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DESERT BLOOMS: UNRAVELING PALYNO-ANATOMICAL DIVERSITY IN ARID BORAGINACEOUS TAXA

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Abstract

The significance of palyno-anatomical features in characterizing Boraginaceous taxa from the arid regions is determined. The pollen micromorphology is carried out utilizing LM and SEM. For petiole anatomy, the sections were prepared using Shandon microtome and visualized under LM. The distinct significant palynological characters are hetropolarity, isocolpate, heterocolpate, porocolpate, polar, equatorial views, shape class, Amb, and exine ornamentation. Similarly, the petiole outline, cell shapes, number of layers, air spaces, and arrangement of vascular bundles are important distinguished anatomical features. Significant diagnostic variations were observed in the analyzed palyno-anatomical features, which efficiently differentiated the species within the same genera of *Heliotropium, Rochelia,* as well as the varieties of the single species *Lappula* spp., *L. spinocarpos*, *L. spinocarpos* subsp. *ceratophora, O. limitanea* var. *limitanea, O. limitanea* var. *major, P. intermedium* var. *intermedium, P. intermedium* var. *calathicarpum*. The quantitative data is compiled into a matrix, and subjected to statistical analysis via NCSS. The boxplot analysis identified the outliers in the data which assisted in taxa discrimination. The species of *Heliotropium* and *Paracaryum* were in the same cluster. In contrast, those of *Onosma, Rochelia,* and *Lappula* were in the different clusters in the hierarchal cluster analysis. The highest positive correlation existed between the polar axis with the equatorial diameter and mesocolpium with the polar length of the colpi. Meanwhile, exine thickness and equatorial width of colpi were negatively correlated. This research will help in the creation of pollen atlas and petiole anatomical documentation for the accurate identification of Boraginaceous taxa.

Key words: Pollen; Heterocolpate; Porocolpate; Anatomy; Petiole.

Introduction

The family Boraginaceae, also called the Borage or forget me not family, has some of the most valuable and extensive anatomical, morphological, ecological, and pharmacological traits of any family in the world (Rabizadeh, 2020; Yousaf *et al*., 2022). This family is worldwide in distribution, most common in temperate regions and is characterized by its vast diversity, with 2,300 species and approximately 130 genera (Buys & Hilger, 2003; Yousaf *et al*., 2022; Attar *et al*., 2019). In Pakistan, there are 32 genera and 135 species represented, including cultivated varieties such as *Anchusa* and *Cordia* (Nasir, 1989). Scorpioid inflorescences, a gynobasic style, and an ovary with two carpels separated into four nutlets are diagnostic traits of this family. Four subfamilies comprise the Boraginaceae family: Boraginoideae, Cordioideae, Ehretioideae, and Heliotropioideae – now treated as an independent family Heliotropiaceae APG (Rabizadeh, 2020). These species also have significant roles in cosmetology and pharmacology (Yousaf *et al*., 2022).

In plant taxonomy, plant anatomy is crucial. The idea is to create a system of classification for plants that will list all of their distinctions and similarities in chronological sequence (Okeke *et al*., 2015). Mabel *et al*., (2013) and Adedeji (2004) have all emphasized the taxonomic significance of anatomical traits, which, in addition to other characteristics, are useful for identifying and classifying plants. Taxonomists use various aspects and disciplines to classify taxa into relevant categories. One significant aspect is the anatomical characters of petiole, which are the key parameter used in identifying and classifying numerous plant families and have been employed to distinguish various genera (Metcalfe & Chalk, 1979). Recently, petiole anatomy is becoming more and more studied as a supplemental tool for plant taxonomy. The classification of plants using this line of evidence has advanced significantly. Additionally, some authors have concluded that the arrangement of vascular bundles in various petiole sections has taxonomic significance (Ekeke & Ogazie, 2020).

Noraini *et al*., (2016) highlighted the potential of petiole vascular patterns in distinguishing certain taxa. The anatomical features found in petiole include vascular tissue patterns, the existence/absence of sclerenchymatous cells encompassing the vascular bundles, medullary vascular bundles, and types of trichomes. Several studies demonstrated the utility of petiole anatomy in grouping genera and identifying species, as evidenced by Kocsis & Borhidi (2003), Noraini & Cutler (2009), and Gürdal & Nath (2022). While limited research exists on petiole anatomy within Boraginaceae. In Boraginaceae species, petiole anatomy variations could be linked to their habitat preferences, water availability, and overall growth strategies. Exploring the internal cellular organization, vascular bundles, and associated tissues within the petioles could shed light on the mechanisms these plants employ to adapt to different environmental conditions.

Another important aspect is palynology, which involves the study of palynomorphs to recognize and categorize closely similar taxa (Umber *et al*., 2022). Palynology has become increasingly captivating in scientific research. Various pollen characteristics, including symmetry, shape, apertural pattern, and exine configuration, play a vital part in plant taxonomy. Today, the study of pollen grains is widely used for taxonomic identification of flowering plants. Pollen morphology has been thoroughly used for the identification and relationship among the flowering plants at the species and variety levels (Hameed *et al*., 2020).

Harmomegathy is a fascinating adaptation observed in the pollen grains in the Boraginaceae family used to stay healthy in different environments. This involves the pollen grains folding when they lose water, helping them avoid drying out too much. The pollen has a strong outer layer and a more flexible inner layer (Sufyan *et al*., 2018). This flexibility allows the pollen to fold up and protect itself. The inner layer also helps the folding process and keeps the pollen alive (Ali & Perveen, 2021). Analyzing the harmomegathy mechanism in various Boraginaceae species can provide valuable information about the evolutionary trajectory of this family, shedding light on how pollen morphology and harmomegathy have shaped their ecological success. Boraginaceae is a eurypolynous family. This diversity in pollen shapes and folding tricks helps these plants survive in dry and wet areas (Volkova *et al*., 2013). The species within the Boraginaceae family display a range of pollen morphologies, allowing for the recognition of many different genera and species. In Pakistan, palynology has examined the appearance of pollen and its therapeutic attributes of medicinally important taxa (Yousaf *et al*., 2022).

This paper describes the petiole anatomy and palynology in determining the taxonomic relationship among 17 species of Borginaceae focusing on the internal cellular organization, vascular bundles, and associated tissues. It elucidates the systematic significance of palynological examination in Boraginaceae. Further studies are needed for a more comprehensive understanding of how Boraginaceae species have evolved and adapted to their respective habitats.

Material and Methods

Specimen collection: Field trips were conducted for the collection of specimens from the various phytogeographically important localities of Baluchistan were carried out from March 2022 to May 2022. The study districts were Loralai, Quetta, Zhob, Mastung, Kalat, Kharan, Lasbella, Khuzdar, and Hingol National Park (Makran). Fresh plant specimens with mature flowers were collected. Plant samples were dried, preserved with mercuric chloride and ethanol (50 ml absolute), and finally fumigated the specimens. Professor Dr. Mushtaq Ahmad (Quaid-e-Azam University), Dr. Amir Sultan (National Herbarium NARC), and the Herbarium of Pakistan (ISL) QAU, the Flora of Pakistan assisted in the taxa identification. International Plant Name Index (IPNI) and the Plant List (TPL) were utilized for plant name confirmation. The accession numbers were assigned to each specimen. Specimens were then mounted on standard herbarium sheets. The accession numbers are assigned to each species and deposited to the Herbarium of Pakistan (ISL) QAU (Table 1).

Pollen exploration via light microscopy: Using forceps and needles, anthers were placed on glass slides to make pollen slides, which were then crushed in the presence of acetic acid and dyed with glycerine jelly (Hameed *et al*., 2020, an improved version of Erdtman's, 1969 acetolysis procedure). Qualitative and quantitative features were noted utilizing the prepared slides and LM at various resolutions. Leica Light Microscope (Model 1000) embedded with the Infinity 1-5 C-MEL (Canada) digital camera was used to take the photomicrographs. Twenty readings for each attribute were recorded.

Table 1. Phytogeography of Boraginaceous taxa from arid regions of Pakistan.

S. No.	Name of species	Locality	Elevation (m)	Voucher No.
1.	*Alkanna tinctoria* subsp. *Tinctoria*	NARC	490	133344(ISL)|QAU
2.	*Buglossoides arvensis* (L.) I.M. Johnst.	Loralai	1400	133345 (ISL)|QAU
3.	*Coccinia mucronanthera* (Vent.) Brand	Loralai	1650	133346(ISL)|QAU
4.	*Cynoglossum lanceolatum* Forssk.	Pathankot	1500	133347(ISL)|QAU
5.	*Gastrocotyle hispida* (Forssk.) Bunge	Loralai	1380	133348(ISL)|QAU
6.	*Heliotropium bacciferum* Forssk.	Kundmalir	160	133349(ISL)|QAU
7.	*Heliotropium campanula* Stocks	Quetta, Zhob	1750	13335 0(ISL)|QAU
8.	*Heliotropium crispum* Desf.	Kundmalir, Beela, Loralai	300, 1350	13335 1(ISL)|QAU
9.	*Heliotropium curassavicum* L.	Kundmalir	204	13335 2(ISL)|QAU
10.	*Lappula spinocarpos* (Forssk.) Asch. ex Kuntze	Otmazai-Zhob	1400-1650	133353(ISL)|QAU
11.	*Lappula* spp.	Quetta	1350	133354(ISL)|QAU
12.	*Lappula spinocarpos* subsp. *ceratophora* (Popov) Y.J. Nasir	Quetta	1280-1980	13335 5 (ISL)|QAU
13.	*Onosma limitanea* I.M. Johnst. var. *limitanea*	Loralai	1800	13335 6(ISL)|QAU
14.	*Onosma limitanea* var. *major* I.M. Johnst.	Quetta	1750	13335 7(ISL)|QAU
15.	*Paracaryum intermedium* var. *intermedium* Y. J. NASIR	Loralai	1350	13335 8(ISL)|QAU
16.	*Paracaryum intermedium* var. *calathicarpum* (Stocks) Y.J. Nasir	Quetta	1600	13335 9(ISL)|QAU
17.	*Rochelia disperma* (L.f.) K.Koch	Loralai	1530	133360(ISL)|QAU
18.	*Rochelia sessiliflora* (Boiss.) Khoshsokhan & Kaz.Osaloo	Kundmalir	252	133361(ISL)|QAU
19.	*Trichodesma indicum* (L.) Sm.	Las Bela	320	133362(ISL)|QAU

Scanning electron microscopy (SEM) analysis: SEM (SEM JEOL made in Japan, model JSM 5910) was conducted using the methodology of Bahadur *et al*., (2019). Two acetic acid droplets were added to the anthers to remove the debris. The specimens were adhered to the stub using double-coated Scotch tape. The specimen received an additional gold palladium sputter coating. SEM experiments on micromorphological traits were conducted at the Department of Physics, Central Resource Library (CRL), University of Peshawar, Pakistan. The pollen images were captured using Polaroid P/N 655 film in the SEM. A standard check sheet was used to examine qualitative and quantitative indicators (for diagnostic features).

