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LEAF, STEM BARK AND FRUIT ANATOMY OF *ZANTHOXYLUM ARMATUM* DC. (RUTACEAE)

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Abstract

Zanthoxylum armatum DC. (Rutaceae) is an important medicinal plant. The present study deals with anatomical exploration of the leaf, stem bark and fruit of this plant. Leaf of *Z. armatum* is bifacial, compound and punctate with glabrous surfaces having a single layer of epidermis and palisade mesophyll. The leaf has a Palisade ratio ranged from 6.00 to 9.00 (8.2±0.32). Vein islets and vein termination number were 14-21 (16.8±0.64) and 17-21 (19.1±0.43) per mm^2 respectively. The vein-islets were quite distinct with squaresh, elongated, polygonal or irregular in shape bounding many forked and unforked vascular branches. Adaxial surface of *Z. armatum* leaf midrib was planoconvex while the abaxial surface was semicircular in appearance. The diagnostic feature of the leaf was the complete absence of any kind of trichomes or any other appendages. The leaf showed prominent oil cavities. Nine types of stomata with different frequencies and other dimensions were observed. Brachparatetracytic stomata was the most frequent stoma (80%) followed by actinostephanocytic (40%) and then straucytic and brachyparacytic (30%) each. Hemiparacytic and stomatal cluster were the rarely occurring stomata (10% each) present on the lower epidermis of the leaf. Stomatal cluster, which is considered to be a special leaf epidermal feature and reported only in few genera of vascular plants, was also recorded in this plant. Bark and fruit anatomy of *Z. armatum* showed different tissue arrangement. The seed was non endospermic and contains an elongated embryo. The present study will be helpful in the phylogeny and taxonomic description of this important medicinal plant.

Introduction

Zanthoxylum armatum DC. (Rutaceae) is a small aromatic tree or shrub with winged petiolate prickly imparipinnate compound leaves. Flowers are minute and polygamous. Male flowers has 6-8 stamens with rudimentary ovary. Female flowers has 1-3 carpels. Fruit is small drupe with red color, splitting into two when ripe. Seeds are rounded and shining black (Hassan-ud-Din & Ghazanfar, 1980). The plant grows in shady or semi shady habitat at altitude starting from about 800m up to 1500m and reported from Malakand, Swat, Dir, Hazara, Buner, Muree hills and Rawalpindi in Pakistan (Shinwari *et al*., 2006). It is also known as prickly ash (English) Dambrary, Tamur (Urdu) and Dambara (Pashtu). It is an important medicinal plant extensively used locally for curing Pneumonia, tick infestation and gum diseases (Iqbal & Hamayun, 2005). Fruit is used for toothache, dyspepsia, as a carminative and for stomachache. Seeds are used as condiment and flavoring agent (Arshad & Ahmad, 2005; Abbasi *et al*., 2010;) Recently the leaves and fruits were investigated for various pharmacological activities and has showed good results including cytotoxic, phytotoxic, antipyretic activities and rationalise its local uses (Barkatullah *et al*., 2011).

Anatomy describes the internal structure of plants and is considered as a source of correct identification of taxa. Anatomy centres on the spatial arrangement of the dermal, ground, and vascular tissue systems (Nancy & Dengler, 2002). Similarly foliar epidermal microscopic features like shape of epidermal cell, type of stomata, presence or absence of pubescence and cell wall thickness are also considered as useful tools for correct taxa identification and their phylogenetic relationship with other taxa (Stace, 1965; Babalola & Victoria, 2009). Now a days plant taxonomists have given much attention towards leaf epidermal and anatomical studies to resolve the taxonomic problems (Taia, 2005). Keeping in view the significance of anatomical and foliar epidermal study for taxonomy and pharmacognosy, anatomy of leaf, stem bark and fruit of *Z. armatum* has been carried out in the present study.

Materials and Methods

Plant collection: Fresh leaves, stem bark and fruit of *Zanthoxylum armatum* were collected from the hills of Batkhela District Malakand.

Anatomy: Large number of thin transverse sections were prepared form leaf, stem bark and fruit with the help of sharp razor. From these fine sections were selected, stained with phloroglucinol and hydrochloric acid, mounted in glycerin on glass slide and then studied under Nikon microscope fitted with camera (Chaffey, 2001).

