viz., 2-Diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)] and to compare with *A. vasica*, (ii) to obtain and analyze a reversed-phase high-performance liquid chromatography (RP-HPLC) fingerprint using anti-bronchial compounds vasicine and vasicinone along with anti-cancer drug betulin in all the samples of *Barleria* in comparison with *A. vasica*, (iii) and finally to provide a complete understanding of the correlations and natural groupings for the analyzed species using statistical tools. Thus, all together to generate information on potential species/variety of *Barleria* about the widely used medicinally important *A. vasica*.

MATERIALS AND METHODS

Plant material

Above-ground parts of *A. vasica* and 16 *Barleria* species were obtained [Table 2] from taxonomically identified field-grown, maintained plants at Botanical Garden, Department of Botany, Shivaji University, Kolhapur. The plant materials were collected from five different individuals for each species and pooled together to minimize the statistical error if any.

Preparation of plant extract

Cleaned plant samples were dried out [at room temperature (RT)] and powdered. This powder was sieved using 20 mm mesh to obtain a uniform powder, which was used for further extraction. Extraction was achieved by adding 1 g of powdered material of all the species to 10 ml of 95% methanol kept on an orbital shaker [Revolutions per minute (RPM): 125; Time: 6 h; Temperature: RT]. The extracts obtained were filtered (Whatman No. 1) and the filtrates were diluted to obtain 0.5% extracts which were used for further analysis.

Chemicals

Solvents and other chemicals were of analytical grade. Aluminum trichloride, ascorbic acid, ferric chloride, Folin–Ciocalteu reagent, sodium

Table 1: Medicinal uses of *Barleria* species under study

Code	Name of species	Uses	References
BAR-2	*B. acuminata*	*No reports*	--
BAR-3	*B. buxifolia*	Cough, anti-inflammatory, anti-feedant activity	Sindhuja *et al.* 2012; Jeyasankar *et al.* 2014
BAR-4	*B. cristata Blue**	Anti-diabetic activity, anemia, toothache, cough, anti-inflammation	Singh *et al.* 2012
BAR-5	*B. cristata Pink**		Sindhuja *et al.* 2012
BAR-6	*B. cristata white**		Gambhire *et al.* 2009
BAR-7	*B. cuspidata*	Stomach disorders	Sindhuja *et al.* 2012
BAR-8	*B. gibsoni*	*No reports*	--
BAR-9	*B. grandiflora*	Mouth ulcer, anti-cancer, antioxidant, anti-fungal activity	Newan *et al.* 2003; Manglani *et al.* 2014; Sawarkar *et al.* 2009; Kumari *et al.* 2015
BAR-10	*B. lawii*	*No reports*	--
BAR-11	*B. lupulina*	Anti-inflammatory, herpes, traditionally used for mental tension, diabetes, rheumatoid, arthritis, and snake bite, anti-inflammatory agent, anti-viral	Kanchanapooma *et al.* 2001; Chopra *et al.* 1968; Suba *et al.* 2005; Wanikiat *et al.* 2007; Lans *et al.* 2001
BAR-12	*B. nitida*	*No reports*	--
BAR-13	*B. prattensis*	*No reports*	--
BAR-14	*B. prionitis*	Antidontalgic property, cough against boils and glandular swellings, tooth ache, anti-inflammatory, hepatoprotective, anti-spermatogenic, anti-nociceptive, anti-diabetic, anti-arthritic, anti-urolithiasis activity	Chopra *et al.* 1996; Khare 2007; Singh *et al.* 2003; Singh *et al.* 2005, Verma *et al.* 2005; Jaiswal *et al.* 2010; Dheer and Bhatnagar 2010; Choudhary *et al.* 2014; Atif *et al.* 2015
BAR-15	*B. sepalosa*	*No reports*	--
BAR-16	*B. strigosa*	Antipyretic and antidote	Kanchanapoom *et al.* 2004
BAR-17	*B. terminalis*	*No reports*	--

*References does not mention variety (blue/pink/white)

Table 2: Accessions of *A. vasica* and 16 *Barleria* species/variety with their betulin, vasicine, and vasicinone contents (mg/g) as determined by RP-UFLC analysis