P/E ratio (Quantitative analysis): The ratio of P/E was calculated via the given formula:

$$P/E \times 100$$

where E is the equatorial diameter and P is the polar axis. Based on the P/E ratio, pollen shapes were investigated (Erdtman, 1969).

Histological studies: The separated petioles were treated for four hours with 10% saline formal solution for fixation (changing the solution twice). The samples were then dehydrated by passing to various concentrations of methanol (70%, 80%, 90%, 100%, and again 100%) for one hour each. Two applications of xylol, each lasting an hour, were made to eliminate the methanol (dealcoholization). The samples were then impregnated with wax at 58–62°C twice for an hour. The samples were implanted by section cutting with a microtome at 3-5 microns thickness. The melting of the prepared slides was finally completed at 62°C. The samples were subjected to 5-minute cycles of xylol deparaffinization twice. The samples were rehydrated by being exposed to methanol at concentrations of 100%, 90%, and 70% for a minute each. Then rinsed for a minute with tap water. After applying the fundamental stain haematoxylin for 5 minutes, the area was rinsed for a minute using tap water. The slides were then submerged in 1% acid alcohol and rinsed with tap water for an entire minute. Slides should be cleaned for a minute with tap water after being treated with 1% eosin for 30 to 60 seconds. Methanol concentrations of 70%, 80%, 90%, 100%, and 100% were administered for 30 seconds. We used xylol to clean the prepared slides twice for one minute. The material used was Dibutylphthalate Polystyrene Xylene (DPX) (Bahadur *et al*., 2019).

Statistical analysis

Evaluation of qualitative and quantitative details is a crucial component of plant systematics for determining the boundaries of species, genus, and tribes. Using SPSS-16, the mean and standard values for the quantitative palynological and anatomical features under study were calculated. Past (ver. 2023) was used for the statistical analysis (Iamonico *et al*., 2023). The correlation, dendrogram Unweighted Pair Group Clustering Method (UPGMA), and principal component analysis (PCA) were analyzed. The overall distribution and analysis of differences between the means are analyzed in the box plot.

Taxonomic key: The palynological and anatomical qualitative attributes were subsequently utilized to create a dichotomous taxonomic key that aids in identifying taxa.

Results

Palynology: Pollen size is mostly small in the examined species. Family Boraginaceae has previously been documented with the smallest pollens. The *Onosma*, *Rochelia,* and *Paracaryum* species were observed with small-sized pollen, except the *Heliotropium* species, which are medium-sized. The shapes of the pollen based on P/E ratio are prolate spheroidal (8), oblate spheroidal (3), suboblate (2), prolate (2), and subprolate (2). All three species *H. bacciferum* (prolate), *H. campanula* (oblate spheroidal), and *H. crispum* (sub oblate) species can be classified based on the pollen shape. The species of *Rochelia* and *Paracaryum* were also observed with distinct shapes (Figs. 1-4).

The pollen qualitative attributes symmetry, polarity, unity, number of apertures, polar view, equatorial view, exine sculpturing, exine surface, aperture membrane, colpi type, arrangement of apertures, Amb, Number, Position and Character (NPC) type of Boraginaceae were investigated and documented. The symmetry and unity of all the studied species were radial and monad. The heteropolarity of Boraginaceous taxa is not only caused by the difference between the size of the two poles but may exist because of the difference in the arrangement of apertures concerning the poles. 12 studied taxa are isopolar, and 5 are heteropolar. The heteropolarity differentiated among the species of the same genus and between the subspecies and variety of the same species. Among the 3 *Heliotropium* species *H. bacciferum* is heteropolar. Whereas *Lappula spinocarpos* subsp. *ceratophora* and *Onosma limitanea* var. *major* were heteropolar while the *L. spinocarpos* and *O. limitanea* var. *limitanea* were isopolar. For other genera, such as *Paracaryum* and *Rochelia* the polarity was isopolar for the examined species.

The aperture type, arrangement, number, aperture membrane, and colpi type are found to be taxonomically important. The porocolpate and heterocolpate arrangement of apertures are noted in 9 taxa. In porocolpate type, around the equator, pollen grains have a pattern of apertures where pores and colpi alternate. While in the heterocolpate, simple and compound colpi are present in pollen grains. These are the specific features of some Boragenous taxa. All of the studied *Heliotropium* and *Paracaryum* species are porocolpate and heterocolpate. Meanwhile, the species of genera *Rochelia* and Lappula species were separated based on the above two traits (Tables 2, 3). Five distinguished apertures types were observed. This trait is assessed to be eminently significant in the delimitation of these Boraginaceous taxa viz. tricolpate, tricolporate, trisynocolporate, hexacolporate, and hexatricolporate. *L. spinocarpos* subsp. *ceratophora* is hexotricolporate whereas *L. spinocarpos* is tricolporate. *O. limitanea* var. *limitanea* was tricolporate while *O. limitanea* var. *major* is trisynocolporate. *R. disperma* was tricolporate but the *R. sessiliflora* was hexotricolporate type. So, the aperture number was also a major palynological character. In the case of genera *Heliotropium* and *Paracaryum*, the number of apertures was significant up to the generic level only. The names of NPC classification were trizonocolpate, trizonocolporate, hexozonocolporate, and hexotrizono-colporate with formula $N_{6,3}P_4C_5$ and $N_{6,3}P_4C_5$.

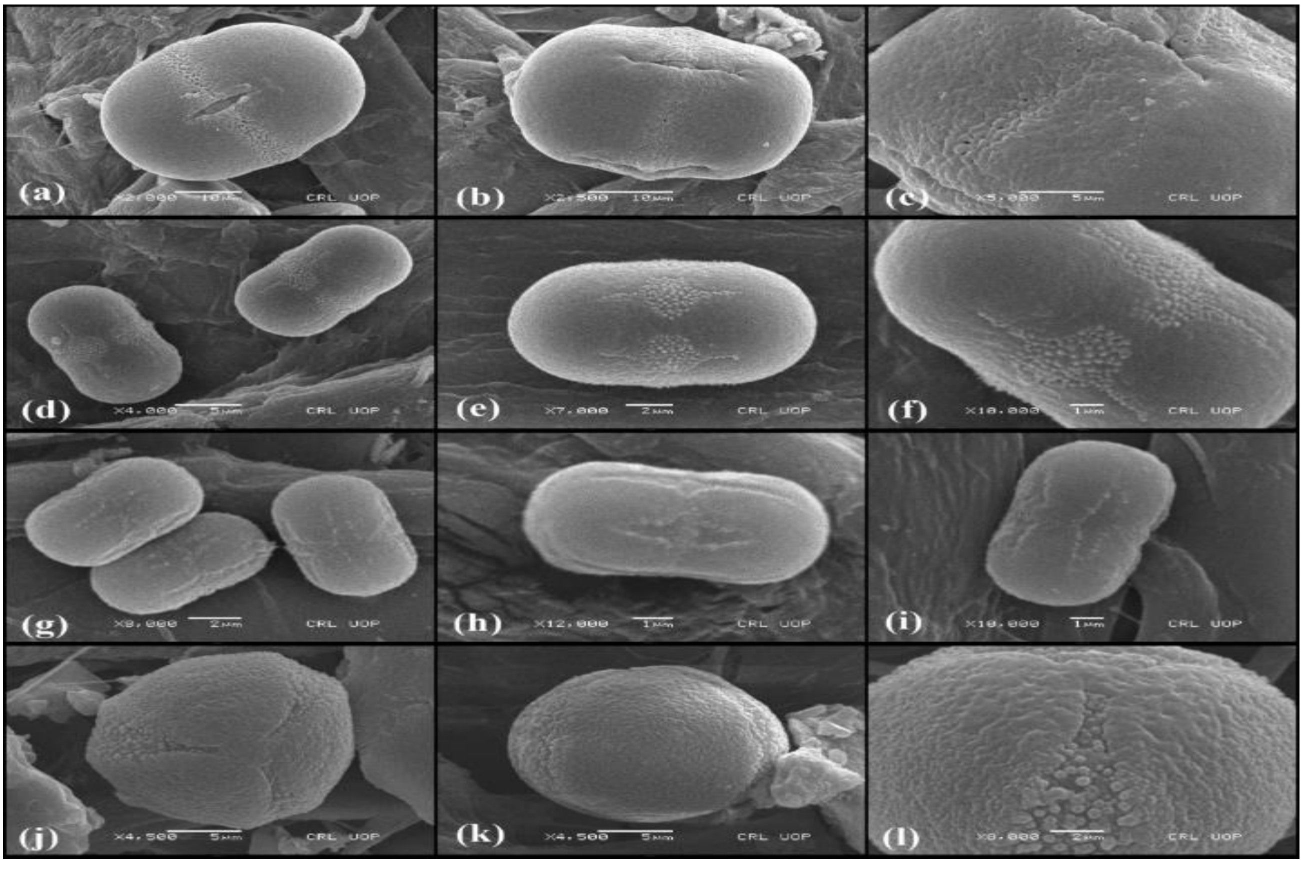

Fig. 1. SEM of *Alkanna tinctoria* subsp. *Tinctoria* (a, b, c) *Buglossoides arvensis* (d, e, f) *Cynoglossum lanceolatum* (g, h, i) *Caccinia mucronanthera* (j, k, l).

