1.72	0.001	0.006	0.009	0.006
CD at 5%	NS	NS	8.18	5.19	NS	NS	0.027	0.017
CV%	10.58	11.40	6.25	4.33	10.579	11.402	6.246	4.330

KJ: *Kunapa jala*, PG: *Pancha gavya*, NS: Nonsignificant, LAI: Leaf area index, FYM: Farmyard manure, NPK: Nitrogen phosphorus potassium, HA: Humic acid, CD: Critical difference, SEM: Standard error of the mean, CV: Coefficient of variations

Table 2: Effect of treatments on physiological attributes viz. leaf area duration, crop growth rate, relative growth rate and net assimilation rate

Treatment	LAD			CGR (g/m^2/day)			RGR (g/g/day)			NAR (g/m^2/day)		
	30-60	60-90	90-120	30-60	60-90	90-120	30-60	60-90	90-120	30-60	60-90	90-120
Control	0.52	1.94	4.67	0.0402	0.0470	0.0126	0.0200	0.0091	0.0017	0.470	0.160	0.028
KJ	0.60	2.22	5.56	0.0464	0.0687	0.0171	0.0202	0.0108	0.0018	0.473	0.199	0.033
PG	0.59	2.19	5.43	0.0467	0.0709	0.0161	0.0201	0.0110	0.0016	0.490	0.211	0.032
FYM	0.53	2.12	4.83	0.0444	0.0479	0.0134	0.0231	0.0090	0.0018	0.485	0.152	0.029
NPK	0.59	2.18	5.18	0.0423	0.0572	0.0106	0.0194	0.0100	0.0013	0.434	0.172	0.022
HA	0.58	2.13	4.82	0.0428	0.0562	0.0080	0.0207	0.0101	0.0010	0.457	0.182	0.018
SEM±	0.02	0.09	0.14	0.0008	0.0010	0.0015	0.0003	0.0002	0.0002	0.021	0.006	0.004
CD at 5%	0.05	NS	0.43	0.0025	0.0029	0.0045	0.0008	0.0007	0.0006	NS	0.018	NS
CV%	6.27	8.69	5.64	3.81	3.32	23.21	2.65	4.37	24.49	8.98	6.80	26.26

KJ: *Kunapa jala*, PG: *Pancha gavya*, FYM: Farmyard manure, NPK: Nitrogen phosphorus potassium, HA: Humic acid, CD: Critical difference, SEM: Standard error of the mean, CV: Coefficient of variations, NS: Nonsignificant, LAD: Leaf area duration, CGR: Crop growth rate, RGR: Relative growth rate, NAR: Net assimilation rate