Leaf surface study: The following cytomicroscopical features of the leaf were carried out:

a. Stomatal Number and Stomatal Index,
b. Vein islets and vein termination number,
c. Palisade cell ratio.

a. Stomatal number and stomatal index: Stomatal number or stomatal density is the number of stomata per square millimetre (mm) of epidermis on each surface of a leaf. Stomatal Index is the percentage of stomata to the total number of epidermal cells in the leaf (Evans, 2002; Bozoglu & Karayel, 2006). Epidermis from both sides of fresh leaf was peeled off with the help of a pair of forceps. The peel was mounted in dilute glycerin and observed under microscope for numerical data i.e. number of epidermal cells and number of stomata per square mm. From this data stomatal index was calculated using the standard formula (Chaudhary & Imran, 1997; Evans, 2002).

$$I = \frac{S}{S + E} \times 100$$

where as
I= Stomatal Index
S= Number of stomata per unit area
E= No. of epidermal cells per unit area

Other features like size of epidermal cell and stomata were also calculated using calibrated Nikon microscope. Similarly presence or absence of stomata, type of stomata and ratio of open and closed stomata were also recorded (Hameed *et al*., 2008).

b. Vein islets and vein termination number: The tiny area of the photosynthetic tissue surrounded by ultimate divisions of veins in leaf is known as vein islet and their number per square mm of the leaf is called vein-islet number. Vein termination is the ultimate free termination of vein-let and their number per square mm is known as vein termination number (Evans, 2002). Small pieces from the leaf of *Z. armatum* were taken midway from margin to midrib and cleared by boiling in 200% Choral hydrate solution in a test tube placed on boiling water bath, in order to make free it from coloration (Choudhary & Kamal, 2004). These were then observed under microscope by focusing one millimetre area. All the vein islets including inside and on the boundary of the square area were counted, whereas vein terminations were counted inside the area. To get exact and standard values, 10 readings for both parameters were taken from different pieces (Evans, 2002).

c. Palisade cell-ratio: The average number of Palisade cells present beneath each upper epidermal cell is called Palisade cell ratio (Evans, 2002). It is an important pharmacognostic parameter for leafy drugs. Small pieces of the leaf were taken and cleared by boiling in 200% Chloral Hydrate solution (Shruthi *et al*., 2010). The cleared pieces were mounted and examined under microscope. A number of groups of each of four upper epidermal cells were first focused. Then by minor rotation of the fine adjustment, the under lying palisade cells were focused within the area of four epidermal cells. Palisade ratio was then obtained by dividing the number of palisade cells by 4. Ten readings were taken from different pieces in order to obtain accurate values (Evans, 2002).

Statistical analysis: All the numerical data relating to stomata and epidermis was statistically analyses following Chaudhary & Kamal (2004).

Results

Microscopic evaluation including leaf anatomy, leaf surface values, stomatal study, stem bark anatomy and fruit anatomy of *Z. armatum* was carried out in the present study.

Leaf anatomy: T.S of leaf lamina of *Z. armatum* showed upper epidermis followed by palisade mesophyll, spongy mesophyle and then lower epidermis. Collateral Vascular bundle was also present (Fig. 1). Upper epidermis was non stomatiferous and covered with thin cuticle, composed of rectangular shaped compactly arranged cells. These cells were 5.9-10.35µ (8.33±1 µ) in length and 2.3-4.6 µ (3.73±0.73 µ) in width. Palisade tissue in the mesophyll region was composed of a single layered compactly arranged cylindrical shaped cells. These cells were 10.35-14.38µ (12.19±1.52 µ) in length and 1.15-2.3 µ (1.95±0.555µ) in width. The spongy mesophyll cells (some of which were idioblast i.e., containing Ca-oxalate crystals) were rounded to somewhat elongated in shape and were arranged loosely with large intercellular spaces. These cells were 3.45-5.18µ (3.85±0.609 µ) in length and 1.7-2.3 µ (2.30±0.271µ) in width. These cells contained schizogenous and lysischizogenous cavities. Lower epidermis was stomatiferous with varied shaped (i.e., rectangular, elongated or irregular shaped) cells. The length of these cells was 4.60-6.33 µ (5.69 ±0.571 µ) in length and 2.01-2.88 µ (2.31 ±0.227 µ) in width.