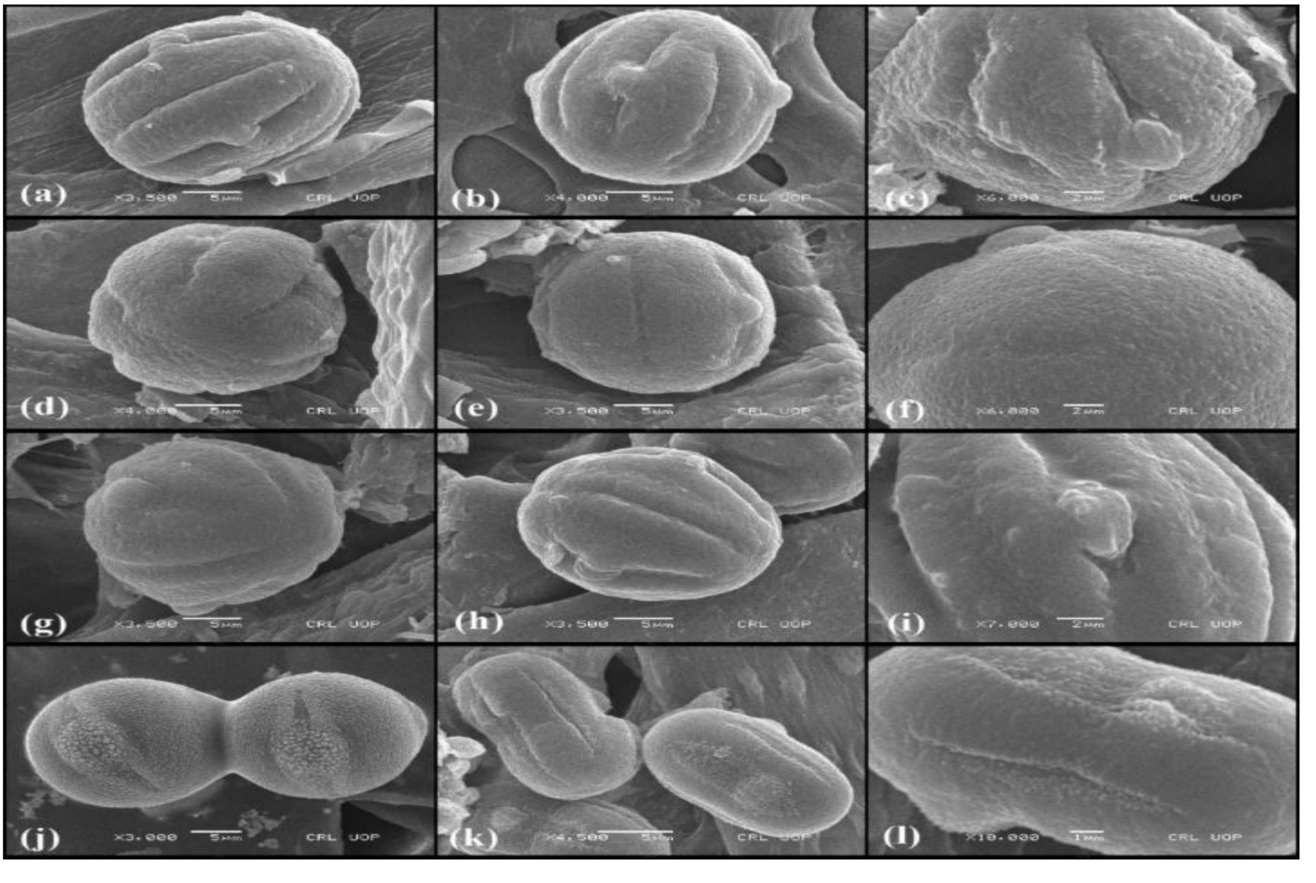

Fig. 2. SEM of *Heliotropium bacciferum* (a, b, c) *Heliotropium campanula* (d, e, f) *Heliotropium crispum* (g, h, i) *Lappula spinocarpos* (j) *Lappula. spinocarpos* subsp. *ceratophora* (k, l).

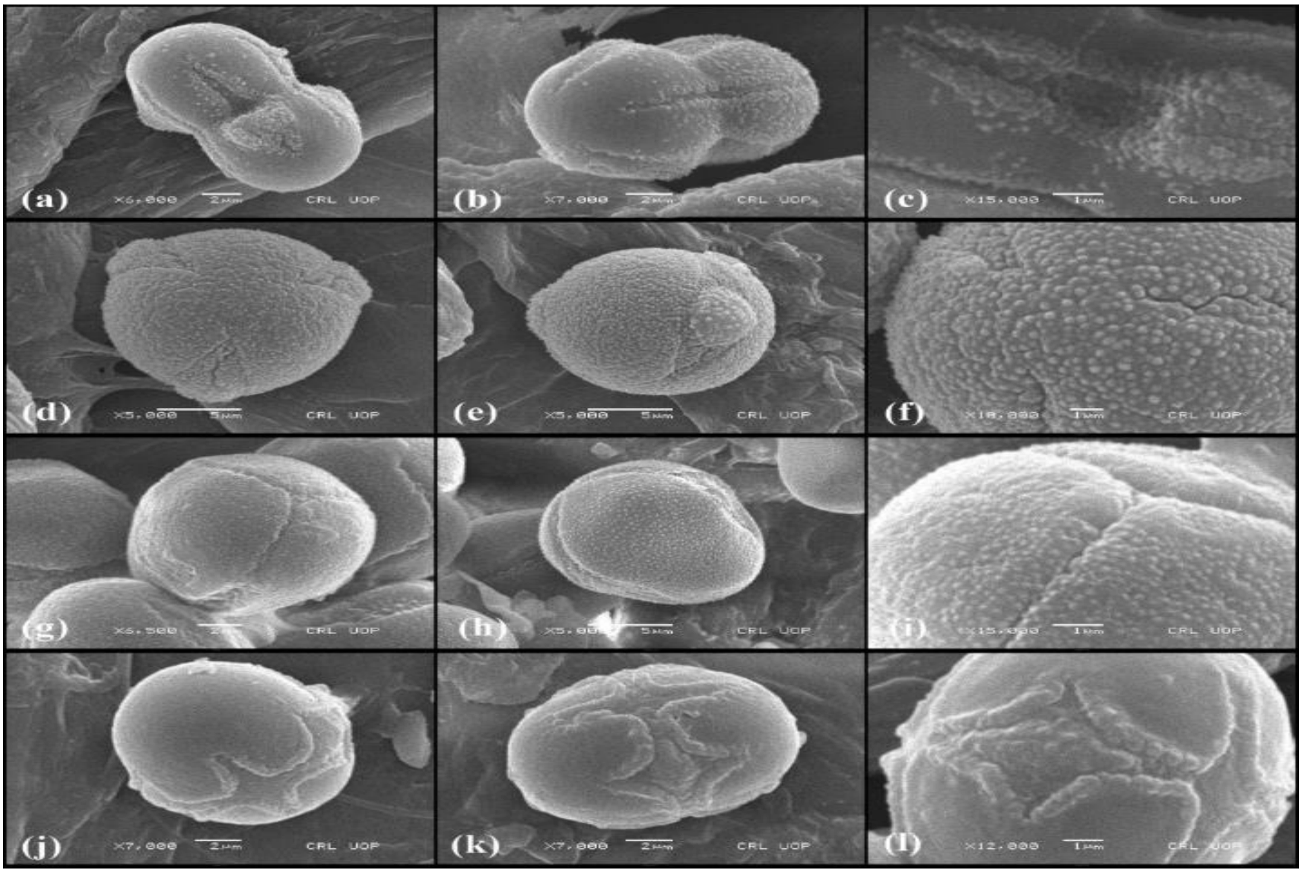

Fig. 3. SEM of *Lappula* spp (a, b, c) *Onosma limitanea* var. *limitanea* (d, e, f) *Onosma limitanea* var. *major* (g, h, i) *Paracaryum intermedium* var. *calathicarpum* (j, k, l).

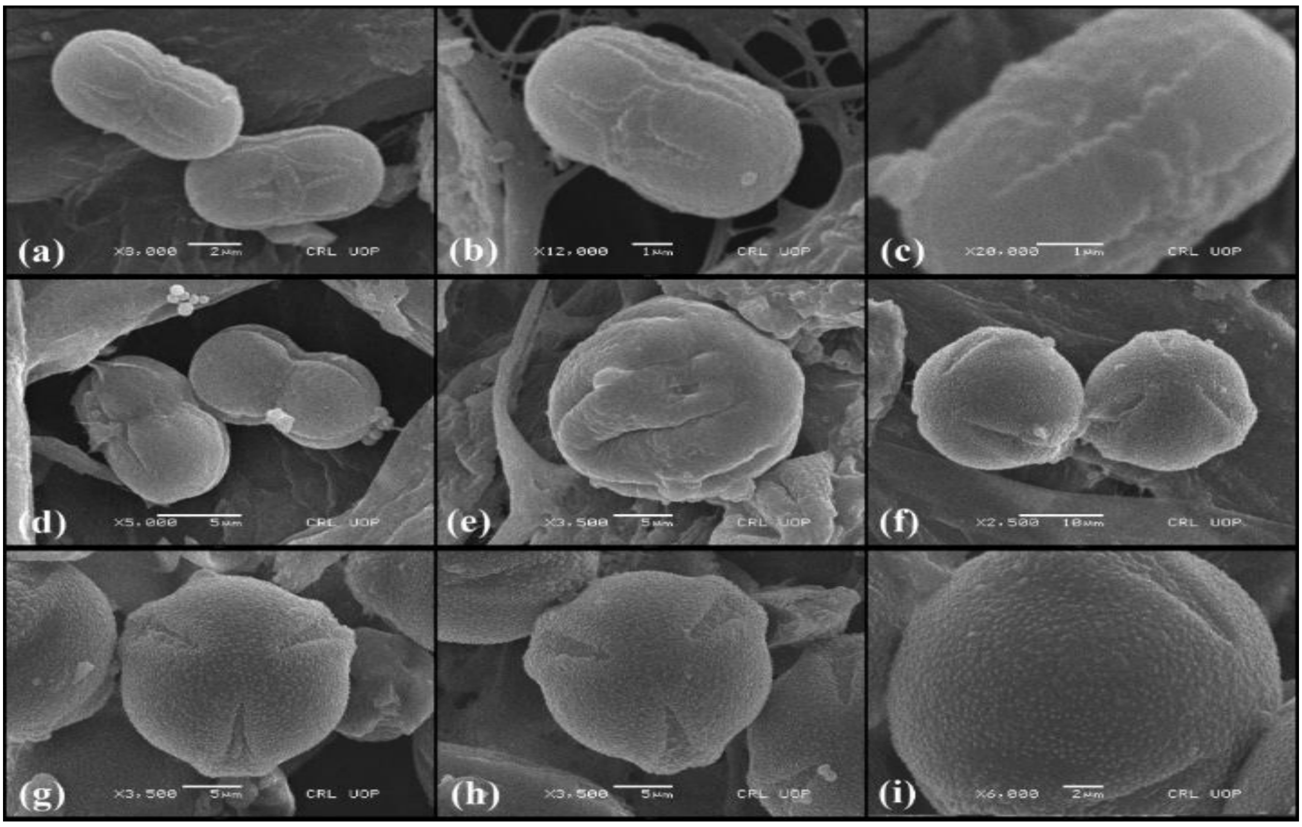

Fig. 4. SEM of *Paracaryum intermedium* var. *intermedium* (a, b, c) *Rochelia disperma* (d) *Rochelia sessiliflora* (e) *Trichodesma indicum* (f, g, h, i).

Table 2. Qualitative traits extracted from the quantitative data of LM of pollens.