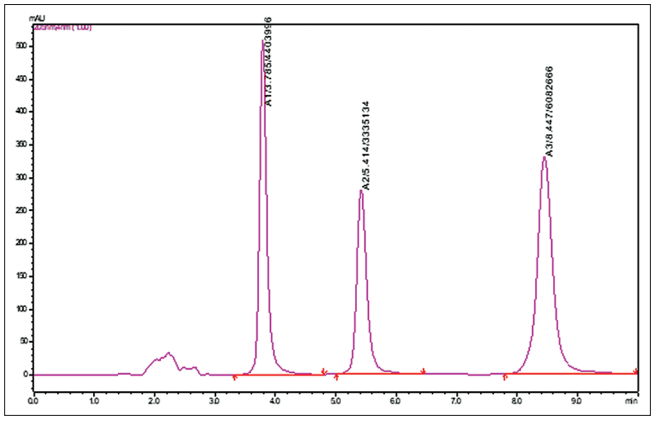

Figure 1: Peaks showing simultaneous detection of A1, A2 and A3

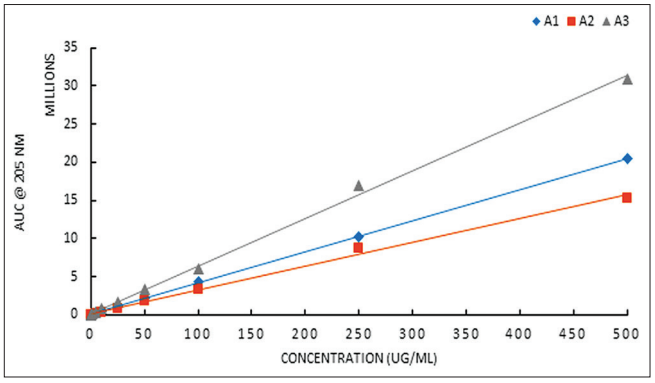

Figure 2: Calibration curve for A1, A2 and A3

spiked samples. The analyzed samples yielded recovery values from the developed method. The percent recovery results of the method are given in Table 4.

The precision was determined by intra- and inter-day repeatability of responses after replicating injections of concentrations 5, 10 and 25 µg/mL which is expressed as Relative Standard Deviation % (RSD) and presented in Table 5.

The LOD and LOQ were determined with the signal: Noise method. Signal: Noise ratios of 3.3 and 10 were used for estimating the LOD and LOQ, respectively. Results are presented in Table 6.

Studied constituents were quantified in whole plant of *A. paniculata* harvested at 30, 60, 90 and 120 DAS. The RP-UFLC analysis demonstrated varied amount of the constituents at different stages of plant growth

and also between treatments. Results are expressed in terms of percentage and presented in Figures 3-6. The maximum amount of constituents were observed at 120 DAS [Figure 6], at this stage maximum amount of A1, A2, and A3 were 5.70 ± 0.28 ($P < 0.01$), 1.36 ± 0.07 ($P < 0.01$) and 0.98 ± 0.05 ($P < 0.01$) %, respectively, were found in PG treated group followed by KJ treated group ([5.43 ± 0.27 {$P < 0.01$}, 1.25 ± 0.06 {$P < 0.01$} and 0.96 ± 0.05 {$P < 0.01$} %, respectively]).

Discussion

The LAD signifies the duration of the functional activity of the leaves. It denotes the ability of the plant with reference to the photosynthetic duration of the plant. Efficient plants will have higher LAD. The CGR is the absolute growth rate per unit area of the ground. It indicates the increase in the dry matter per unit land area per unit time. The increase in the CGR at 60–90 DAS was due to the maximum plant height, number of branches and leaves per plant resulting in maximum dry matter accumulation. These results are in accordance with the study conducted on *Plantago ovata* Forsk.[30] The RGR is a measure of the ability of the plant to produce newer plant materials and it is also called as efficiency index. Plants with higher efficiency index will perform better. The RGR denotes the rate of increase in dry matter per unit dry matter present, it decreases with the advancement of crop growth,

Table 3: Retention time and linearity of andrographolide, neoandrographolide and 12-didehydroandrographolide

Standards	Retention time (min)	Linearity		
		Slope (m)	Intercept (c)	Correlation coefficient (r^2)
A1	3.82±0.02	40,762	95,410	0.999
A2	5.47±0.04	31,166	137,255	0.996
A3	8.46±0.05	62,645	113,367	0.998

A1: Andrographolide, A2: Neoandrographolide, A3: 12-didehydroandrographolide

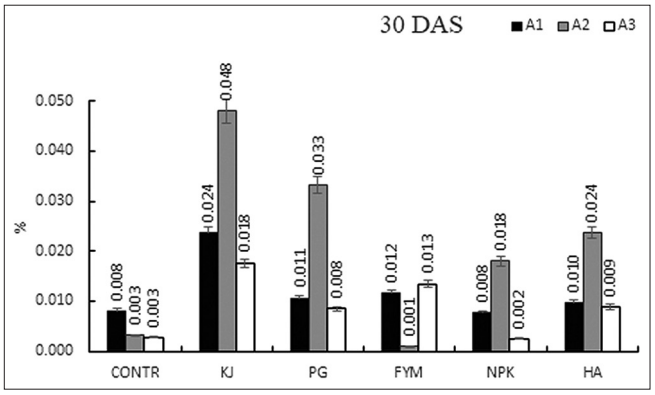

Figure 3: Percentage of A1, A2 and A3 at 30 days after sowing

Table 4: Accuracy of andrographolide, neoandrographolide and 12-didehydroandrographolide