Code	Name of species/varieties	Betulin	Vasicine	Vasicinone
BAR-1	*A. vasica*	54.941±2.747***	3.710±0.186***	0.043±0.002***
BAR-2	*B. acuminata*	55.856±2.793^{ns}	0.982±0.049**	0.036±0.002^{ns}
BAR-3	*B. buxifolia*	63.222±3.161**	0.313±0.016**	ND
BAR-4	*B. cristata* (Blue)	67.706±3.385**	0.606±0.030**	0.280±0.014**
BAR-5	*B. cristata* (Pink)	51.579±2.579^{ns}	1.314±0.066**	0.005±0.000^{ns}
BAR-6	*B. cristata* (White)	57.884±2.894^{ns}	0.109±0.005**	0.022±0.001^{ns}
BAR-7	*B. cuspidata*	07.700±0.385**	0.329±0.016**	0.104±0.005^{ns}
BAR-8	*B. gibsoni*	32.062±1.603**	ND	1.103±0.055**
BAR-9	*B. grandiflora*	40.303±2.015**	ND	2.752±0.138**
BAR-10	*B. lawii*	50.273±2.514^{ns}	ND	ND
BAR-11	*B. lupulina*	61.306±3.065*	0.118±0.006**	ND
BAR-12	*B. nitida*	28.735±1.437**	0.092±0.005**	0.025±0.001^{ns}
BAR-13	*B. prattensis*	08.289±0.414**	ND	0.293±0.015**
BAR-14	*B. prionitis*	63.740±3.187**	0.122±0.006**	0.034±0.002^{ns}
BAR-15	*B. sepalosa*	32.362±1.618**	ND	0.056±0.003^{ns}
BAR-16	*B. strigosa*	73.447±3.672**	0.104±0.005**	ND
BAR-17	*B. terminalis*	65.157±3.258**	0.302±0.015**	0.147±0.007*

Values in table are results of mean obtained by compiling data from three independent injection±SD; ns=not significant; *P<0.05;**P<0.01; ***P<0.001

carbonate, DPPH, potassium acetate, and 2,4,6-tripyridyl-s-triazine, were procured from Hi-media chemicals, India. Analytical standards Betulin was obtained from Sigma Chemical Co., USA, vasicine, and vasicinone from Natural remedies, Bangalore, India, whereas standards gallic and tannic were from Hi-media, India. All HPLC grade solvents such as glacial acetic acid (GAA), methanol, ethanol, and acetone were purchased from Qualigens, India.

Analysis methods

Quantification of total phenolic content (TPC)

TPC was quantified using the Folin–Ciocalteu method described by Upadhya *et al.*[30] with some modifications. The plant extracts (0.125 ml) with distilled water 0.5 ml were mixed with 0.125 ml Folin–Ciocalteu reagent and kept for 10 min for incubation at 25°C to it 1.25 ml of 7% sodium carbonate was added and kept for 90 min and absorbance was taken at 760 nm. Gallic and tannic acid (10–200 mg/l) were used to plot standard curves $y = 0.003x + 0.025$, $R^2 = 0.999$; $y = 0.002x + 0.019$, $R^2 = 0.999$, respectively, and the results were represented as mg/g dry powder as gallic acid equivalent (GAE) and tannic acid equivalent (TAE).[31]

Quantification of total flavonoid content (TFC)

TFC was quantified using the method described by Deshmukh *et al.*[32] An aliquot of 1.5 ml extract was added to 1.5 ml 2% aluminum chloride, vortexed and the reaction was kept for 10 min at RT in the dark, and absorbance was measured at 367 nm using a UV-visible spectrophotometer. Quercetin and ellagic acid (10–200 mg/l) were used to plot the calibration curve to obtain an equation $y = 0.007x - 0.046$, $R^2 = 0.999$; $y = 0.003x + 0.012$, $R^2 = 0.998$, respectively, and the total flavonoid was calculated as mg/g dry powder as quercetin acid equivalent (QUE) and ellagic acid equivalent (EAE).

DPPH radical scavenging activity

DPPH assay described by Upadhya *et al.*[33] was employed during the present study. Plant extract (0.1 ml) was added to 2.9 ml of DPPH reagent and was allowed to stand in dark at RT for 30 min. Absorbance was measured at 517 nm on a UV-visible spectrophotometer. Ascorbic acid (100–900 µM) and Trolox® (100–1000 µM) were used as standards for calibration $y = 0.0009x + 0.0543$, $R^2 = 0.9930$; $y = 0.0004x + 0.0326$, $R^2 = 0.9970$, respectively, and control (without extract) was also analyzed and the amount of DPPH activity was obtained and represented as µM dry powder as ascorbic acid equivalent antioxidant capacity (AEAC) and/or trolox equivalent antioxidant capacity (TEAC).

