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CHROMATOGRAPHIC EVALUATION OF OPTIMIZATION AND SEASONAL VARIATION OF BACOSIDE A CONTENT FROM WILD AND CULTIVATED BRAHMI (*BACOPA MONNIERI* LINN.) PLANT

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ABSTRACT: *Brahmi* (*Bacopa monnieri* Linn.) is a miraculous Ayurvedic drug used commercially in memory-enhancing formulations. Its nootropic action is due to Bacoside A, which modern parameters have proved. Bacoside A content fluctuated during the course of the whole year. In the present study, High-Performance Liquid Chromatography was conducted to evaluate total Bacoside A content (expressed as the sum of Bacoside A3, Bacopaside II, Jujubogenin and Bacopasaponin C) on a monthly basis in methanolic extract of *Bacopa monnieri* collected from the wild and cultivated area during Summer, Rainy and Winter season of a year. Instead of the entire herb, 10 cm apex part and the remaining part of *Brahmi* were used. Chromatographic analysis was performed with Shimadzu Prominence HPLC instrument; equipped with a quaternary pump, LC2010CHT, degasser DGU-20A3R, Column oven CTO-10As, Autosampler SIL-20 HT, Diode-Array-Detector SPD-M20A with Prime SIL C18 column. Among individual plant parts, maximum Bacoside A content was observed in the remaining part of a plant in July for the wild sample (38.86%) and in the remaining part of a plant in September for the cultivated sample (25.1%). The present study estimated gross as well as individual components of Bacoside A investigated the influence of seasonal temperature and humidity on different plant parts in wild and cultivated accessions. This study based on guidelines of Good Agricultural and Collection Practices concluded that drug manufacturers should collect wild as well as cultivated *Brahmi* plants in appropriate months with a focus on specific part-oriented harvesting rather than gross harvesting, and it will ensure the provision of standard raw material with maximum efficacy.

INTRODUCTION: There is a huge surge in medicinal plants, and it has become a subject of intensive research for various aspects [1].

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Overburdening on natural resources and negligent attitude for its conservation in past few years has resulted into over-exploitation and damage of this valuable wealth [2].

Standardization and quality control of herbal medicines is another burning issue of the current decade. Quality of raw drug directly impacts safety and efficacy of the product and ultimately the health of consumers [3]. Hence, raw material used in manufacturing of medicines must be authentic, rich with its biologically active components, and quality certified [4]. Impact of season, rainfall, and temperature is higher in plant material [5]. The entire plant is not always potent medicinally. Certain parts carry more active constituents than others, so these parts of plant should be isolated and used for treatment. It enhances the quality of raw material as well as finished products [6, 7]. *Brahmi* (*Bacopa monnieri* Linn.) is an ancient and renowned medicinal plant with a legendary reputation as a memory vitalizer. It belongs to Scrophulariaceae family, commonly growing in damp and marshy places throughout India [8]. Ayurveda treatises have explored the benefits of *Brahmi* some 3000 years before [9]. It is globally used in therapeutics for enhancing memory and intellect.

Brahmi has been enlisted among 178 species of medicinal plants of India with high trade requirements (≥ 100 million tons per year). More than 90 percent of raw material used by industry is collected from wild sources which include 70 percent unorganized harvesting that is invariably destructive [10]. According to NMPB (National Medicinal Plant Board) guidelines, *Brahmi* plant can be ideally harvested by ratooning so that the upper portion of the stem, 4-5 cms from the base, is removed and the rest left for subsequent regeneration [11].

Hence, manufacturers should collect wild as well as cultivated plants of *Brahmi* in the suitable season with focus on specific part-oriented harvesting rather than gross harvesting. The present study was designed to show comparison and quantification of memory enhancer molecule- Bacoside A in wild and cultivated *Brahmi* with respect to apex and remaining plant part in Summer, Rainy, and Winter season.