Plant name	Polar axis	Equatorial diameter	Size class	P/E ratio	Shape class
A. tinctoria subsp. tinctoria	47.5	43.85	Medium	1.083	Prolate spheroidal
B. arvensis	15.85	12.4	Small	1.278	Subprolate
C. lanceolatum	20.15	22.5	Small	0.895	Oblate spheroidal
C. mucronanthera	24.85	24.55	Small to Medium	1.012	Prolate spheroidal
H. bacciferum	28.45	20.5	Medium	1.387	Prolate
H. campanula	24.1	25.15	Medium	0.958	Oblate spheroidal
H. crispum	23.95	27.05	Medium	0.885	Suboblate
L. spinocarpos	25	27.95	Medium	0.894	Oblate spheroidal
L. spinocarpos subsp. *ceratophora*	15.2	14.1	Small	1.078	Prolate spheroidal
Lappula spp.	15	13.3	Small	1.127	Prolate spheroidal
O. limitanea var. *limitanea*	22.65	20.65	Small	1.096	Prolate spheroidal
O. limitanea var. *major*	14.9	14	Small	1.064	Prolate spheroidal
P. intermedium var. *calathicarpum*	14.15	13.3	Small	1.063	Prolate spheroidal
P. intermedium var. *intermedium*	13.2	9.2	Small	1.434	Prolate
R. disperma	15.4	19.75	Small	0.779	Suboblate
R. sessiliflora	14.95	13	Small	1.15	Subprolate
T. indicum	26	22.85	Medium	1.137	Prolate spheroidal

The exine sculpturing of 12 species was psilate. Other observed types were scabrate, foveolate, and gemmate. Sculpturing was found helpful in the discrimination of *L. spinocarpos* subsp. *ceratophora* psilate from *L. spinocarpos* scabrate. Similarly, *O. limitanea* var. *limitanea* gemmate and *O. limitanea* var. *major* scabrate are significantly distinguished. For the genera *Heliotropium, Rochelia,* and *Paracaryum,* exine ornamentation was taxonomically nonsignificant. The foveolate exine was present singly in *C. mucronanthera.* Perforation in the exine surface was observed in 4 taxa. *H. campanula* has perforated exine among the three *Heliotropium* species. The aperture membrane was smooth, granular, operculate, and granular-operculate types. Considerable variations occurred in the studied taxa's polar, equatorial view, and Amb. The Amb was of three forms i.e., peritreme (8), ptychotreme (7), and goniotreme (2). *Onosma* and *Lappula* species were separated on the basis of Amb form. Pollen grains appeared as circular, triangular obtuse convex, and quinquangular obtuse convex in the polar view.

For the hierarchical cluster analysis, the two-way dendrogram was created based on quantitative data on pollens. The results are presented in the (Fig. 5). The dendrogram separated *A. tinctoria* subsp. *tinctoria* being the largest of all others. The 2 major clusters were then further delineated into two subclusters each. The *Heliotroipium* species were in the same cluster. Similarly, the species of *Paracaryum* were also in the same cluster. The *Onosma* and *Rochelia* species were found in different clusters, and this revealed the quantitative variations in the palynological traits. The *Lappula spinocarpos* is separate from two other *Lappula* species. The principal component analysis ordination (PCA) of 17 Boraginaceous species were observed with grouping by correspondence to the polar axis, equatorial diameter, polar and equatorial length, and width of colpi, exine diameter, mesocolpium (Fig. 6). Score 1 and Score 2 accounted for the variance among the mean values of quantitative parameters. The overall data distribution was statistically presented in a box plot (Fig. 7). The data dispersion range was given along the Y-axis, and the analyzed characters are represented along the X-axis. The outliers in each group were represented as dots. The correlation plot determined the possible association among the means of different traits. The maximum correlation was found between two pairs of mesocolpium and the polar length of colpi and the polar axis and equatorial diameter. Meanwhile, a negative correlation was observed between the exine thickness and equatorial width of the colpi (Fig. 8).

Petiole anatomy: Most of the studied petioles were winged (Figs. 9-11). *Heliotropium curassavicum* and *H. bacciferum* had winged petioles. The presence or absence of grooves in the petiole discriminated the taxa. The outline of the petiole varied from sulcate in 8 species, flat in 4 taxa, and oval in 2 members. Prominent trichomes were observed in 6 species. Unicellular trichomes were present in 3, uniseriate in 2, and multiseriate in 1 taxon. The cuticle was either undulated, e.g. in *Heliotropium crispum* (Fig. 12), or smooth in other Boraginaceous species.

The epidermal cells were round, oval, rectangular, angular, and isodiametric or the combination of these shapes. Collenchyma was 1-layered in 11 taxa. In *A. tinctoria* subsp. *tinctoria, H. crispum*, and *Lappula* spp., collenchyma was 2-layered. The subepidermal ring of collenchyma was observed in *H. crispum* and *H. campanula.* Angular collenchyma was present in 9 species followed by lamellar (2), annular (2), and lacunar (1). *H. campanula* was distinguished from other *Heliotropium* species for having lamellar collenchyma. The maximum parenchymatous layers was 7 in *H. campanula,* whereas the minimum,1, was observed in *O. limitanea* var. *limitanea.* The shape of parenchyma was mostly irregular to angular and isodiametric. 1-2 layers of chlorenchyma were observed in studied species. There was no prominent layer in *C. lanceolatum.*

Vascular bundles are arranged in bicollateral, collateral closed, amphicribral, and collateral open. The vascular bundle in *H. bacciferum* was amphicribral, while the collateral closed type was present in the remaining three *Heliotropium* taxa. The number of Vascular bundles was 1 in all the studied species. This feature cannot be used to delimit the studied Boraginaceous taxa. The vascular bundle size was suitable for the differentiation of the species. The xylem vessel shape varied from round, oval to square, rectangular, and angular. Whereas the phloem cells were mostly angular, tri to hexagonal (Tables 4, 5).

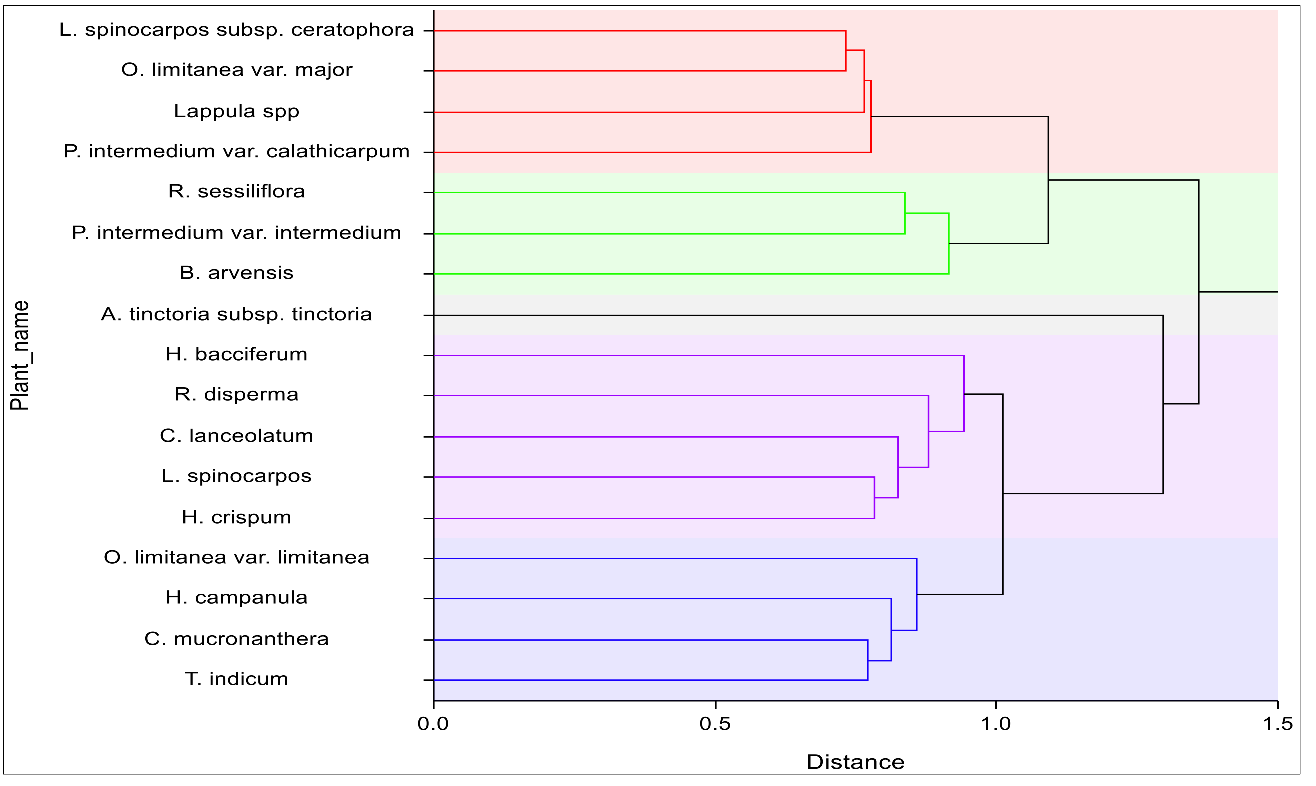

Fig. 5. UPGMA cluster analysis based on Polar axis, Equatorial Diameter, polar width and length of colpi, equatorial width and length of colpi, Mesocolpium, and Exine thickness.