Leaf midrib anatomy: Adaxial surface of *Z. armatum* leaf midrib was planoconvex while the abaxial surface was semicircular in appearance. There was complete absence of any kind of appendage on it. T.S of midrib showed upper epidermis, hypodermis, vascular bundle, cortex and lower epidermis (Fig. 2). Upper epidermal was a single layered with oval to rectangular shaped cells, 1.72-2.30 µ (2.05±0.275µ) in length and 1.5-2.0 µ (1.93±0.379µ) in width. The upper epidermis was covered with cuticle. Epidermis was followed beneath by 3-5 layered hypodermis consisting of thick walled collenchymatous cells, 1.73- 2.88 µ (2.16±0.35 µ) in diameter. Next was many layered cortical region of round thin walled parenchymatous cells with diameter of 2.88-5.18 µ (with a mean of 4.07±0.75 µ). Shizogenous oil cavities were present in this region. The vascular bundle was Arc-shaped in which xylem was adaxial in position followed by phloem in the abaxial position. Xylem vessel appeared in radial rows ranged from 2.07- 3.45 µ (2.62±0.618 µ) in diameter. Phloem was characterized by rounded cells with almost the same dimensions as that of xylum. Vascular bundle was surrounded by iodioblast cells having calcium oxalate crystals which were either present solitary or either aggregated in clusters. The lower epidermis of the midrib region was composed of rectangular cells, 2.30-4.03 µ (3.34±0.890 µ) in size.

Leaf surface characters: Various quantitative microscopic features of leaf surface such as palisade ratio, vein islets number and vein termination number of *Z. armatum* were also carried out in the present study (Table 1). The leaf has a Palisade ratio from 6.00 to 9.00 with average of 8.2±0.32 (Fig. 3a). Vein iselet and vein termination number were 14-21 (16.8±0.64) and 17 - 21 (19.1±0.43) per mm^2 respectively. The vein-islets were quite distinct and were squaresh, elongated, polygonal or irregular in shape, provided with many forked and unforked vascular branches (Fig. 3b). Leaf was hypostomatic as the stomata were present only on the lower epidermis (Fig. 4). Stomatal number and stomatal Index were worked out along with its statistical parameter i.e., variance, co-efficient of variance, standard deviation and standard error were worked out (Table 1). The stomatal index was 10.28 to 13.58(12.32±0.26) while the stomatal density was 175 to 210 (196.1±3.07).

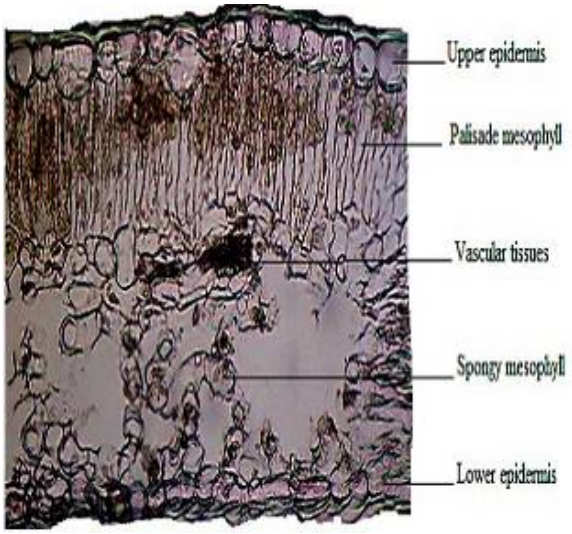

Fig. 1. T. S. of *Zanthoxylum armatum* leaf.

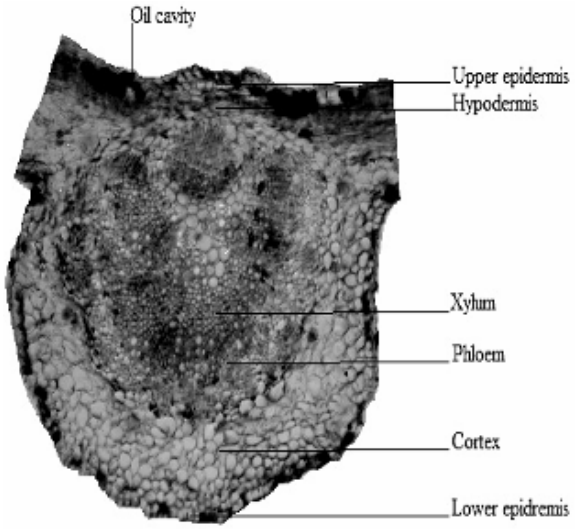

Fig. 2. T. S. of *Zanthoxylum armatum* leaf midrib.