MATERIALS AND METHODS:

Collection and Authentication of Plant Material: Wild sample of *Brahmi* was collected from wild locality of Salod (dist. Wardha), Maharashtra in 12 months of summer, Rainy, and winter season of the year 2016-17 **Fig. 1.** Cultivated sample of *Brahmi* was collected in 12 months of summer, Rainy, and winter season from the herbal garden of Mahatma Gandhi Ayurved College, Hospital and Research Centre, Salod (dist. Wardha), Maharashtra. Identification and Authentication of *Brahmi* were done in Foundation for Revitalisation of Local Health Traditions (FRLHT), Bengaluru, Karnataka Herbarium specimen no. FRLH 119707. Collection period was confined to mid of the month (from 10 to 15 of every month), and collection timing was in the early morning hours. Sample grouping and labeling was done as following (plate 2, 3). Sample 1. Wild Remaining (WR) - Leftover *Brahmi* after cutting 10 cm part of the whole creeper was labelled as remaining part. Sample 2. Wild Apex (WA) -10 cm apex part of *Brahmi* creeper was measured using scale and cut into pieces. Sample 3. Cultivated Remaining (CR)-Leftover *Brahmi* after cutting 10 cm part of whole creeper was labelled as remaining part. Sample 4. Cultivated Apex (CA)-10 cm apex part of *Brahmi* creeper was measured using scale and cut into pieces.

Chemicals: Two units of Standard marker compound of Bacoside A (10 mg) were purchased from Natural Remedies Pvt. Ltd., Bangalore, before the commencement of the study and were stored at room temperature in a cool, dry place. Bacoside A is a clinically active component, a mixture of Bacoside A3, Bacopaside II, Jujubogenin isomer of Bacopasaponin C and Bacopasaponin C. Methanol, Acetonitrile and Orthophosphoric acid (HPLC grade) were supplied by National Environmental Engineering Research Institute (NEERI), Nagpur.

Extraction of Brahmi Samples: All four samples were washed under running water for removing physical impurity, and roots of plant were removed. All samples were dried under shade for 12 h. Since *Brahmi* leaves are fleshy and require too much time for shade drying procedure, after shade dry samples were kept in Hot air oven at 50 ºC for 1 h. Dried samples were coarsely grounded to make powder.

Dried powders were stored in four separate labelled airtight containers. Pre-dried coarse *Brahmi* powder of each sample (WR, WA, CR, CA) weighed separately, and 5 gm amount was taken for extraction. Then 20 ml of methanol was added in each beaker using a volumetric pipette, and aluminium foil was wrapped immediately around each beaker to prevent evaporation. All 4 beakers containing *Brahmi* powder and methanol were kept inside Ultra sonicator (with working heating and vibration mode) with proper spacing in between samples **Fig. 4.** The extraction process continued for 1 hour with sonicator temperature 40 °C. Tripod stands mounted with glass filters containing filter papers were arranged and labelled properly. Filter papers (Pall science, quantitative, P-42, 150 mm diameter) were pre- washed with methanol. Filtration of individual sample was done. Filtrate so obtained was again filtered using 0.2 µm Millipore and BD syringe to prevent entry of any suspended particles into HPLC system. Using BD syringe, filtrate quantity of each sample was measured, labelled and recorded. This extraction method was chosen as it is the most efficient extraction method with maximum extract quantity and minimum time span.

Preparation of Mobile Phase A and B: Some quantity of liquid KH2PO4 (potassium dihydrogen orthophosphate) was taken in petry dish with lid. Then it was kept in hot air oven for 1 hour on 250° C. Then allowed it to cool for 10 min. Then using an electronic weighing balance, 140 mg (0.140 gm) of anhydrous KH2PO4 was taken. 0.140 g of anhydrous potassium dihydrogen orthophosphate (KH2PO4) dissolved in 900 ml of HPLC grade water and 0.5 ml of Orthophosphoric acid added in it. It was made up to 1000 ml with distilled water. The solution was filtered through a 0.45 µ membrane filter and degassed in a sonicator for 3 min. This 500 ml phosphate buffer solution with salt pH 4.3 – 4.5 was used as mobile phase A. 500 ml Acetonitrile (HPLC grade) was taken as Mobile phase B.

HPLC of Standard Marker Compound Bacoside A and Brahmi Samples: Preparation of stock solution was done using standard marker compound of Bacoside A and Methanol (HPLC grade). 2 mg of standard Bacoside A compound was added in 10 ml Methanol, and it was shaken well, and stock solution of 200 ppm was prepared.

This was further serial diluted to create 4 – point calibration curve across a concentration range of 100, 125, 150, 175 ppm. All five concentration solutions (100, 125, 150, 175, 200 ppm) were subjected to the HPLC system. Chromatographic analysis was performed with the Shimadzu Prominence HPLC instrument. It was equipped with a quaternary pump, LC2010CHT, degasser DGU-20A3R, Column oven CTO-10As, Autosampler SIL-20 HT, Diode-Array-Detector SPD-M20A. Standard solutions were analyzed with PrimeSIL C18 column (250 x 4.6 mm. ID).20 µl of freshly prepared stock solution of 200 ppm of marker compound Bacoside A was injected into the C18 column and eluted at the flow rate of 1 ml/min. 205 nm UV wavelength and column oven temperature 27°C with a run time of 55 minutes was considered for demonstrating better peak shape and peak area. Data acquisition was performed by class LC solution software.