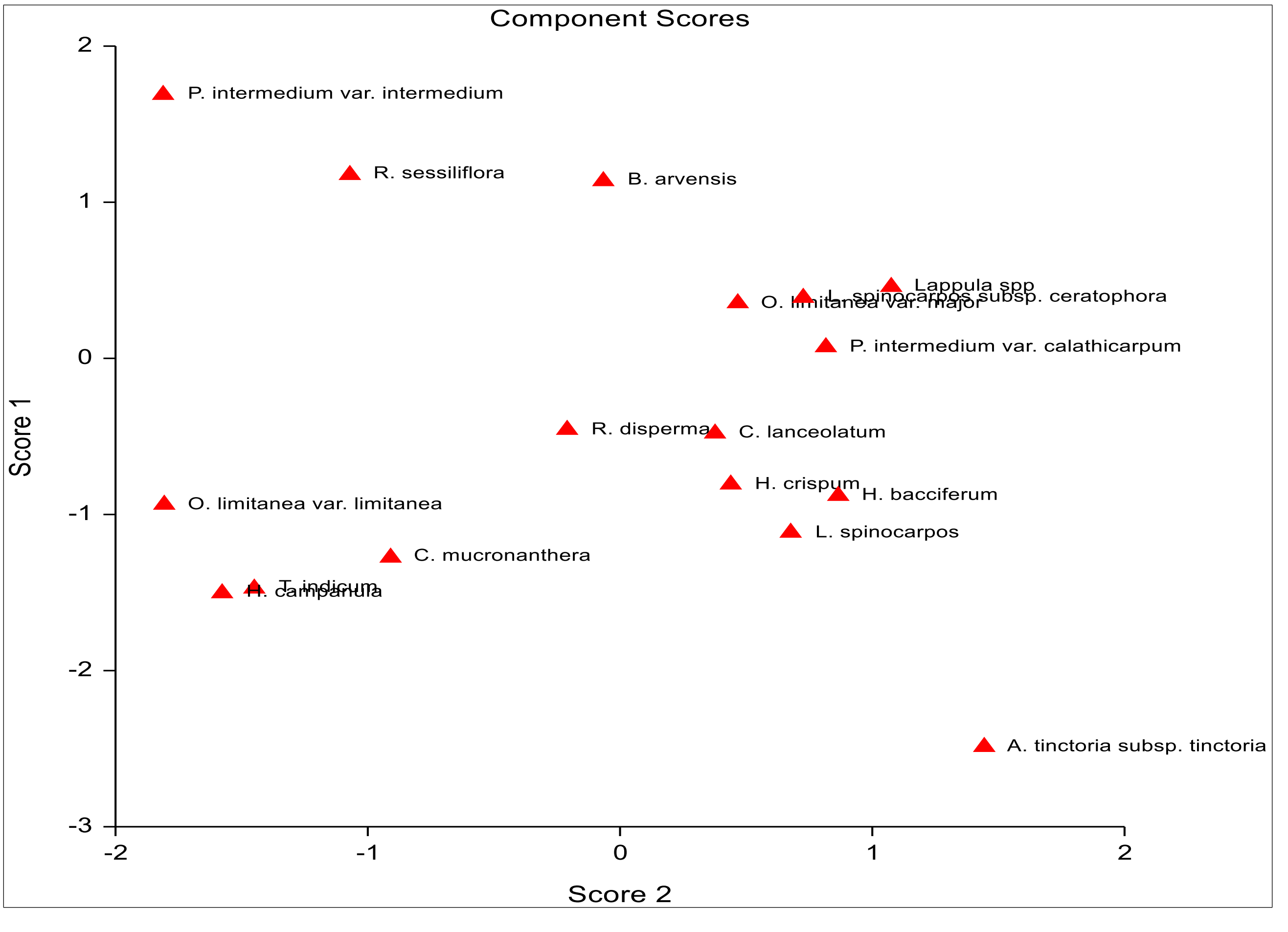

Fig. 6. Multivariate analysis via PCA plot for Polar axis, Equatorial Diameter, polar width and length of colpi, equatorial width and length of colpi, Mesocolpium, and Exine thickness.

Table 3. Qualitative traits based on SEM of pollens.

Taxa	Symmetry	Polarity	Unity	No. of apertures	Polar view	Equatorial view	Exine sculpturing
A. tinctoria subsp. *tinctoria*	Radial	Isopolar	Monad	Tricolporate	Circular	Rectangular obtuse convex	Psilate
B. arvensis	Radial	Heteropolar	Monad	Hexacolporate	Circular	Rectangular obtuse convex	Psilate
C. lanceolatum	Radial	Isopolar	Monad	Hexotricolporate	Circular	Rectangular obtuse convex	Psilate
C. mucronanthera	Radial	Isopolar	Monad	Tricolpate	Triangular obtuse convex	Elliptic truncate	Foveolate
H. bacciferum	Radial	Heteropolar	Monad	Hexotricolporate	Quinquangular obtuse convex	Elliptic truncate	Psilate
H. campanula	Radial	Isopolar	Monad	Hexotricolporate	Quinquangular obtuse convex	Circular	Psilate
H. crispum	Radial	Isopolar	Monad	Hexotricolporate	Quinquangular obtuse convex	Elliptic truncate	Psilate
L. spinocarpos	Radial	Isopolar	Monad	Tricolporate	Circular	Circular	Scabrate
L. spinocarpos subsp. *ceratophora*	Radial	Heteropolar	Monad	Hexotricolporate	Circular	Rectangular obtuse concave	Psilate
Lappula spp.	Radial	Heteropolar	Monad	Hexotricolporate	Triangular obtuse convex	Rectangular obtuse concave	Psilate
O. limitanea var. *limitanea*	Radial	Isopolar	Monad	Tricolporate	Circular	Circular	Gemmate
O. limitanea var. *major*	Radial	Heteropolar	Monad	Trisynocolporate	Triangular obtuse convex	Rhombic obtuse truncate	Scabrate
P. intermedium var. *calathicarpum*	Radial	Isopolar	Monad	Hexotricolporate	Circular	Elliptic truncate	Psilate
P. intermedium var. *intermedium*	Radial	Isopolar	Monad	Hexotricolporate	Circular	Rectangular obtuse convex	Psilate
R. disperma	Radial	Isopolar	Monad	Tricolporate	Triangular obtuse convex	Rectangular obtuse concave	Psilate
R. sessiliflora	Radial	Isopolar	Monad	Hexotricolporate	Quinquangular obtuse convex	Elliptic truncate	Psilate
T. indicum	Radial	Isopolar	Monad	Tricolporate	Circular	Circular	Scabrate

Table 3 (Cont'd.).

Taxa	Exine surface	Aperture membrane	Colpi type	Arrangement of apertures	Amb	NPC classification	
						Name	Formula
A. tinctoria subsp. *tinctoria*	Perforate	Smooth	Isocolpate	-	Peritreme	Trizonocolporate	$N_3P_4C_5$
B. arvensis	Non-perforate	Granular	Isocolpate	-	Peritreme	Hexazonocolporate	$N_{6,3}P_4C_5$
C. lanceolatum	Non-perforate	Smooth	Heterocolpate	Porocolpate	Peritreme	Hexotrizonocolporate	$N_{6,3}P_4C_5$
C. mucronanthera	Perforate	Granular	Isocolpate	-	Goniotreme	Trizonocolpate	$N_3P_4C_5$
H. bacciferum	Non-perforate	Operculate	Heterocolpate	Porocolpate	Ptychotreme	Hexotrizonocolporate	$N_{6,3}P_4C_5$
H. campanula	Perforate	Operculate	Heterocolpate	Porocolpate	Ptychotreme	Hexotrizonocolporate	$N_{6,3}P_4C_5$
H. crispum	Non-perforate	Operculate	Heterocolpate	Porocolpate	Ptychotreme	Hexotrizonocolporate	$N_{6,3}P_4C_5$
L. spinocarpos	Non-perforate	Granular	Isocolpate	-	Peritreme	Trizonocolporate	$N_3P_4C_5$
L. spinocarpos subsp. *ceratophora*	Non-perforate	Granular operculate	Heterocolpate	Porocolpate	Ptychotreme	Hexotrizonocolporate	$N_{6,3}P_4C_5$
Lappula spp.	Non-perforate	Granular operculate	Heterocolpate	Porocolpate	Ptychotreme	Hexotrizonocolporate	$N_{6,3}P_4C_5$
O. limitanea var. *limitanea*	Non-perforate	Granular operculate	Isocolpate	-	Peritreme	Trizonocolporate	$N_3P_4C_5$
O. limitanea var. *major*	Non-perforate	Granular	Isocolpate	-	Goniotreme	Trizonocolporate	$N_3P_4C_5$
P. intermedium var. *calathicarpum*	Non-perforate	Operculate	Heterocolpate	Porocolpate	Peritreme	Hexotrizonocolporate	$N_{6,3}P_4C_5$
P. intermedium var. *intermedium*	Non-perforate	Operculate	Heterocolpate	Porocolpate	Peritreme	Hexotrizonocolporate	$N_{6,3}P_4C_5$
R. disperma	Non-perforate	Operculate	Isocolpate	-	Ptychotreme	Trizonocolporate	$N_3P_4C_5$
R. sessiliflora	Non-perforate	Operculate	Heterocolpate	Porocolpate	Ptychotreme	Hexotrizonocolporate	$N_{6,3}P_4C_5$
T. indicum	Perforate	Granular	Isocolpate	-	Peritreme	Trizonocolporate	$N_3P_4C_5$

amb: Circumference, NPC: Number, Position, Characteristic

Table 4. Qualitative anatomical observations of the Petiole of Boraginaceous taxa.

Plant	Petiole wing	Grove in the upper surface	Co (No of layers)	Subepidermal ring of Co	Ch (No of layers)	Pa (No of layers)	Sc Presence in VB	Air spaces As	No. of VB
A.tinctoria subsp. *tinctoria*	+	+	2	-	2	2	+	-	1
C. lanceolatum	+	-	1	-	-	2	-	+	1
C. mucronanthera	+	-	1	-	1	3	-	-	1
G. hispida	+	+	1	-	1	4	-	+	1
H. bacciferum	+	+	1	-	1	3	-	-	1
H. campanula	-	+	1	+	2	7	-	-	1
H. crispum	-	-	2	+	1	3	-	-	1
H. curassavicum	+	-	1	-	2	4	-	-	1
Lappula spp.	-	+	2	-	2	4	-	-	1
O. limitanea var. *limitanea*	+	+	1	-	1	1	-	-	1
O. limitanea var. *major*	+	-	1	-	1	2	-	+	1
P. intermedium var. *intermedium*	-	+	1	-	1	2	-	+	1
R. sessiliflora	-	+	1	-	2	3	-	-	1
T. indicum	+	+	1	-	2	4	+	+	1

Co: Collenchyma, Ch: Chlorenchyma, Sc: Sclerenchyma, VB: Vascular bundles, Pa: parenchyma

Table 5. Qualitative anatomical observations of the Petiole of Boraginaceous taxa.

Plant	Outline	Trichome	Ep shape	Pa shape	Co Shape	Sc shape	VB arrangement	Xy vessel shape	Ph shape
A.tinctoria subsp. *tinctoria*	Sulcate	-	Oval to angular and irregular	Irregular	Annular	Irregular	Bicollateral	Isodiametric to round	Angular to irregular
C. lanceolatum	Sulcate	-	Square to angular	Irregular	Lamellar	-	Collateral closed	Round	Tri to hexagonal
C. mucronanthera	Sulcate	Multiseriate	Irregular	Isodiametric	Lacunar	-	Collateral closed	Round to oval	Angular
G. hispida	Sulcate	-	Angular to irregular	Irregular	Angular	-	Amphicribral	Round to oval	Angular
H. bacciferum	Sulcate	-	Angular to irregular	Irregular	Angular	-	Amphicribral	Round to angular	Angular
H. campanula	Flat	-	Rectangular to square and round	Irregular	Lamellar	-	Collateral closed	Round to oval	Angular
H. crispum	Oval	Uniseriate	Square to angular	Isodiametric	Annular	-	Collateral closed	Round	Angular
H. curassavicum	Sulcate	-	Square to angular	Angular to isodiametric	Angular	-	Collateral closed	Round to angular	Tri to hexagonal
Lappula spp.	Flat	-	Square to oval	Tri to hexagonal	Angular	-	Amphicribral	Round to angular	Angular
O. limitanea var. *limitanea*	Sulcate	Unicellular	Angular	Angular to irregular	Angular	-	Amphicribral	Round	Tri to hexagonal
O. limitanea var. *major*	Sulcate	Uniseriate	Isodiametric to angular	Irregular to isodiametric	Angular	-	Collateral closed	Round to angular	Tri to hexagonal
P. intermedium var. *intermedium*	Flat	Unicellular	Angular	Irregular	Angular	-	Collateral closed	Round to oval	Angular
R. sessiliflora	Oval	-	Angular	Tri to hexagonal	Angular	-	Amphicribral	Round, angular, oval	Tri to hexagonal
T. indicum	Flat	Unicellular	Round to angular	Angular to isodiametric	Angular to lamellar	Angular	Collateral open	Rectangular to square	Irregular