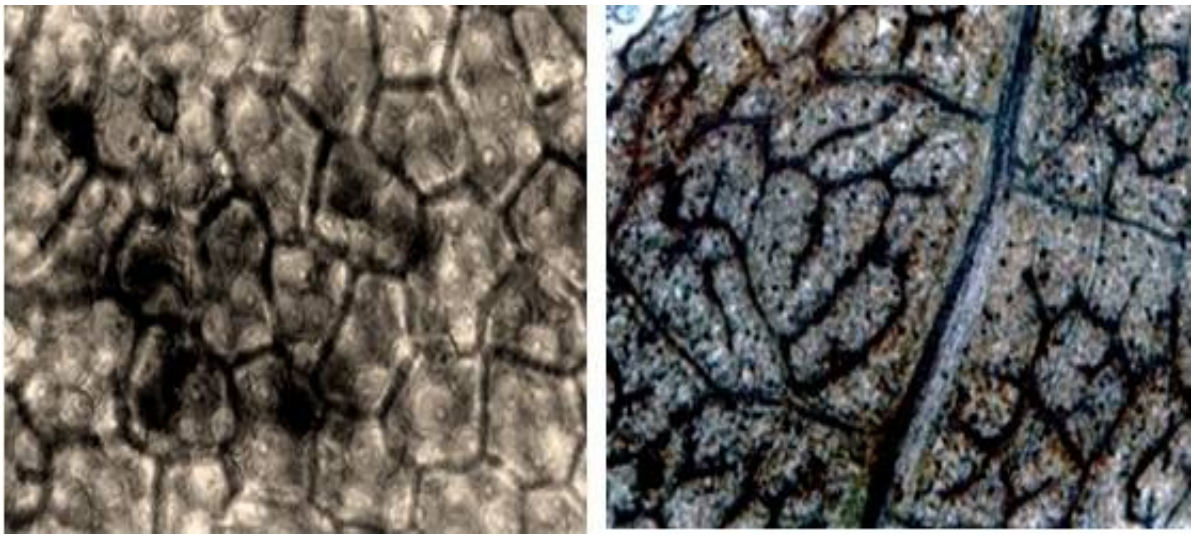

Fig. 3. a. Palisade cells arrangement under epidermal cells; b. veins arrangement in *Zanthoxylum armatum* leaf.

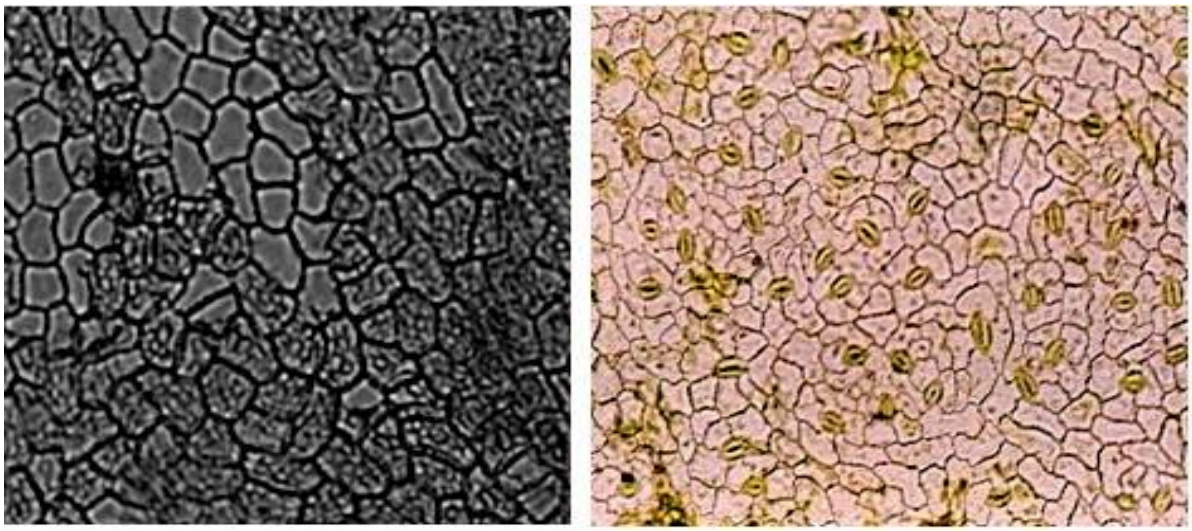

Fig. 4. a. Upper and lower epidermis of *Zanthoxylum armatum* leaf.

Table 1. Leaf constant values of *Zanthoxylum armatum*.