Peak I with retention time 23.436 was identified as Bacoside A3, peak II with retention time 24.163 was Bacopaside II, peak III with retention time 26.011 was Jujubogenin, and peak IV with retention time 26.899 was Bacopasaponin C. The standard calibration curve was established by plotting the area of Peak against different concentrations of 100, 125, 150, 175 ppm. Quantification of Bacoside A in the monthly collected samples was determined using the regression equation of the calibration curve.

HPLC profiling of *Brahmi* extracts of 4 samples in each month of summer, rainy, and winter season was done using Shimadzu High-Performance Liquid Chromatography system LC 2010 CHT equipped with PDA detector in combination with Class LC solution software. 20 µl of freshly prepared *Brahmi* extract of each sample was injected to the C18 column and eluted at the flow rate of 1 ml/min. 205 nm UV wavelength and column oven temperature 27 °C with run time of 55 minutes was considered for demonstrating better peak shape and peak area 500 ml of phosphate buffer (with 0.5 ml H3PO4) was taken as mobile phase A and 500 ml of Acetonitrile was taken as mobile phase B. By comparing Brahmi extract chromatograms with standard graphs, interpretation was done. Quantification of unknown concentration of Bacoside A in all 4 samples was done. This procedure was repeated for twelve months in Summer, Rainy, and Winter seasons of a year.

Statistical Analysis: All data were analyzed by applying Analysis of Variance (ANOVA) and paired t-test using SPSS software.

RESULTS AND DISCUSSION: Observations obtained with the analytical study of wild and cultivated are summarized as follows.

FIG. 1: BRAHMI (BACOPA MONNIERI LINN.) PLANT

FIG. 2: SAMPLING OF BRAHMI PLANT

FIG. 3: SAMPLING AND CODING OF BRAHMI PLANT

FIG. 4: EXTRACTION USING ULTRA SONICATOR

Effect of Temperature on Bacoside A content: The temperatures ranged from 28 ºC-43 ºC during all three seasons **Table 1**. The percentage of humidity ranged from 41-88% during all three seasons. The synthesis and accumulation of the active ingredients of medicinal plants(secondary metabolism) is an extremely complex process affected by a series of ecological factors, comprising a multivariable system. A significant decrease in temperature (as in winter) may possibly lead to inactivation of metabolic enzymes of the plant, thereby hampering the cascade of production of secondary metabolites in plants. In this study, significant differences were observed in the active ingredient of *Brahmi*- Bacoside A in the apex and remaining parts of *Bacopa monnieri* Linn. planted in all three seasons of a year.

Effect of Season on Vegetative Growth of Brahmi: The metabolism and accumulation of active ingredients is strongly affected, either directly or indirectly, by environmental factors. Most plants regulate the types and amount of active ingredients according to environmental variations. *Brahmi* showed enhanced vegetative growth in terms of quality and quantity during the rainy season among all three seasons.

TABLE 1: TEMPERATURE & HUMIDITY RECORDED PER MONTH AT THE TIME OF SAMPLE COLLECTION

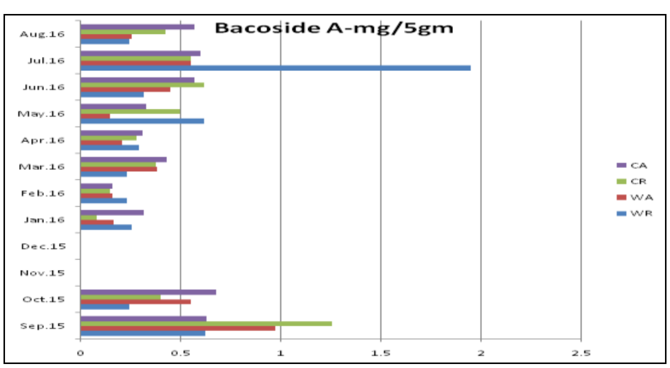
S. no.	Month	Season	Temperature (ºC)	Humidity (%)
1	July	Rainy	28	66
2	August	Rainy	31	70
3	September	Rainy	35	43
4	October	Rainy	37	41
5	November	Winter	30	88
6	December	Winter	28	87
7	January	Winter	29	69
8	February	Winter	33	70
9	March	Summer	39	54
10	April	Summer	42	57
11	May	Summer	43	50
12	June	Rainy	40	72