FRAP activity

The method of Ankad and coworkers[34] was employed for FRAP analysis. FRAP reagent and plant extracts were mixed in the proportion of 2.9:0.1 ml, respectively, incubated at RT (15 min) and absorbance was recorded at 595 nm. Ascorbic acid and Trolox (100–800 µM) were used to plot the graph for standard to obtain equations ($y = 0.001x + 0.173$, $R^2 = 0.970$; $y = 0.001x + 0.0131$, $R^2 = 0.954$, respectively) and the amount of FRAP activity was represented as above.

ABTS assay

Method of Subramanya *et al.*[35] was used during the study. ABTS reagent formed by assimilation of 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution as equal amount, put it to dark for 12 h. Incubated reagent diluted in methanol (1.1 ± 0.02 absorbance at 734 nm) which gives ABTS reagent for reaction. The plant extracts (0.1 ml) were reacted with 2.9 ml of the ABTS reagent, the mixture was incubated in dark for 2 h, and absorbance was taken at 734 nm. Ascorbic acid and Trolox (100–1000 µM) were used for calibration of a standard curve ($y = 0.0006x + 0.0105$, $R^2 = 0.9965$; $y = 0.0005x + 0.0212$, $R^2 = 0.9926$), respectively, and the amount of ABTS activity was calculated as above.

RP-HPLC fingerprint analysis using betulin, vasicine, and vasicinone

HPLC has widely been used to detect and determine nutrients,[31,36] and/or medicinal components from various plants.[37] For RP-HPLC analysis, methanolic extracts of all *Barleria* species under study were used. The column used during the analysis was Lichrospher 100, C18e (5 µm) column (250–4.6 mm) for betulin, Waters Nova-Pak, C_{18} (5 µm) column (250-4.6 mm) for vasicine and vasicinone. Mobile phase: A: methanol, B: water with pH 5.0 (by GAA) (A: B; 90:10) was used for the separation of betulin. Acetonitrile, 0.1 M phosphate buffer (pH 4.0), and GAA (14.5:84.5:1 *v/v/v*) were used for the separation of vasicine and vasicinone. A flow rate of 1 ml/min, 20 µl injection volume with a detection wavelength of 210 nm for betulin and 300 nm for vasicine, vasicinone were used. A system suitability test was performed considering the peak area for method repeatability and peaks for resolution.

Statistical and hierarchical cluster analysis (HCA) multivariate analysis

GraphPad Ver. 3.06 software compatible with windows 10 was used for statistical analysis. One-way analysis of variance (ANOVA) was performed to understand significant differences (at the significance level of $P < 0.05$). The HPLC profiles for all the extracts were evaluated on LC software (Shimadzu, Version 1.25). To understand correlations and study the groupings multivariate analysis was performed using Biodiversity-pro (version 2) eco-statistical software. The principal component analysis (PCA) and HCA were performed considering (i) antioxidant activities and (ii) the content of the different chemical constituents in samples.

RESULTS AND DISCUSSION

Quantification of TPC and TFC

It is an accepted experience that phenolics are the widely obtained natural resources having concern due to their antioxidant potential in human nutrition and medicine. Similarly, many workers have reported antioxidant activity of flavonoids; hence, interest has been increased greatly to obtain them from natural resources. TPC and TFC in methanolic extracts of different *Barleria* species using various standards are presented in Figure 1a and b. The results suggest that the species under study had higher TFC than TPC. The results revealed that *B. grandiflora* possesses the highest TPC (3.05% TAE and 2.00% GAE) and TFC (4.01% QUE and 9.14% EAE) among the species analyzed. While *B. prattensis* exhibited the lowest TPC (0.53% TAE and 0.31% GAE) and TFC (0.81% QUE and 1.66% EAE) content than other *Barleria* species. *B. cuspidata, B. gibsoni, B. grandiflora, B. lawii,* and *B. sepalosa* were detected with higher TPC and TFC in comparison to *A. vasica*.