Concentration (μg/mL)	Standard	Area ($n=3$)	Mean recovery (μg/mL)	Percentage recovery
5	A1	299232.0	5.00±0.09	100.01±1.73
	A2	293336.0	5.01±0.03	100.16±0.58
	A3	426463.7	5.00±0.09	99.96±1.62
10	A1	502432.3	9.99±0.05	99.85±0.51
	A2	448059.3	9.97±0.04	99.73±0.42
	A3	738673.7	9.98±0.08	99.82±0.83
25	A1	1097645.3	24.59±0.31	98.35±1.25
	A2	911733.3	24.85±0.13	99.40±0.52
	A3	1674131.3	24.91±0.09	99.66±0.35

A1: Andrographolide, A2: Neoandrographolide, A3: 12-didehydroandrographolide

Table 5: Precision of andrographolide, neoandrographolide, and 12-didehydroandrographolide

Concentration (μg/mL)	Standard	Intra day			Inter day		
		Area ($n=3$)	Mean recovery (μg/mL)	Percentage RSD	Area ($n=3$)	Mean recovery (μg/mL)	Percentage RSD
5	A1	299232.0	5.00±0.09	1.73	295820.0	4.92±0.08	1.65
	A2	293336.0	5.01±0.03	0.58	292743.7	4.99±0.02	0.40
	A3	426463.7	5.00±0.09	1.65	424652.7	4.97±0.04	0.86
10	A1	502432.3	9.99±0.05	0.51	502078.7	9.98±0.08	0.80
	A2	448059.3	9.97±0.04	0.42	446532.0	9.92±0.08	0.80
	A3	738673.7	9.98±0.08	0.83	744852.7	10.08±0.06	0.58
25	A1	1097645.3	24.59±0.31	1.27	1098711.3	24.61±0.43	1.75
	A2	911733.3	24.85±0.13	0.53	899862.3	24.47±0.27	1.12
	A3	1674131.3	24.91±0.09	0.35	1670033.0	24.85±0.14	0.57

A1: Andrographolide, A2: Neoandrographolide, A3: 12-didehydroandrographolide, RSD: Relative standard deviation