Variation of Bacoside A in Wild and Cultivated Brahmi: In this study, significant differences were observed in the active ingredient of *Brahmi*-Bacoside A in the apex and remaining parts of *Bacopa monnieri* Linn. planted at different production locations (wild and cultivated).In the months of August, September, October, January, March, April, May, and June; maximum Bacoside A content was seen in Cultivated *Brahmi*. In the month of February and July, maximum Bacoside A content was seen in Wild *Brahmi* **Table 2, 3** and **Graph 1, 2.** Soil factors were significantly different owing to different production locations, and climate factors changed with geographical conditions of study sites, which caused the differences in terms of the growth environment of *Bacopa monnieri* Linn.

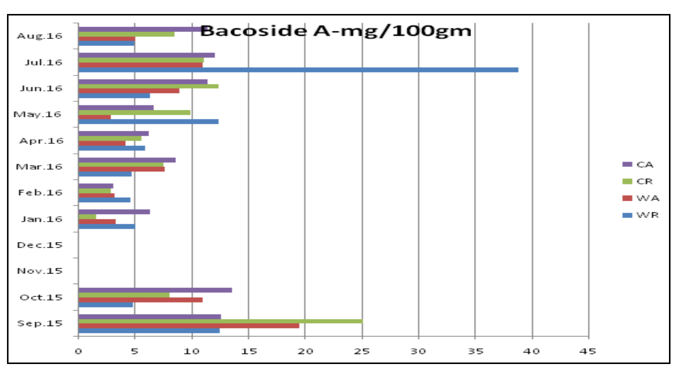
Variation of Bacoside an in Apex and Remaining Part of Brahmi: Ratooning method for collection of Brahmi plant material described by National Medicinal Plant Board was applied in this study. In months of August, October, January, and March, Bacoside A content was more than remaining part in both wild and cultivated variety.

TABLE 2: SEASON WISE QUANTIFICATION OF BACOSIDE A IN DRY WEIGHT/ 5gm

Month	Wild (Total Bacoside-A in mg/ 5 gm)		Cultivated (Total Bacoside-A in mg/ 5 gm)	
	Remaining Part	Apex	Remaining part	Apex
September	0.623	0.972	1.255	0.628
October	0.240	0.548	0.4	0.675
November	00	00	00	00
December	00	00	00	00
January	0.25	0.162	0.076	0.312
February	0.227	0.158	0.143	0.154
March	0.230	0.378	0.373	0.429
April	0.29	0.207	0.275	0.306
May	0.614	0.143	0.491	0.328
June	0.316	0.444	0.617	0.569
July	1.943	0.546	0.55	0.598
August	0.241	0.251	0.422	0.568



GRAPH 1: SHOWING THE BACOSIDE A IN mg / 5 gm IN ALL THE SAMPLES AND ALL SEASONS



GRAPH 2: SHOWING BACOSIDE A in mg / 100 gm IN ALL THE SEASONS AND ALL THE SAMPLES

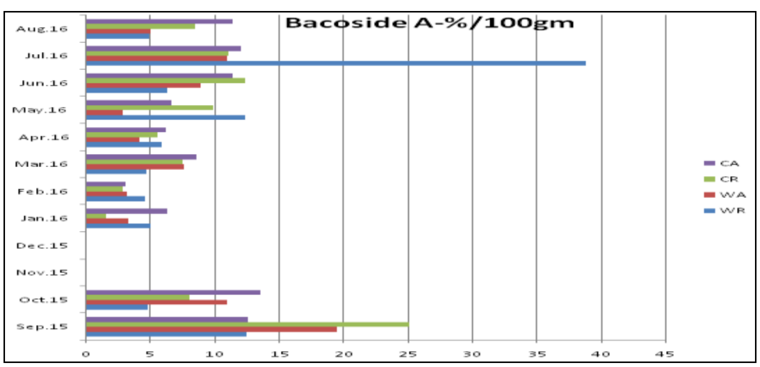
TABLE 3: SEASON WISE QUANTIFICATION OF BACOSIDE A IN DRY WEIGHT / 100 gm

Month	Wild (Total Bacoside-A in mg/ 100 gm)		Cultivated (Total Bacoside-A in mg/ 100 gm)	
	Remaining part	Apex	Remaining part	Apex
September	12.46	19.44	25.1	12.56
October	4.8	10.96	08	13.5
November	00	00	00	00
December	00	00	00	00
January	05	3.24	1.52	6.24
February	4.54	3.16	2.86	3.08
March	4.6	7.56	7.46	8.58
April	5.8	4.14	5.5	6.12
May	12.28	2.86	9.82	6.56
June	6.32	8.88	12.34	11.38
July	38.86	10.92	11	11.96
August	4.82	5.02	8.44	11.36