Ep: Epidermis, Pa: Parenchyma, Co: Collenchyma, Sc: Sclerenchyma, VB: Vascular bundles, Xy: Xylem, Ph: Phloem

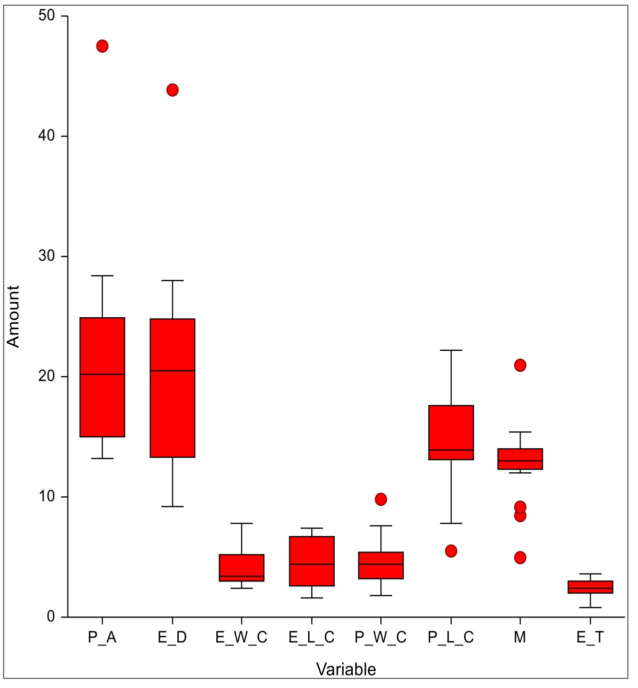

Fig. 7. Statistical analysis via boxplot of the mean values of Polar axis (P.A), Equatorial Diameter (E.D), polar width and length of colpi (P.W.C, P.L.C), equatorial width and length of colpi (E.W.C, E, L, C), Mesocolpium (M), Exine thickness (E.T).

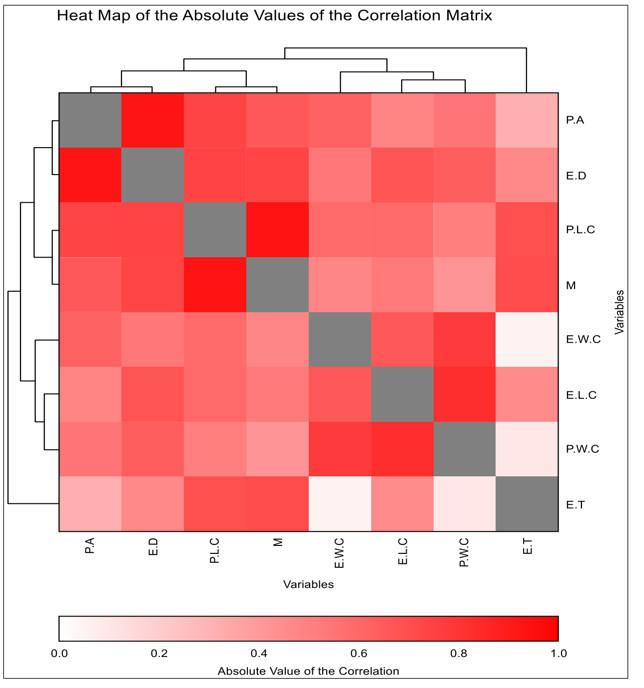

Fig. 8. Correlation among the mean values of Polar axis (P.A), Equatorial Diameter (E.D), polar width and length of colpi (P.W.C, P.L.C), equatorial width and length of colpi (E.W.C, E, L, C), Mesocolpium (M), Exine thickness (E.T).

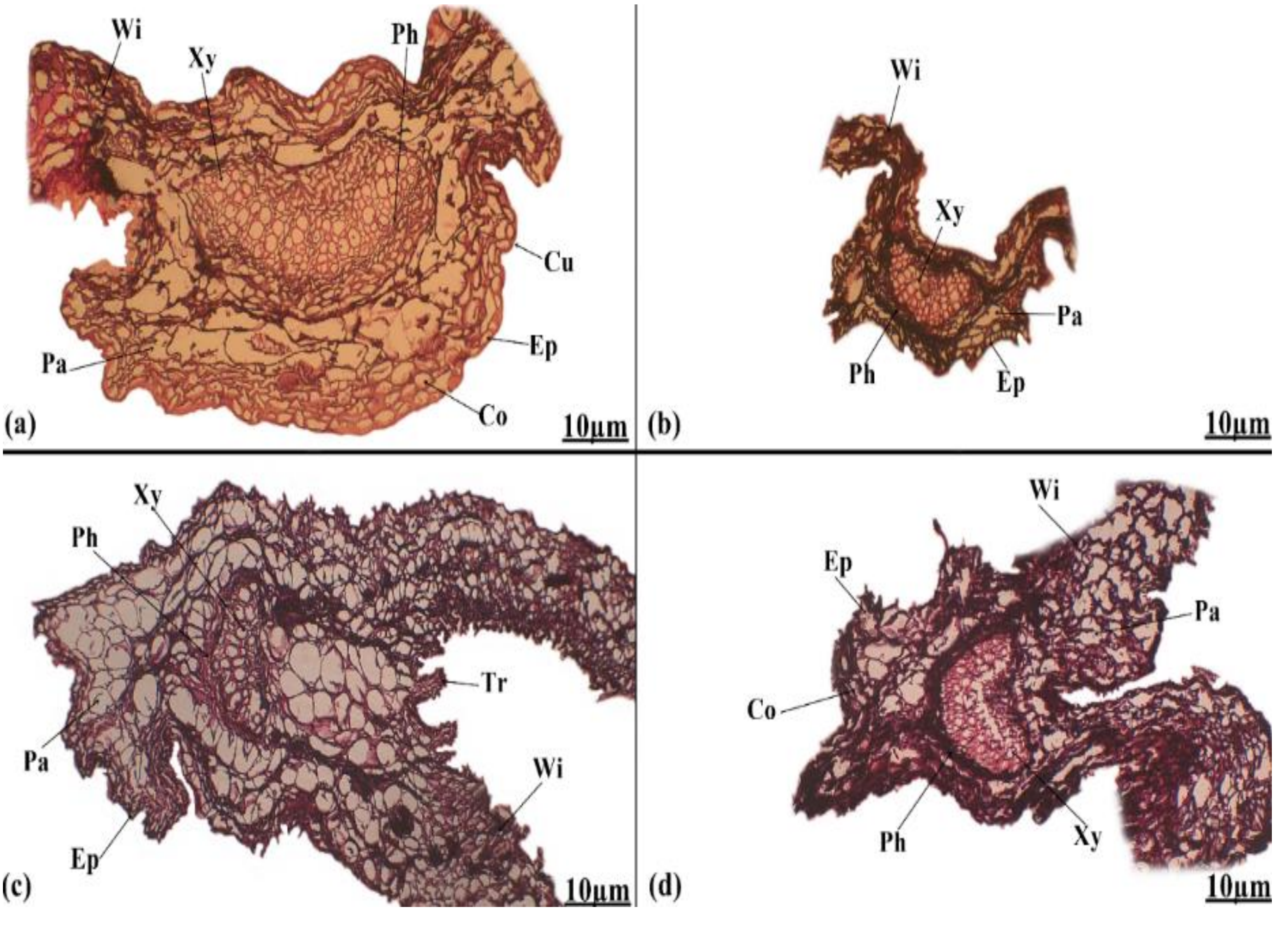

Fig. 9. Petiole anatomy of (a) *Alkanna tinctoria* subsp. *Tinctoria* (b) *Cynoglossum lanceolatum* (c) *Coccinia mucronanthera* (d) *Gastrocotyle hispida* (Cu: cuticle, Ep: Epidermis, Pa: parenchyma, Co: collenchyma, Ch: chlorenchyma, Sc: slerenchyma, Xy: xylem, Ph: Phloem, Tr: trichome, Wi: wing).

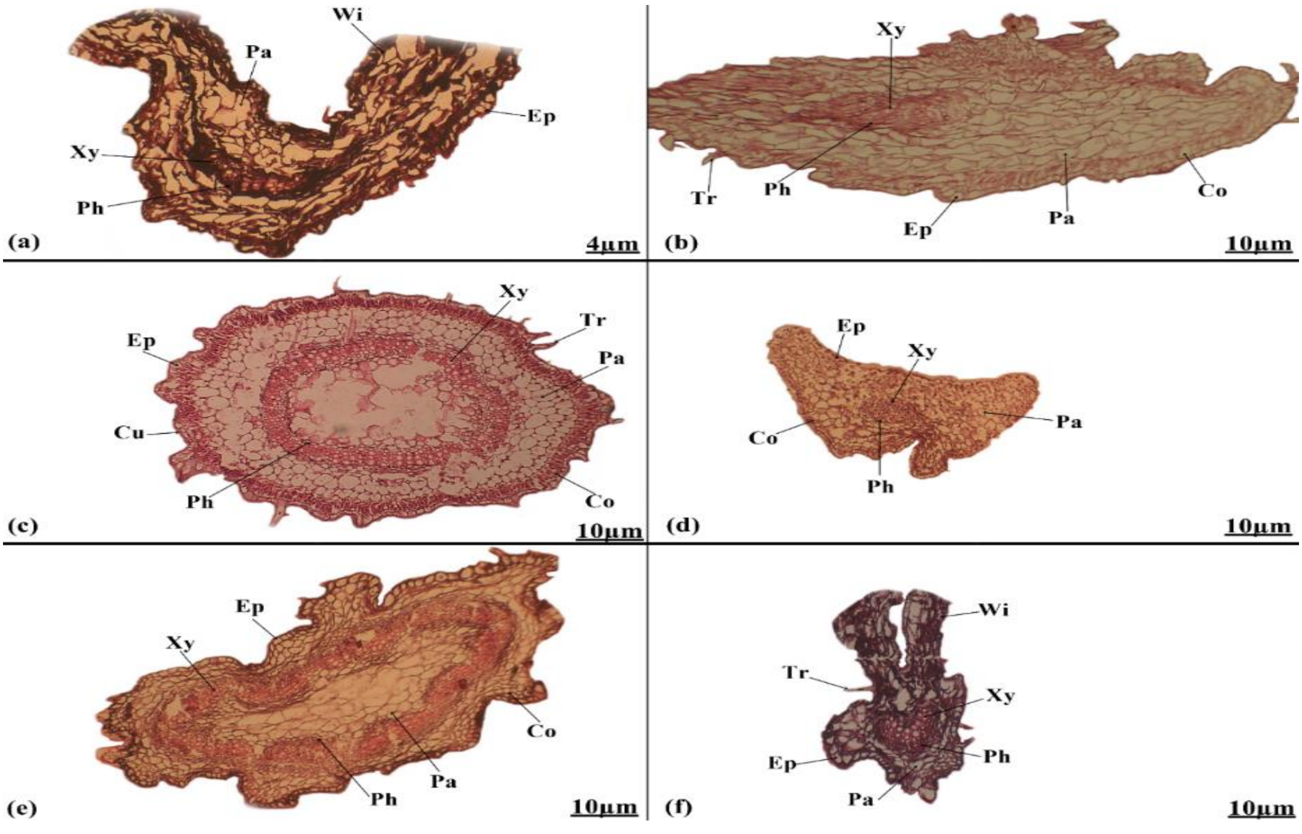

Fig. 10. Petiole anatomy of (a) *Heliotropium bacciferum* (b) *Heliotropium campanula* (c) *Heliotropium crispum* (d) *Heliotropium curassavicum* (e) *Lappula* spp (f) *Onosma limitanea* var. *limitanea* (Cu: cuticle, Ep: Epidermis, Pa: parenchyma, Co: collenchyma, Ch: chlorenchyma, Sc: sclerenchyma, Xy: xylem, Ph: Phloem, Tr: trichome, Wi: wing).