The calibration graph for standards TPC and TFC had equations with $R^2 \leq 0.999$. TPCs using TAE were higher compared to gallic acid. The higher reducing power of tannic acid over gallic acid at similar assay concentrations resulted in higher absorbance. This is further reflected in graphs with a higher slope for the tannic acid curve compared to gallic acid. Therefore, a higher yield of TAE was observed; however, the % variation between them (TAE and GAE) remained the same. Further as inferred by Upadhya *et al.* 2015,[33] and Mujica *et al.* 2009,[38] in two independent studies, the absorbance of these compounds is about their structures, where one is hydrolyzable tannin (tannic acid) and the other is a trihydroxy benzoic acid (gallic acid). Similarly, quercetin equivalent TFC was higher than ellagic acid equivalent.

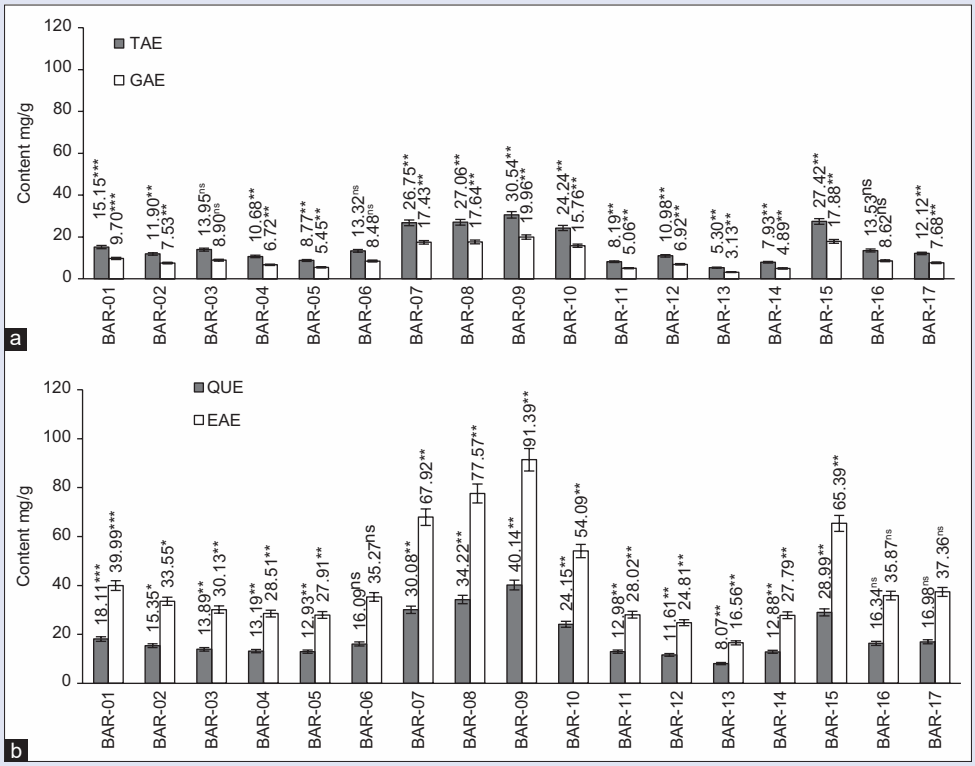

Figure 1: (a) Total phenolic, (b) TFC determined in eleven *Barleria* species/varieties in comparison with *A. vasica* (BAR-1)

Antioxidant activities

The DPPH assay has been a popular choice for determining reactive oxygen species. Figure 2a represents a comparative result obtained for antioxidant activity determined using DPPH. The results revealed that *B. grandiflora* possesses the strongest (626.22 µM AEAC and 1472.25 µM TEAC) DPPH radical scavenging activity among the different species studied. Whereas *B. lupulina* exhibited the lowest (110.00 µM AEAC and 310.75 µM TEAC) DPPH radical scavenging activity than other species *Barleria* with equivalent to both standards' ascorbic acid and Trolox. It was observed from the results that except *B. cristata* (Pink), *B. lupilina, B. prattensis* and *B. prionitis*, all other *Barleria* species showed higher DPPH radical scavenging activity in comparison to *A. vasica*. All TEAC values were higher compared to AEAC and the differences were more than 50%.

The FRAP assay evaluates antioxidant properties based on their reducing ability. Results obtained from FRAP assay for species under study are depicted in Figure 2b. The results revealed that *B. gibsoni* possesses the strongest (910.40 µM AEAC and 952.40 µM TEAC) FRAP activity among the other species. While *B. prattensis* exhibited the lowest (72.40 µM AEAC and 114.40 TEAC) FRAP activity. Even here TEAC values were higher than AEAC with a difference not exceeding 40%.