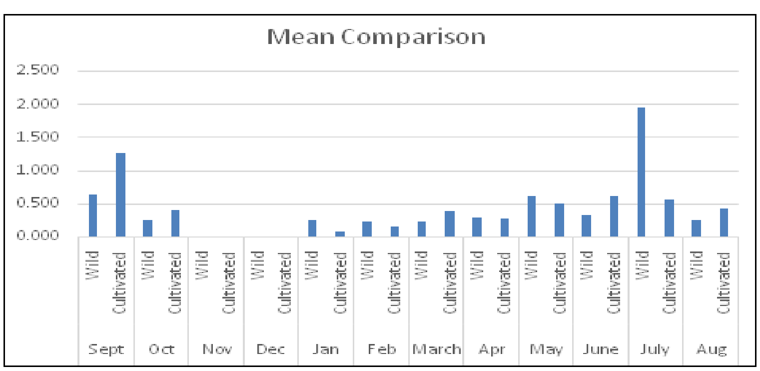
The percentage for quantitative distribution of bacoside A content in different parts of *B. monnieri* collected during twelve months of a year are presented in **Table 4, Graph 3.**

TABLE 4: SEASON WISE PERCENTAGE OF AVERAGE TOTAL BACOSIDE A CONTENT % PER 100 gm)

Month	Season	Wild		Cultivated	
		Remaining Part	Apex	Remaining Part	Apex
September	Rainy	12.46	19.44	25.1	12.56
October	Rainy	4.8	10.96	08	13.5
November	Winter	00	00	00	00
December	Winter	00	00	00	00
January	Winter	05	3.24	1.52	6.24
February	Winter	4.54	3.16	2.86	3.08
March	Summer	4.6	7.56	7.46	8.58
April	Summer	5.8	4.14	5.5	6.12
May	Summer	12.28	2.86	9.82	6.56
June	Rainy	6.32	8.88	12.34	11.38
July	Rainy	38.86	10.92	11	11.96
August	Rainy	4.82	5.02	8.44	11.36



GRAPH 3: SHOWING THE BACOSIDE A-%/100 GM IN ALL THE SAMPLES AND ALL SEASONS



GRAPH 4: COMPARISON OF MEAN BACOSIDE A IN REMAINING PART OF WILD AND CULTIVATED

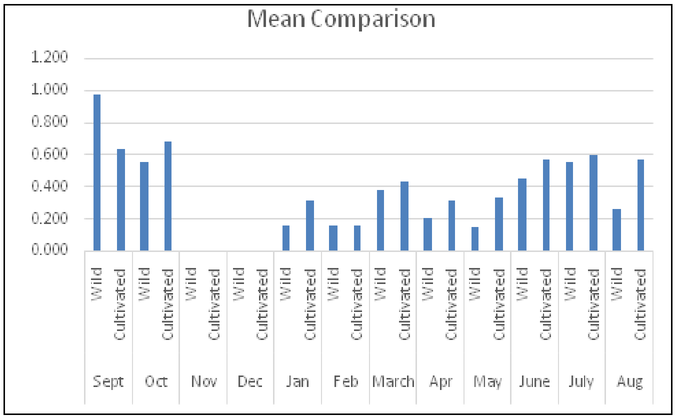
Statistical Analysis: of 48 samples using ANOVA test was done. There was a significant difference in mean Bacoside A content in wild and cultivated samples **Table 5, 6** and graph **4, 5**.