S. No.	Parameter	Range	Average
1.	Palisade ratio	6.00 to 7.75 to 9.00	8.25 ± 0.32
2.	Vein islets number	14 to 18 to 21	16.8 ± 0.64
3.	Vein termination number	11 to 13 to 15	13.1 ± 0.43
4.	Stomatal number	175 to 197 to 210	196.1 ± 3.07
5.	Stomatal Index	10.28 to 12.33 to 13.58	12.32 ± 0.26
6.	Variance	94.22	
7.	Co-efficient	4.95	
8.	Standard error	3.07	
9.	Standard deviation	9.71	

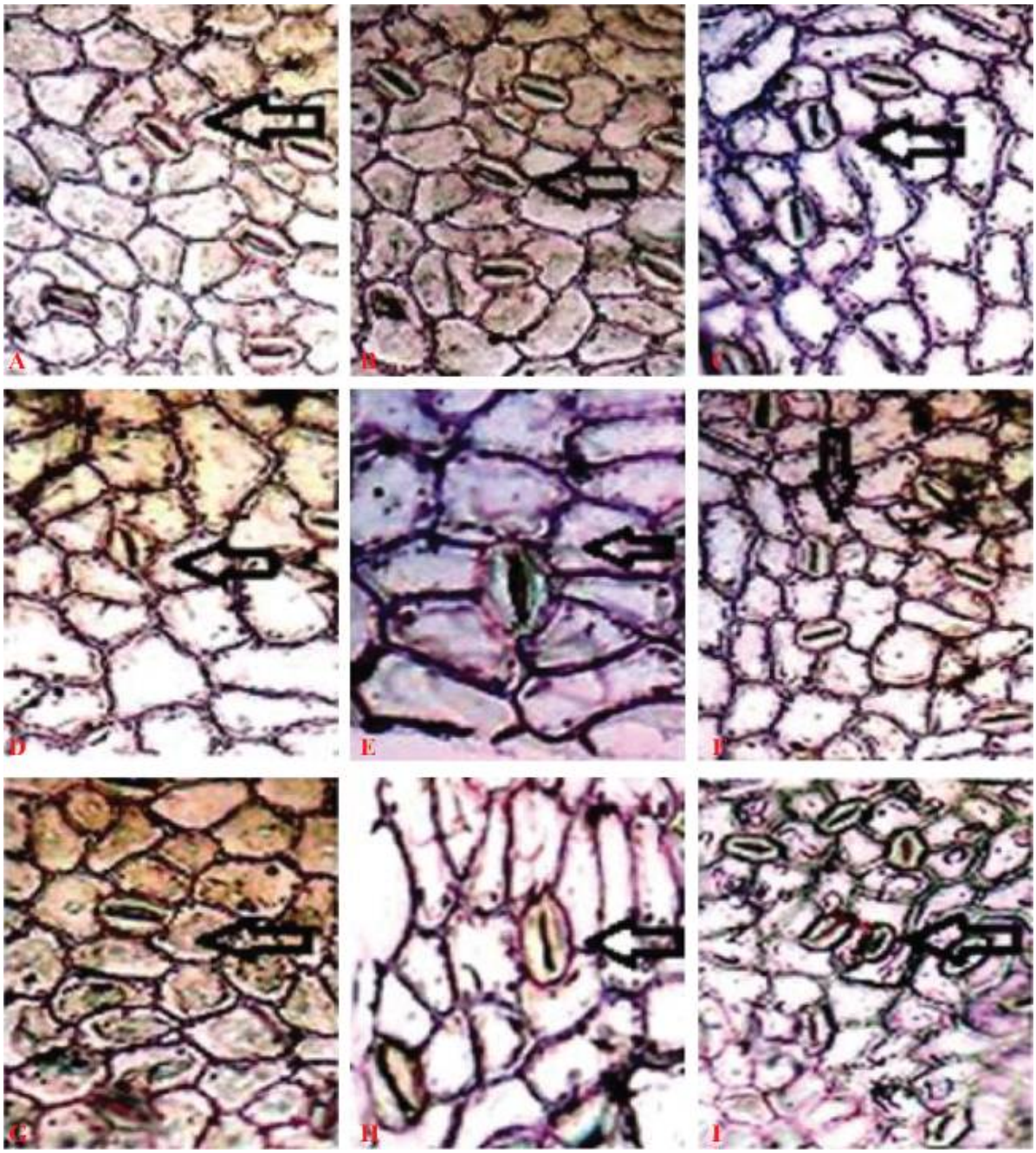

Fig. 5. Various types of stomata in the lower epidermis of *Zanthoxylum armatum* leaf, a. Anomocytic; b. Actinocytic; c. Actinostephanocytic; d. Staurocytic; e. Laterocyclocytic; f. Brachyparacytic; g. Brachyparatetracytic; h. Hemiparacytic; i. Stomatal cluster.