Similarly, Figure 2c depicts species wise comparison of antioxidant activity determined using the ABTS method. Results here indicate that *B. cuspidata* show higher (381.83 µM AEAC and 436.80 µM TEAC) ABTS radical scavenging activity among all other species. Interestingly, the results of the ABTS assay for *B. cuspidata, B. gibsoni, B. lawi,* and *B. sepalosa* are represented for 20 µl unlike the normal of 100 µl extract during the setting up of the reaction. Here, *B. prattensis* had shown lower (349.83 µM AEAC and 398.40 µM TEAC) ABTS radical scavenging activity.

RP-HPLC fingerprint analysis using betulin, vasicine, and vasicinone

Two different systems were identified for quantification of anti-cancer compound betulin and bronchodilator drugs vasicine and vasicinone from the species under study. All the sample extracts and standards were mixed in appropriate concentrations for RP-HPLC analysis. Seven concentrations of betulin (10, 20, 40, 80, 100, 200, and 400 µg/ml) and nine concentrations each of vasicine and vasicinone (0.01, 0.1, 0.5, 1, 5, 10, 50, 75, and 100 µg/ml) were injected and calibration data were obtained as $y = 10526x - 30282$, $R^2 = 0.999$ (betulin); $y = 36948x + 20146$, $R^2 = 0.995$ (vasicine); $y = 38855x - 22685$, $R^2 = 0.995$ (vasicinone). None of the R^2 values were < 0.995 indicating good linearity and there was a considerable relation between the concentrations of analyte with the corresponding peak areas. These equations were used for quantifying analytes in the plants under study.

The retention time observed for the analytes were 11.906 ± 0.077 min (betulin); 3.712 ± 0.032 min (vasicine) and 6.244 ± 0.103 min (vasicinone) using respective systems as described above with relative standard deviation values <2%. Method validation was achieved by injecting a spiked sample (50 µl each of 40 µg/ml betulin and 10 µg/ml vasicine and vasicinone) separately, to obtain recovery within 95–100%. The results obtained from the RP-HPLC study for betulin, vasicine, and vasicinone are tabulated in Table 2. Peaks of the standards were sharp with no tailing or shouldering, indicating good purity (98%) and no mixture of compounds. This made sure that there was no compatibility between the analytes, samples, extraction solvents, and mobile phase.

Betulin content in the samples ranged from 7.700 ± 0.385 to 73.447 ± 3.672 mg/g with a difference of ~ 89.51%. It is observed that *B. strigosa* (73.447 ± 3.672 mg/g) possesses higher content of betulin than others and this content was ~ 25.20% more than the content of