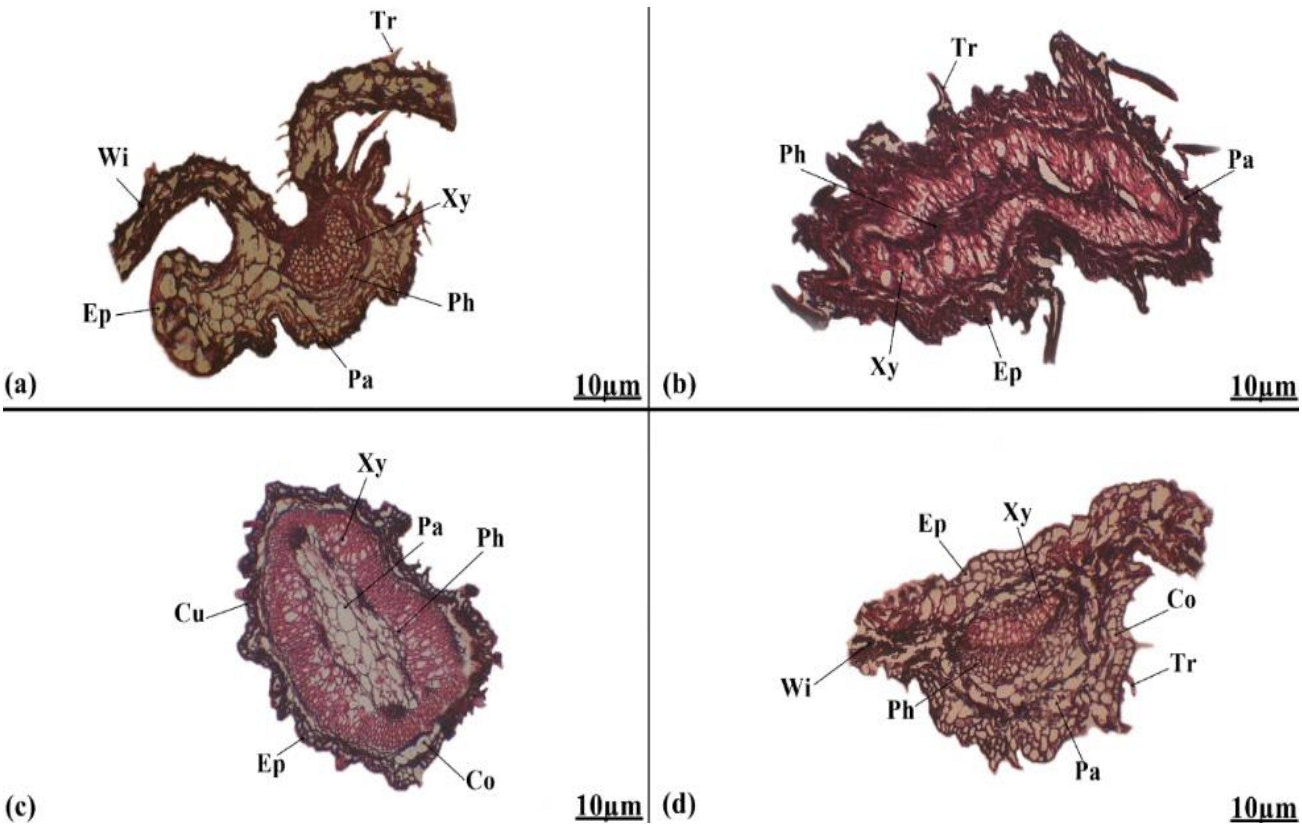

Fig. 11. Petiole anatomy of (a) *Onosma limitanea* var. *major* (b) *Paracaryum intermedium* var. *intermedium* (c) *Rochelia sessiliflora* (d) *Trichodesma indicum* (Cu: cuticle, Ep: Epidermis, Pa: parenchyma, Co: collenchyma, Ch: chlorenchyma, Sc: sclerenchyma, Xy: xylem, Ph: Phloem, Tr: trichome, Wi: wing).

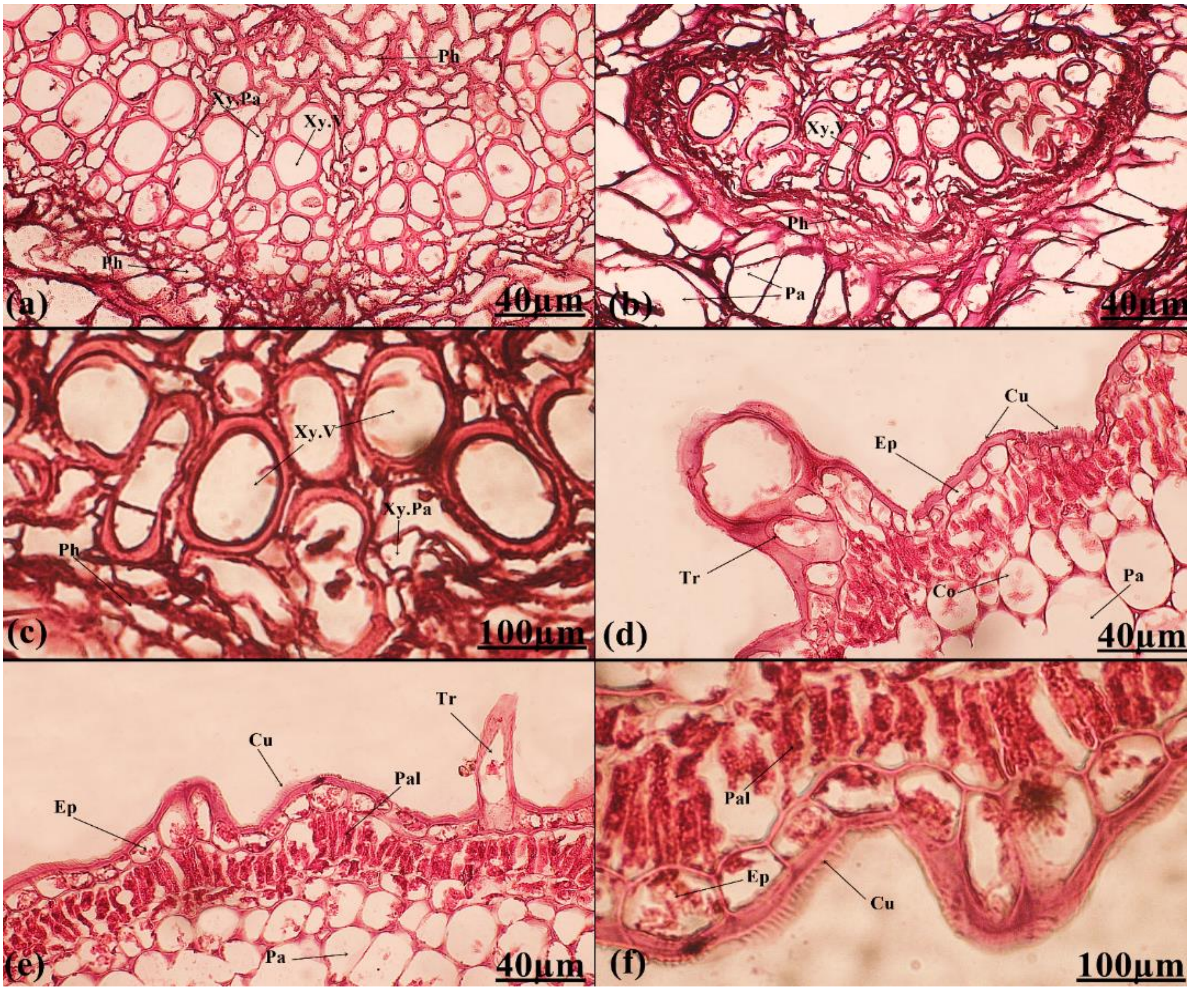

Fig. 12. Magnified photomicrographs of petiole cross-section of (a) *Alkanna tinctoria* subsp. *Tinctoria* (b, c) *Coccinia mucronanthera* (d, e, f) *H. crispum* (Cu: cuticle, Ep: Epidermis, Pa: parenchyma, Co: collenchyma, Ch: chlorenchyma, Sc: sclerenchyma, Xy: xylem, Ph: Phloem, Tr: trichome, Pal: palisad).

Discussion

Palynology is one of the important fields of plant research. It has made significant contributions by offering helpful data for phylogenetic analysis (Perveen, 2000). In systematics, all pollen characteristics are essential. Generally, many pollen characteristics, including the aperture type, exine sculpturing, size, shape, polarity, and symmetry are used to categorize pollen grains. Understanding linkages across tribes and genera based on pollen features can also be done by looking at the patterns of pollen grain evolution (Mazari & Liu, 2019).

The palynological characteristics of the Boraginaceae are heterogeneous (Umber *et al*., 2022), and it is a eurypalynous family; many different Boraginaceous species were previously identified by this aspect (Díez & Valdés, 1991). For the identification of morphotypes, palyno-anatomical data is now frequently used. In numerous recent pollen morphological and petiole anatomical studies, it has been demonstrated as a useful tool for the delimitation of species. These characteristics have been used to ze many species in Boraginaceae (Teke & Binzet, 2017). For Boraginaceae taxa, the existence of pollen grains that are both heterocolpate and isocolpate is a distinguishing palynological characteristic. The Boraginaceae's heterocolpate pollen grains are often dumbbell or rectangular, either with or without equator-based constriction (Mazari *et al*., 2018).