Table 2. Stomatal diversity with frequency and quantitative features in the lower epidermis of *Zanthoxylum armatum*.

S. No.	Type of stomata	Frequency	Length (µ)	Breadth (µ)	Opening (µ)
1.	Anomocytic	20	25.08 ± 0.001	12.3 ± 0.001	0 ± 0
2.	Actinocytic	20	25 ± 0.000	11.7 ± 0.000	1 ± 0.001
3.	Actinostephanocytic	40	24.7 ± 0.001	12.2 ± 0.001	1 ± 0.001
4.	Staurocytic	30	24.78 ± 0.001	12.2 ± 0.001	1 ± 0.001
5.	Laterocyclocytic	20	25.2 ± 0.003	11.9 ± 0.001	1 ± 0.001
6.	Brachyparacytic	30	26 ± 0.001	12.3 ± 0.001	0 ± 0
7.	Brachyparatetracytic	80	25 ± 0.001	11.7 ± 0.001	0 ± 0
8.	Hemiparacytic	10	24.7 ± 0.00	12.5 ± 0.00	0 ± 0
9.	Stomatal cluster	10	24.78 ± 0.00	12.3 ± 0.00	1 ± 0

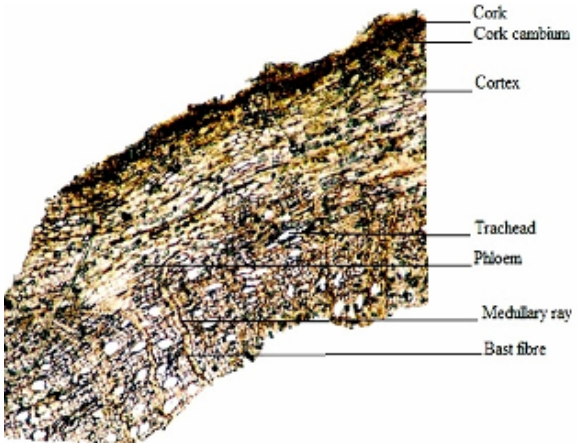

Fig. 6. T. S. of *Zanthoxylum armatum* stem bark.

Various types of stomata were identified (Table 2). These included Anomocytic, actinocytic, actinostephanocytic, staurocytic, brachyparacytic, brachyparatetracytic, laterocyclocytic and stomatal cluster (Fig. 5a-5h).

Brachparatetracytic stomata was the most frequent stoma (80%) followed by actinostephanocytic (40%) and then staurocytic and brachy paracytic (30%). Hemiparacytic and stomatal cluster were the rarely occurring stomata (10% each) (Table 2). In spite of great variation in the frequencies, no significant differences were observed in the length and breadth of these various types of stomata. The orientation of stomata was such that they were either parallel or at right angle to each other.

Bark anatomy: Bark anatomy of *Z. armatum* showed different tissues layers identified as cork (Outer phellem), cork cambium (middle phelogen), cortex (Pheloderm), medullary rays, phloem, bast fibers and tracheids (Fig. 6). Cork was composed of a few layers of closely packed brown color lignified thick walled rectangular or squared cell, 2.88-4.03 µ (3.39±0.571 µ) in length and 1.73-3.45 µ (2.01±0.729 µ) in breadth. Cork cambium was a continuous layer of small elongated rectangular thin walled parenchymatous cells, ranged from 3.45-5.75 µ (4.72±473 µ) in length and 1.15-2.3 µ (1.78±0.424µ) in breadth. The cortex was composed of closely packed large parenchymatous cells. The length of the cells ranged from 3.45 – 5.18 µ (4.54±0.63 µ) in length and 1.15- 2.88 µ (1.84±0.706 µ) µ in width. Most of these cells were idioblasts. Medullary rays were also present passing through the phloem and were 2 to 5 cells wide. The phloem consisted of intact and crushed phloem elements. The bast fibers and large treachery cells were also present.