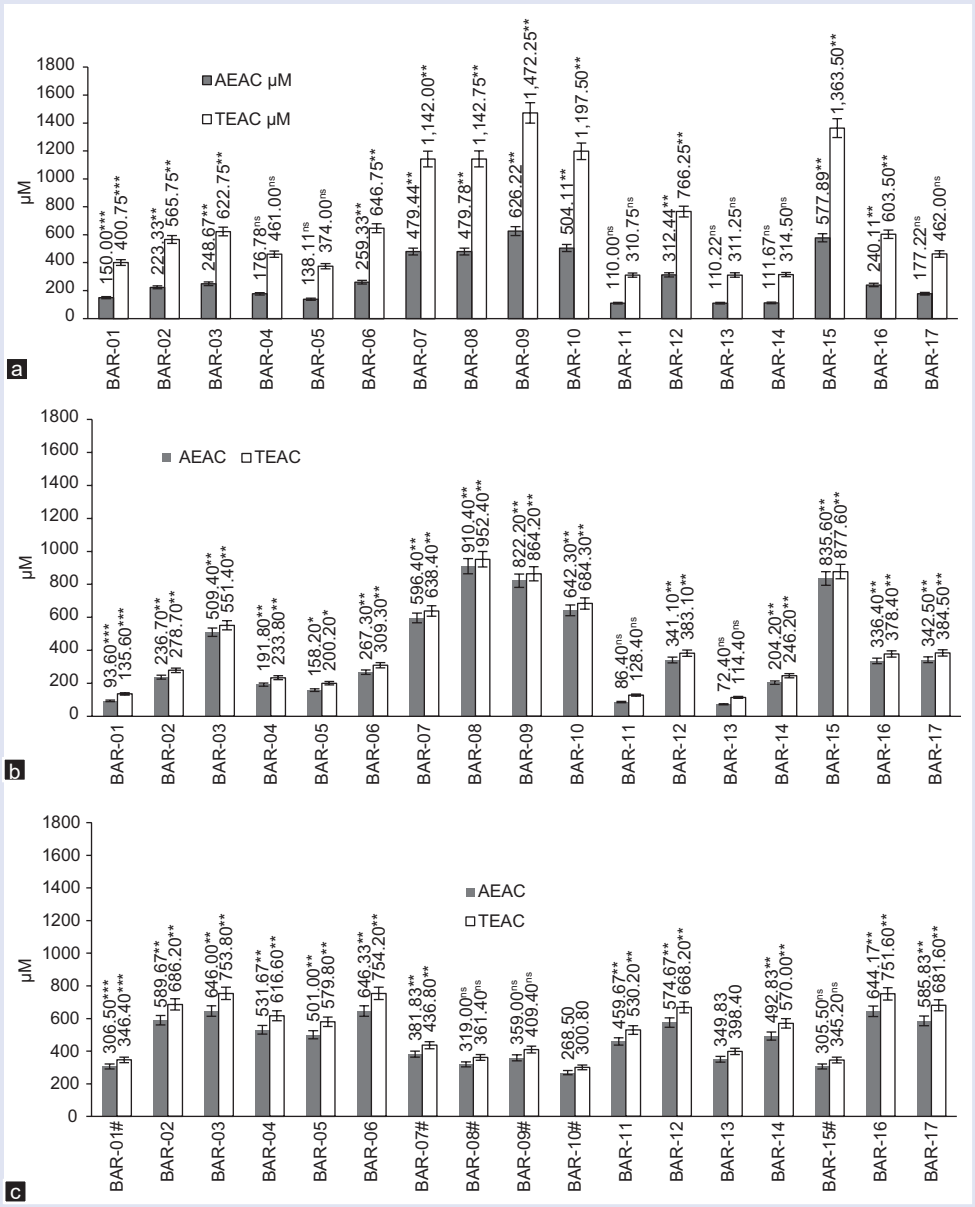

Figure 2: Antioxidant activities as determined by (a) DPPH; (b) FRAP and (c) ABTS assays for various *Barleria* species/varieties in comparison to *A. vasica* (BAR-1)

A. vasica (54.941 ± 2.747 mg/g). Whereas seven species and one variety of *Barleria* showed betulin content higher than *A. vasica* [Table 2].

Similarly, vasicine and vasicinone content ranged from 3.710 ± 0.186 to 0.092 ± 0.005 mg/g and 2.752 ± 0.138 to 0.005 ± 0.000 mg/g, respectively. *A. vasica* (3.710 ± 0.186 mg/g) certainly accounts for the highest vasicine content, followed by *B. cristata* (pink) (3.710 ± 0.186) with a 64.58% difference. On the other hand, *A. vasica* (0.043 ± 0.002 mg/g) was eighth highest after *B. grandiflora* (2.752 ± 0.138 mg/g) >*B. gibsoni* (1.103 ± 0.055 mg/g) >*B. prattensis* (0.293 ± 0.015 mg/g) >*B. cristata* (Blue) (0.022 ± 0.001 mg/g) >*B. terminalis* (0.147 ± 0.007 mg/g) >*B. cuspidate* (0.104 ± 0.005 mg/g) >*B. sepalosa* (0.056 ± 0.003 mg/g). It was observed that vasicine was absent in five and vasicinone in four *Barleria* species. *B. lawii* was the only species in which both vasicine and vasicinone were absent.

The data obtained were subjected to understanding the statistical significance, for which, a one-way ANOVA using the Dunnett test was performed. Data sets at $P < 0.05$ were considered significant within and in between the groups.