Heliotropium pollen grains were examined by Perveen (2000), Scheel *et al.* (1996), Gasparino *et al*., (2014) and Kamel *et al*., (2018). Kasem (2015) and Yousaf *et al*., (2022) used the palynological and anatomical data using LM to discriminate the *Heliotropium* species. Mazari *et al*., (2018) distinguished the pollen of *Heliotropium* according to the presence or absence of constriction at the equatorial region. In this study, 4 *Heliotropium* species have been distinguished based on the differences in both palyno-anatomical features. The amphicribral vascular bundles are noted singly in *H. bacciferum*. Other significantly different anatomical traits are petiole outline and collenchyma shapes. *H. curassavicum* and *H. bacciferum* are sulcate in outline. *H. campanula* is flat, while *H. crispum* is an oval-shaped outline. The exine sculpturing, apertures number, and Amb are found to be non-significant for the distinction of *Heliotropium* species. All species are observed with psilate exine, amb ptychotreme, hexotricolporate type apertures. The same was reported by Landi *et al*., (2022) that *Heliotropium* L., and *Myriopus*, the two Heliotropiaceae species, did not exhibit morphological variety in their pollen grains concerning the kind and quantity of their apertures or sculpturing of exine.

However, among the Heliotropiaceae taxa, differences in pollen grains' exine surface and a number of apertures were found useful for the taxonomic identification. Similar results were reported by Landi *et al*., (2022), that Boraginaceous species displayed differences in the size, shape, type, and number of apertures (heterocolpate, 3-porate, 3-colporate, and3-colpate) in the pollen characters, along with their exine ornamentation (rugulate, echinate, psilate, reticulate, and microechinate-verrucate), allow the *Heliotropium* species to be identified. Smaller diameter values, aperture, and exine ornamentation distinguish the Heliotropiaceae species. Currently, the polarity and shape of the pollen proved to be important taxonomic markers for the studied *Heliotropium* species. Pollen in *H. bacciferum* is heteropolar prolate, whereas in *H. campanula* oblate spheroidal and *H. crispum* suboblate.

It is challenging to research the systematics of the unique genus *Onosma* (Koyuncu *et al*., 2013). The two studied *Onosma* varieties of the same species *O. limitanea*, differ clearly in palyno-anatomical traits. The variety *limitanea* had isopolar, gemmate and peritreme pollen with angular epidermis, round xylem vessel, and amphicribral vascular bundles arrangement. The variety *major* is heteropolar, scabrate, goniotreme, angular to isodiametric epidermis, round to angular xylem vessel, and collateral closed vascular bundles. The distinct vascular bundles proved to be highly useful in taxonomic studies (Elkiran, 2023). The pollen were trisyncolporate in the variety *major*. Teke & Binzet (2017) reported that pollen of *O. discedens* were spheroidal while *O. nana* was subprolate. As demonstrated by the investigations on the genus Onosma, the information collected from palynological studies is sufficient to identify species. Koyuncu *et al*., (2013) identified a new *Onosma* species *Onosma atila-ocakii.* The morphological identity of the new species was difficult from the closely related species *O. roussaei* and *O. aucheriana*. However, the distinct palynological features such as heteropolar, syncolporate, subprolate, smallest pollen, and granulate-scabrate exine separated this new species.

Maggi *et al*., (2008) reported 5 *Onosma* species as tiny, 3-syncolporate, subprolate, heteropolar, with microechinate tectums, round to rounded triangular polar shapes, and ovate equatorial contours. They concluded that the genus *Onosma* was highly homogenous, there were no taxonomic significant differences seen in the micromorphology of pollen grains across the *Onosma* taxa studied, except pollen size. In contrast, substantial differences in the characteristics of pollen were reported by Perveen (2000).

The *Lappula* and *Rochelia* belong to the tribe Eritrichieae, whereas *Paracaryum* is a member of Cynoglosseae. The two tribes were studied by Díez & Valdés (1991). Currently, the three Lappula taxa were studied are *Lappula* spp., L. *spinocarpos*, and *L. spinocarpos* subsp. *ceratophora.* Although the classification of the genus is difficult, the palynological examination showed clear differences among them. This knowledge will assist in the taxonomic placement and separation of species of the genus *Lappula*. *L. spinocarpos* spp., is isopolar, whereas the *subsp. ceratophora* is heteropolar. Other differences are that *L. spinocarpos* is tricolporate, scabrate, and peritreme Amb, while *L. spinocarpos* subsp. *ceratophora* is hexotricolporate, psilate, ptychotreme Amb. The one unidentified *Lappula* sp., was quite similar to *subsp. ceratophora,* but the variations occurred in the polar view. Triangular obtuse convex polar view was observed in the *Lappula sp,* but *subsp. ceratophora* appeared to have circular a polar view. The *Rochelia* species *R. disperma* and *R. sessiliflora* were isopolar, psilate, operculate, and ptychotreme Amb, with non-perforated exine. *R. disperma* is tricolporate, isocolpate, suboblate in contrast *R. sessiliflora* was hexotricolporate, heterocolpate, porocolpate, subprolate. Both species differed in the polar and equatorial views. In the former species, the polar and equatorial views were triangular obtuse convex and rectangular obtuse concave, while the latter, quinquangular obtuse convex and elliptic truncate. In contrast, Díez & Valdés (1991) reported that Eritrichieae taxa cannot be separated on the pollen shape and apertures, the distinction can only be carried up to the generic level.

The members of the Cynoglosseae tribe have been separated based on their micromorphological characteristics (Attar *et al*., 2019). The varieties of *Paracaryum* – var. *intermedium,* var. *calathicarpum* and var. *intermedium* shared all the palynological traits besides the pollen shape. Similar results were documented by Díez & Valdés (1991). The petiole of *P. intermedium* var. *intermedium* is flat in shape, with unicellular trichomes. The cuticle is undulated. The vascular bundles are closed collaterally. The rearrangements of Paracaryum and Cynoglossum species were done utilizing pollen traits (Ovchinnikova *et al*., 2021). Cynoglosseae cannot be separated on pollen characteristics due to the similarities in shape and apertural system observed in some genera, including certain species (Díez & Valdés, 1991).

Furthermore, one of the accepted methods in palynology is numerical analysis. In contrast to classical taxonomy, Binzet *et al*., (2018) emphasized the value of numerical taxonomy and recommended using it for taxa with comparable morphologies (like *Onosma*). They highlihgted that the best method for determining the identities and morphological correlations among the genus *Onosma* was through quantitative taxonomy. Several techniques are used in numerical analysis, but PCA, and dendrogram are the most popular. PCA evaluates the best qualities for taxonomy and permits multicollinear statistics to identify the traits. Heteropolar, and trisynocolporate, are more advanced traits (Mazari & Liu, 2019; Teke & Binzet, 2017). The multivariate analysis of equatorial diameter, exine thickness, polar axis, mesocolpium, colpus polar and equatorial length, and width via PCA, phylogeny, correlation, and boxplot. The variations in the data separated the taxa and varieties.

PCA is the most popular quantitative technique for identifying pollens from different plant species and investigating the most important pollen characteristic with the highest proportion of variability. PCA is typically represented graphically as two-dimensional or occasionally three-dimensional axes-based plans of sample data. Using parameters such as mesocolpium, polar axis, equatorial diameter, exine thickness, colpus length, and width, our study uses PCA to examine pollen variability in 16 different plant species. Among the angiosperms the smallest pollen grains ca. 5 × 2 mm are found in the Boraginaceae, *Myosotis*, and *Trigonotis*. The largest pollen grains 55 × 40 mm were observed in *Anchusa* in the tube, Boragineae (Attar *et al*., 2019). The variations in the pollen size significantly differentiated the examined species. *A. tinctoria subsp. tinctoria* had the largest pollen, 47.5 µm, and 43.85µm. Previously, pollen size was the most significant feature for separating *Onosma* species (Maggi *et al*., 2008). The boxplot determined the outliers in the data and the deviation. The evolutionary association among the quantitative variables is captured in the phylogenetic tree. The positive and negative association among the data set is determined via a correlation plot. The detailed multivariate numerical analysis is discussed in the results section. Taxonomic key based on the palynological traits (Table 6) efficiently delimited the Boraginaceous taxa up to the species level. The variations in the polarity, number of apertures, arrangement of apertures, Amb, polar and equatorial views, sculpturing, and pollen shape proved to be fair enough for the taxonomic distinction of the examined species.

Table 6. Qualitative traits extracted from the quantitative data of LM of pollen.

Link Character	Leads	Characters	Taxa/ Go to link character
1	+	Pollen heteropolar	2
	-	Pollen isopolar	6
2	+	Amb peritreme	*B. arvensis*
	-	Amb, not peritreme	3
3	+	Amb Goniotreme	*O. limitanea* var. *major*
	-	Amb Ptychotreme	4
4	+	Aperture membrane non granular operculate	*H. bacciferum*
	-	Aperture membrane granular operculate	5
5	+	Polar view circular	*L. spinocarpos* subsp. *ceratophora*
	-	Polar view triangular obtuse convex	*Lappula* spp.
6	+	Exine sculpturing foveolate	*C. mucronanthera*
	-	Exine sculpturing non foveolate	7
7	+	Gemmate exine	*O. limitanea* var. *limitanea*
	-	Exine not gemmate	8
8	+	Scabrate ornamentation of exine	9
	-	Pislate ornamentation of exine	10
9	+	Oblate spheroidal	*L. spinocarpos*
	-	Prolate spheroidal	*T. indicum*
10	+	Tricolporate	11
	-	Hexotricolporate	12
11	+	Circular polar view	*A. tinctoria* subsp. *tinctoria*
	-	Triangular obtuse convex polar view	*R. disperma*
12	+	Perforated exine surface	*H. campanula*
	-	Non-perforated exine surface	13
13	+	Oblate spheroidal	*C. lanceolatum*
	-	Shape class not oblate spheroidal	14
14	+	Amb ptychotreme	15
	-	Amb peritreme	16
15	+	Suboblate	*H. crispum*
	-	Subprolate	*R. sessiliflora*
16	+	Prolate spheroidal	*P. intermedium* var. *calathicarpum*
	-	Prolate	*P. intermedium* var. *intermedium*

Conclusions

The morphological knowledge is not enough to identify differences among the closely related species and varieties. The present Palyno-anatomical data provides new information for the characterization of Boraginaceous taxa. Characters such as number of apertures, sculpturing, aperture arrangement, membrane of aperture, shape class, polar and equatorial view, diameter, colpus length and width, petiole outline, shapes of cells, number of layers, vascular bundles type, trichome type are found significant for the separation of Boraginaceous taxa. However, there was no significant difference in the shape of the phloem, number of vascular bundles, symmetry, or unity of the pollen. The TEM of pollen will add to future taxonomic examination of these Boraginaceous species.

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