Fruit anatomy: T.S of fruit showed two portion i.e., fruit wall and seed. Fruit wall is clearly distinguished into three layers i.e. epicarp, mesocarp and endocarp (Fig. 7). The outer most layer, epicarp is composed of closely arranged thick walled rectangular cells, 2.3-4.03µ (3.22±0.55 µ) in length and 0.75-1.13 µ (0.86±0.25 µ) in width. The middle layer, mesocarp is composed of irregular thin walled parenchymatous cells, 2.30-4.31 µ (3.31±0.70 µ) in length and 2.30-4.6 µ(3.74±0.82 µ) in width. This layer contained large schizogenous and lysoschizogenous oil cavities, about 30 µ in diameter. The inner layer of fruit wall (endocarp) was composed of two layers. The outer layer is composed of small isodiametric or rectangular, thin walled parenchymatous cells, 4.60-7.48µ (6.04±1.24 µ) in length and 2.30-3.00 µ (2.69±0.33 µ) in width. While the inner layer of mesocarp is composed of comparatively larger, thin walled rectangular cells, having a length of 8.05-13.8 µ (10.12±2.35 µ) and width of 3.45-5.18 µ (4.31±0.48 µ). There was a single, somewhat oval shaped seed in the fruit. T.S of seed showed an outer pigmented layer of testa followed by a layer of thin walled small cells. The seed was non endospermic and contained small elongated embryo.

Discussion

Study of different types of tissues and other microscopic techniques like linear measurements, determination of leaf constants and quantitative microscopy are indispensable in the initial identification of plants materials and for drug evaluation in pharmacognosy (Jarald & Jarald, 2007). Leaf epidermal studies are of immense significance in finding phylogeny and taxonomy of closely related species. Taxonomists have given prime importance to leaf epidermal features to resolve taxonomic conflicts (Taia, 2005). In the present study different features of leaf surface like palisade ratio, vein islets and vein termination number were evaluated for the purpose to set parameter for this important plants so as to use this information as reference for correct identification and authentication in future. Comparing this data with other workers like Bhagwat *et al*., (2008), Venkatesh *et al*., (2008), Gupta *et al*., (2010) and Chidambaram & Aruna (2013) clearly indicated that these features showed variation in different species. Further these values are found in constant range for a particular species and is not affected by geographical variation or age (Shruthi *et al*., 2010); therefore these leaf surface features are considered diagnostic for characterization, standardisation and identification of particular species.