TABLE 5: COMPARISON OF MEAN BACOSIDE A IN REMAINING PART OF WILD AND CULTIVATED SAMPLE (MONTH WISE)

	Sample	N	Mean	SD	SE	t	P-Value	Result
Sept	Wild	4	0.625	0.38	0.19	-1.133	0.301	NS
	Cultivated	4	1.258	1.05	0.53			
Oct	Wild	4	0.240	0.28	0.14	-0.575	0.586	NS
	Cultivated	4	0.400	0.48	0.24			
Nov	Wild	4	0.000	.00000[a]	0.00	NA	NA	NA
	Cultivated	4	0.000	.00000[a]	0.00			
Dec	Wild	4	0.000	.00000[a]	0.00	NA	NA	NA
	Cultivated	4	0.000	.00000[a]	0.00			
Jan	Wild	4	0.255	0.28	0.14	1.210	0.272	NS
	Cultivated	4	0.078	0.07	0.04			
Feb	Wild	4	0.230	0.37	0.19	0.387	0.712	NS
	Cultivated	4	0.145	0.23	0.12			
March	Wild	4	0.230	0.30	0.15	-0.606	0.567	NS
	Cultivated	4	0.373	0.37	0.18			
Apr	Wild	4	0.290	0.37	0.18	0.067	0.948	NS
	Cultivated	4	0.275	0.25	0.12			
May	Wild	4	0.618	0.36	0.18	0.605	0.567	NS
	Cultivated	4	0.493	0.20	0.10			
June	Wild	4	0.315	0.13	0.07	-1.232	0.264	NS
	Cultivated	4	0.618	0.47	0.24			
July	Wild	4	1.945	1.55	0.77	1.763	0.128	NS
	Cultivated	4	0.550	0.33	0.16			
Aug	Wild	4	0.240	0.18	0.09	-1.228	0.265	NS
	Cultivated	4	0.423	0.24	0.12			

TABLE 6: COMPARISON OF MEAN BACOSIDE A IN APEX PART IN WILD AND CULTIVATED SAMPLE (MONTH WISE)

	Sample	N	Mean	Std. Deviation	Std. Error Mean	t	P-Value	Result
Sept	**Wild**	**4**	**0.975**	**0.55**	**0.27**	**0.774**	**0.468**	**NS**
	Cultivated	4	0.630	0.70	0.35			
Oct	Wild	4	0.548	0.54	0.27	-	0.737	NS
	Cultivated	4	0.678	0.51	0.25	0.352		
Nov	Wild	4	0.000	.00000[a]	0.00	NA	NA	NA
	Cultivated	4	0.000	.00000[a]	0.00			
Dec	Wild	4	0.000	.00000[a]	0.00	NA	NA	NA
	Cultivated	4	0.000	.00000[a]	0.00			
Jan	Wild	4	0.160	0.17	0.09	-	0.274	NS
	Cultivated	4	0.315	0.19	0.09	1.205		
Feb	Wild	4	0.160	0.25	0.12	0.029	0.977	NS
	Cultivated	4	0.155	0.23	0.12			
March	Wild	4	0.378	0.30	0.15	-	0.864	NS
	Cultivated	4	0.430	0.51	0.25	0.178		
Apr	Wild	4	0.205	0.25	0.12	-	0.613	NS
	Cultivated	4	0.308	0.29	0.15	0.533		
May	Wild	4	0.145	0.16	0.08	-	0.093	NS
	Cultivated	4	0.330	0.09	0.04	1.994		
June	Wild	4	0.445	0.17	0.09	-	0.600	NS
	Cultivated	4	0.568	0.41	0.20	0.553		
July	Wild	4	0.548	0.37	0.19	-	0.857	NS
	Cultivated	4	0.598	0.38	0.19	0.188		
Aug	Wild	4	0.253	0.18	0.09	-	0.157	NS
	Cultivated	4	0.568	0.35	0.17	1.616		



GRAPH 5: COMPARISON OF MEAN BACOSIDE A IN APEX PART IN WILD AND CULTIVATED SAMPLE (MONTH WISE)

Bacoside, A content in the remaining part of wild sample, collected during July month (rainy season) was38.86%, and in September for the cultivated sample was 25.1%. Rainy season preferred plant growth as well as bacoside accumulation. These observations were similar to those of previous studies, wherein they have reported higher bacoside A content in different accessions of *B. monnieri* during monsoon period (June to September). It might be due to moderate humidity, high temperature, and availability of abundant water during the rainy season, which are satisfactory conditions for the growth of *B. monnieri* plants.

CONCLUSION: Present research work intended to aid inputs to guidelines regarding the influence of seasons on Bacoside A content for the maximum therapeutic potential of the drug. Results of the present study showed that the highest bacoside content was obtained from the remaining part of the wild sample in July month as well as the cultivated sample in September month of the Rainy season. In conclusion, our study might be beneficial for ascertaining the suitable time for commercial collection and harvesting of this significant medicinal plant *Bacopa monnieri*, for getting maximum Bacoside A content to prepare commercial formulations.

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