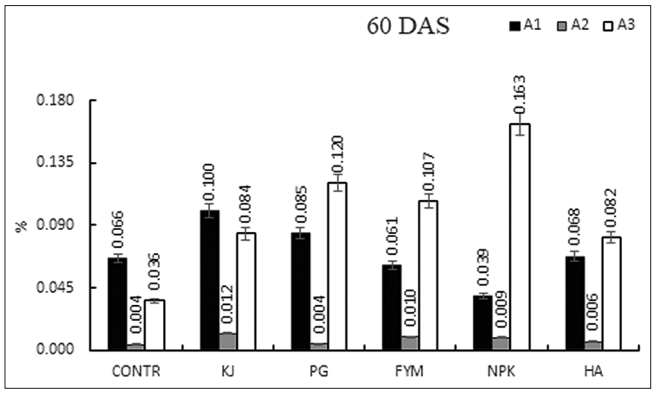

Figure 4: Percentage of A1, A2 and A3 at 60 days after sowing

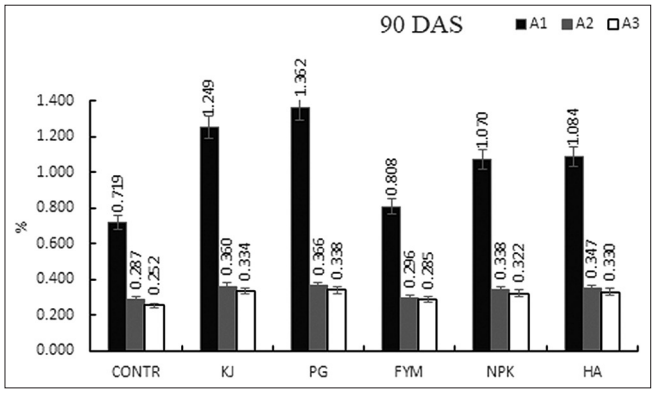

Figure 5: Percentage of A1, A2 and A3 at 90 days after sowing

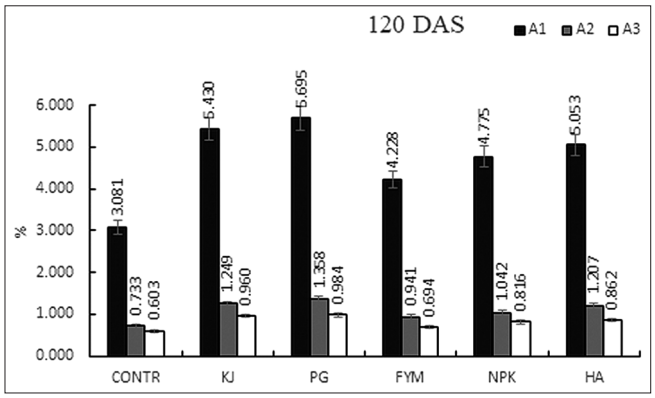

Figure 6: Percentage of A1, A2 and A3 at 120 days after sowing

similar observations were made in *Pimpenella anisum* and *Coleus* sp.[31,32] The NAR is the measure of photosynthetic product that is partitioned to form the plant materials. Thus the NAR gives an estimate of net photosynthesis in plants, maximum NAR was observed at the initial stage (30–60 DAS) and was declined in subsequent stages when dry matter accumulation reached to its maximum. Similar changes in NAR curve were reported in *Brassica campestris*.[33] As stated by Azarpour *et al*.[26] and also evident from the present study, the declination in values of CGR, RGR and NAR were due to acceleration in leaf production and early closure of canopy which enables absorption of less solar radiation by

Table 6: Limit of detection and limit of quantification (μg/mL)

Standard	LOD	LOQ
A1	0.017	0.051
A2	0.071	0.216
A3	0.051	0.155

LOD: Limit of detection, LOQ: Limit of quantification, A1: Andrographolide, A2: Neoandrographolide, A3: 12-didehydroandrographolide

the leaves, resulting in decreased values. It was observed that fertilizers containing the appropriate amount of nutrients can influence the quantity of secondary metabolites, which is in agreement with previous reports.[6,34,35] It was observed that the highest amount of A1, A2and A3 were reported in plants at 120 DAS which is considered as flowering and seed setting stage.[36] Earlier reports also suggested that the plants at this stage contain the highest levels of A1.[37,38] Pholphana *et al*. reported maximum amount of A1 (2.39%) at different growth stages in leaves and Sharma *et al*. reported maximum amount of A1 (2.4%) in marketed samples of leaves,[39,40] however our findings revealed maximum amount of A1 was 5.7% in PG treated group. On the basis of the findings of our study, it can be interpreted that fertilizers with the appropriate quantity of nutrients can enhance the amount of secondary metabolites, which is in accordance with the studies conducted by Hassan[34], Ormeno and Fernandez.[35] Inappropriate application of the chemical fertilizer hampered the quality of the products and also hazardous to health and the environment, however, the fertilizers or manures of organic nature have increased the quality and quantity of yield, they also maintain the fertility of the soil.[41] Similar observations have been made in our study, wherein PG being organic in nature has yielded higher production of secondary metabolites than the inorganic fertilizer (NPK) treated group. Similar remarks were also made by Ibrahim *et al*.[6] wherein the application of chicken dung (organic fertilizer) produced the maximum amount of secondary metabolites in *Labisia pumila*. There are reports suggesting that synthetic fertilizer can enhance plant growth with increased cultivation cost and affecting adversely the ecosystem.[42]

Conclusion

The present study forms the first comprehensive report on effect of *Kunapa jala* and *Pancha gavya* treatment, indicating the effect was better or at par as equated to other treatments on physiological parameters and production of selected diterpenoids from *A. paniculata*. Based on the reports, it is evident that traditional horticulture treatments can be implemented in the cultivation of medicinal plants.

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Conflicts of interest

There are no conflicts of interest.

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