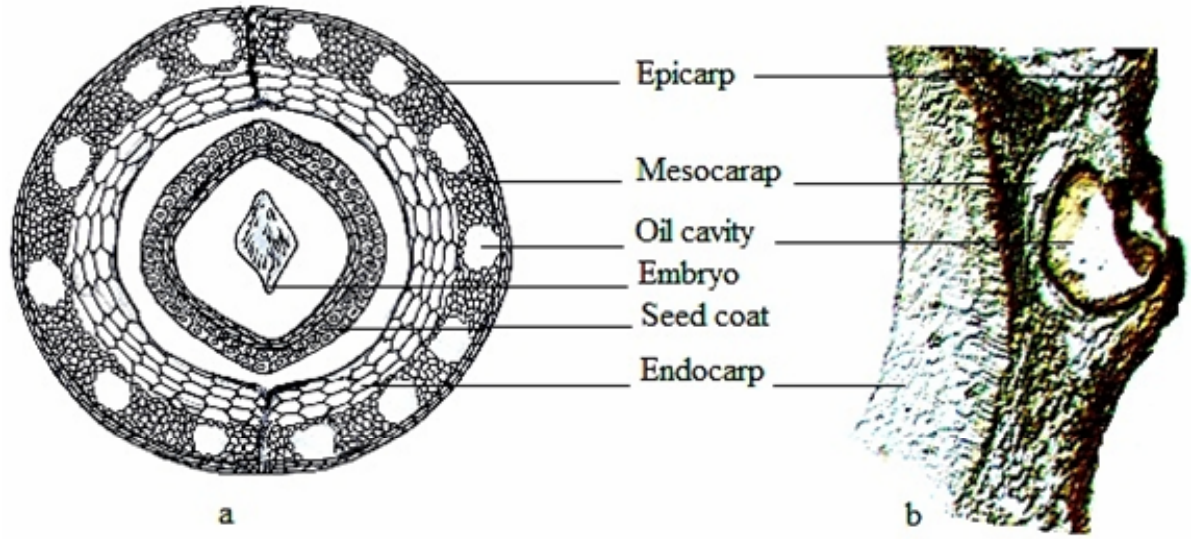

Fig. 7. *Zanthoxylum armatum* fruit. a. T. S of fruit; b. T. S. of fruit wall

Stomata mostly present on the leaf epidermis. On the basis of its occurrence leaf may be amphistomatic (Stomata present on both epidermises) or epistomatic (stomata present only on upper epidermis) or hypostomatic as (stomata only present on the lower epidermis) (Perveen *et al*., 2007). Stomatal arrangement and types are considered best taxonomic criteria and provide efficient basis for exploring phylogenetic relationship in taxonomic hierarchy (Hameed *et al*., 2008). Some workers like Sen & Hennipman (1981) had the idea that they may not be so an effective tool in taxonomy because of their inconsistent arrangement in epidermises, however stomatal values like stomatal number and stomatal index are of great value in the evaluation of leaf origin crude drugs (Evans, 2002). The leaf of *Z. armatum* was hypostomatic. The occurrence of stomata on lower epidermis is regarded to be a xerophytic trait (Esau, 1977). Various features like the stomatal size, stomatal index and stomatal pore size are of great significance in differentiating the taxa at specific and interspecific level (Nabin *et al*., 2000). Ferris *et al*., (2002) also reported the usefulness of co-efficient of variance, stomatal density, stomatal index, epidermal cell area and number of epidermal cells per leaf of poplar plant. Similarly Hameed *et al*., (2008) carried out statistical evaluation, including mean, standard deviation, variance and coefficient of variance of stomata in the epidermises of some members of family polygonaceae. In the present study stomatal density and stomatal index along with statistical parameter were investigated for the purpose of pharmacognostic evaluation.

Metacalfe & Chalk (1950) reported that stomatal size and stomatal density are inversely proportional to each other. A Similar situation was found in the present study, as stomatal size was small and its density was quite high.

Nine different types of stomata were identified showing great diversity in the arrangement of epidermal cells around the guard cells. Stoamtl types were (i) anomocytic characterized by four or more undifferentiated cells (Metacalfe & Chalk, 1950), (ii) actinocytic, having radial elongation of subsidiary cells (Wilkinson, 1974), (iii) actinostephanocytic having slight radial elongations of some or all cells (Carpenter, 2005) and (iv) Staurocytic, stomata surrounded by four or more cells, having differently oriented cell wall to the stomatal pore (Van Cotthem, 1970) the later three are consider subtypes of stephanocytic type stomata. (v) Brachyparacytic, having two lateral epidermal cell, oriented parallel to guard cell (Carpenter, 2005). (vi) Brachyparatetracytic having four subsidiary cells, of which two were polar and two lateral (Chengqi *et al*., 2007) (vii) Hemiparacytic, characterized by a single parallel cell. (Carpenter, 2005). (v) to (vii) were subtypes of Paracytic stomata. (viii) laterocyclocytic in which subsidiary cells were arranged in the form of a circle around the stoma (Carpenter, 2005) (ix) Stomatal cluster, where two stomata are arranged side by side without any common subsidiary cell in between them (Tang *et al*., 2002). Stomatal cluster appeared to be a special leaf epidermal feature reported only in very limited number of genera of vascular plants (Tang *et al*., 2002). Chengqi *et al*., (2007) reported similar pattern of stomatal apparatus in *Camellia henryana* and *C. tsingpienensis*. As stomatal cluster was very rare feature; therefore further work is required to consider it as a taxonomic tool.

Adaptation skills of plants depend on stomatal arrangement on the epidermises, as transpiration and photosynthesis are closely related to Stomata. These are also useful in taxonomic categorization and detection of future clues for observing environmental factors. Micro and macro elements in plants are also closely related to stomatal density (Nabin *et al*., 2000; Brownlee, 2001). Very little work has been done on the stomatal study of family Rutaceae. Ogunkunle & Oladele (1997) reported paracytic, hemiparacytic, brachy paracytic, brachy paratetracytic and anomocytic stomatal complexes with uniform size from abaxial epidermises of various *Citrus* species (Rutaceae) and also proved that in spite of high stomatal density, they have relatively low transpiration rate as compared to species with low stomatal density. Similar observations were also recorded in the present study, showing that these might be a future finger printing in exploring phylogenetic relation of family Rutaceae.

Conclusion

Z. armatum is important medicinal plant used locally for various ailments. This study will be helpful in the phylogenetic and taxonomic identification of this important plant. Also epidermal study and identification of various tissues in different parts will be fingerprints for the proper identification of crude drugs from this plant, which will provide basis for pharmacological preparations in future.

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