Statistical and HCA multivariate analysis

To justify the selection of data for statistical analysis and to maintain asynchrony in the results to be obtained, we distributed the data sets into two (i) antioxidant activities and (ii) content of different chemical constituents. HCA and PCA minimize the visual mistakes done by simply studying the data obtained. HCA produces a one-dimensional view of the relation of one sample with another on basis of the data provided. In PCA the score values give the projection in the graph and loadings determine the direction. The principal component (PC1) is linear whereas PC2 is orthogonal to the first. PC1 is for original variables with the highest variability, whereas PC2 is the next in terms of the amount of variability.[39]

Dendrogram clusters were obtained using the Bray–Curtis cluster analysis method. The results of (i) antioxidant activity were subjected

to obtaining a dendrogram. Wherein, at a percent similarity of 57.83%, 17 samples were divided into two major clades, with a range of 57.83–96.60% similarity between them. Clade 1 is comprised of *Barleria* species with high antioxidant activities as observed in *B. grandiflora* (DPPH); *B. gibsoni* (FRAP) and *B. cuspidata* (ABTS). Clusters 1 and 2 were merged into single clade 2, due to their moderate and lower antioxidant activities, respectively. Clusters 2 in clade 2 had samples with lower activity comprised of *A. vasica, B. lupulina,* and *B. prattensis*. Similarly, on other hand, dendrogram for the content of chemical constituents in the study. This dendrogram had the major two clades at 40.23% with 16 clusters and the similarity ranged from 40.23 to 98.51. The dendrogram for antioxidant activity was spread over the 38.77% range whereas it was 58.28% for the dendrogram of chemical contents suggesting compactness in the dendrogram of earlier over the latter one. Lower content of betulin, vasicine, vasicinone along with TPC and TFC in *B. prattensis* and *B. cuspidata* resulted in the formation of the separate cluster at the top. *A. vasica* was at the bottom along with *B. acuminata*, *B. cristata* (white), *B. cristata* (pink), and *B. lawii.* All these species had betulin content in the range of 50–58%, linked to another cluster with a betulin range between 60 and 74% (*B. buxifolia*, *B. prionitis*, *B. lupulina*, *B. cristata* (blue), *B. terminalis*, and *B. stigosa*).

Predictive modeling of the groups was obtained for PCA in similar lines as discussed above HCA. The unknown members in PCA are classified based on Eigenvalues.[40] The plots for loadings of the variables for (i) antioxidant assays and (ii) phytochemical data. All the species in the PCA fall in the positive quadrant of *x: y*-axis, exception of *B. lupulina* (near the negative *x*-axis) possibly due to lower antioxidant activity. Out of the three antioxidant activities tested *B. lupulina* had shown lower activity in two. The two groups toward the right of the positive *x*- and *y*-axis were of species with higher antioxidant activities which were in concurrence with clade 1 of the dendrogram. *A. vasica* was among the other 11 samples of *Barleria* with moderate to lower antioxidant activity.

On the same lines, PCA for phytochemical data showed species distribution mostly on the negative, positive (*x*, *y*) axis of the scattered plot. Only three species, *B. cuspidata*, *B. gibsoni,* and *B. grandiflora* were distributed in the positive quarter of the plot, whereas *B. prattensis* and *B. sepalosa* were on the *y*-axis distinguishing positive *x* and negative *x*. An interesting observation was *B. nitida* and *B. lawii* along with *B. prattensis* and *B. cuspidata* were scattered and did not make any grouping with any species, suggesting dissimilar behavior within the phytochemical contents in comparison to other species. Two groups circled left of the scattered plot were of 6 [*B. crsitata* (Blue), *B. prattensis*, *B. prionites*, *B. strigosa* and *B. terminalis*] and 4 species [*A. vasica*, *B.acuminata*, *B. cristata* (Pink) and *B. cristata* (White)]. The results of PCA make are in close agreement with that of HCA.

CONCLUSION

Conclusively, it was observed that *Barleria* and *Adathoda* differed in their activities as well as phytochemical profiles. *B. grandiflora* needs more attention due to its significant antioxidant activity and higher phytochemical content. Rich variations were observed in *Barleria* samples concerning phytochemical contents and antioxidant activities. Higher phenolics and antioxidants also justify their pharmacological properties and ethnobotanical use. Apart from identifying the analytes (betulin, vasicine, and vasicinone), the data will also improve understanding of its distribution in various species of *Barleria*. RP-HPLC method proved to be an effective and accurate tool in the quality assessment of *Barleria* species. HCA and PCA predictive modeling provide insights to find and statistically signify appropriate *Barleria* species close to *Adathoda*. It also helped in clustering higher yielding species together based on data generated. In conclusion, the results of the study suggested that *B. grandiflora* is the closest candidate to *A. vasica*, contributing to higher antioxidant activities and with higher vasicinone content.

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Conflicts of interest

There are no conflicts of interest.

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