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MICROSCOPIC STUDIES ON *LENS CULINARIS* MEDIKUS SEEDS USING BRIGHT-FIELD MICROSCOPE AND POLARIZED LIGHT MICROSCOPE

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Abstract

Lens culinaris Medikus seeds, also known as *L. esculenta* Moench and lentils, belong to Leguminosae family. They are widely cultivated due to their high nutritional value. In the recent years, they have also gained popularity due to their dynamic therapeutic potential. However, before conducting research, it is essential to authenticate and standardize them. Botanical microscopy is the most convenient techniques for authenticating a plant material. Literature survey did not provide sufficient information on microscopic profile of lentil seeds. Therefore, the present study was conducted to obtain a detailed microscopic profile of lentil seeds and seed coat using bright-field microscope and polarized light microscope. The healthy seeds were processed for macroscopic and microscopic examination. The seeds were elliptical, reddish brown, smooth and shiny with long pale white raphe. Seed coat contained outer thin smooth cuticle followed by macrosclereids, osteosclereids and parenchymatic cells. It broke open to the seed's hilum, followed by tracheid-bar. The embryo, between the cotyledons, was cylindrical and vertically oblong. The thick conical radicle was directed towards the micropylar end at one end and plumule shoot apex at the other end. Powder microscopy revealed the presence of cylindrical, oval and elongated starch grains with a polarimark, fragments of seed coat and cotyledonary tissue. The present study is the first comprehensive microscopic study conducted on lentil seeds which may be used for identification as well as standardization of the plant material.

***Keywords*:** Botanical microscopy, lentils, *Lens culinaris*, standardization, authentication

Introduction

Medicinal plants, especially nutraceuticals, are gaining popularity due to their magnificent nutritional as well as therapeutic profile (Chauhan, Kumar, Kalam, and Ansari, 2013). Prior to their use for research or formulation, their authentication is essential. Herbal authentication refers to accurate identification of the plant material in terms of species, variety and plant part (Kumar and Lalitha, 2017).

Botanical microscopy, as introduced by Schleiden, has been a significant tool used by a pharmacognosist at an early stage of plant analysis (Upton, 2010). Observing and reporting of detailed anatomical structures, tissue arrangement, and illustrations of the medicinal part of the plant, in its whole as well as powdered form, is still being followed today for authentication and standardization of a plant material (Kumar and Lalitha, 2017; Upton, 2010). In addition to the structural characteristics, microscopy helps in identifying the botanical family and species, adulterating species, and quality of the plant part (Chu *et al*., 2009; Upton, 2010; Xia *et al*., 2008).

Seeds of *Lens culinaris* Medikus, a leguminous plant, belong to Papilionaceae sub-family and Leguminosae or Fabaceae family. They are also known as *L. esculenta* Moench and lentil seeds (Rastogi and Mehrotra, 1991). They are generally grown in tropical, temperate and subtropical regions with cool climate (Chu *et al*., 2009). India is also well known for its lentil cultivation (Kirtikar and Basu, 2003). The seeds are biconvex lens shaped and have a soft seed coat. They are edible and are characterized by high protein and carbohydrate content (Jood, Bishnoi, and Sharma, 1998; Satya, Kaushik, and Naik, 2010; Solanki, Kapoor, and Singh, 1999).

Off lately, extensive research has been conducted on lentil seeds due to their high nutritional and therapeutic value (Kāśyapa and Ayachit, 2002; Nene, 2006). However, insufficient information is available related to its microscopy. Preliminary microscopic studies on lentil seeds have been reported earlier, but further investigation is needed (Vohra and Gupta, 2012). Due to small size of seed, thin and soft seed coat, microscopy of lentils has been challenging. Therefore, the present study focused to investigate the structure of seed coat and seed of lentils using bright-field and polarized light microscopy.

Materials and Methods

Collection of specimens

The seeds specimen was obtained from healthy *L. culinaris* plants. They were dried and stored in clean and dry containers. They were taxonomically authenticated by CSIR-NISCAIR, New Delhi, India under reference number NISCAIR/RHMD/Consult/2018/3155-04.

Macroscopic examination

Morphological examination revealed that the seeds were reddish brown in color with smooth and shiny texture (Fig. 1). Further, the seeds were measured (diameter in cm), and examined for taste, and odor.

Fig. 1: *Lens culinaris* seeds

Microscopic examination

Anatomical structure

The microscopic examination of the lentil seeds focused to identify the anatomical structure of seed as well as seed coat. To attain the same, the seeds were processed by following three steps, namely fixation, dehydration, and embedding.

- **Fixation :** The seeds were fixed in a solution containing formalin (5 ml), acetic acid (5 ml) and ethyl alcohol (70% v/v; 90 ml) (FAA solution) for 24 hours.
- **Dehydration :** Dehydration was performed to replace the water present in the tissues with a solvent miscible with embedding media such as paraffin or resin. Post fixing, the specimens were dehydrated with graded series of tertiary butyl alcohol (TBA) (Sass, 1940).
- **Embedding :** The specimens were further infiltered by gradual addition of paraffin wax with a melting point of 58°-60°C. The process was carried out until TBA solution attained the stage of super-saturation. The specimens were further embedded into paraffin blocks for sectioning.
- **Sectioning :** The cast containing paraffin embedded specimens were sectioned with the help of Rotary Microtome, followed by dewaxing of the sections (Johansen, 1940).
- **Staining :** The sections were stained with toluidine blue (O'Brien, Feder, and Mc Cull, 1964). The sections were also stained with safranin, fast-green and iodine-potassium iodide solution. All the sections were mounted with glycerine (Evans, 2002).

Powder microscopy

The seeds were powdered and were further cleared with sodium hydroxide and mounted in glycerin medium after staining with suitable staining agents.

Photomicrographs

Photographs of different magnifications were taken with Nikon Lab Photo 2 microscopic unit. Bright field microscope was used for normal observations while polarized light microscope was used for the study of starch grains (Easu, 1964; Easu, 1979).

Results

Macroscopic examination

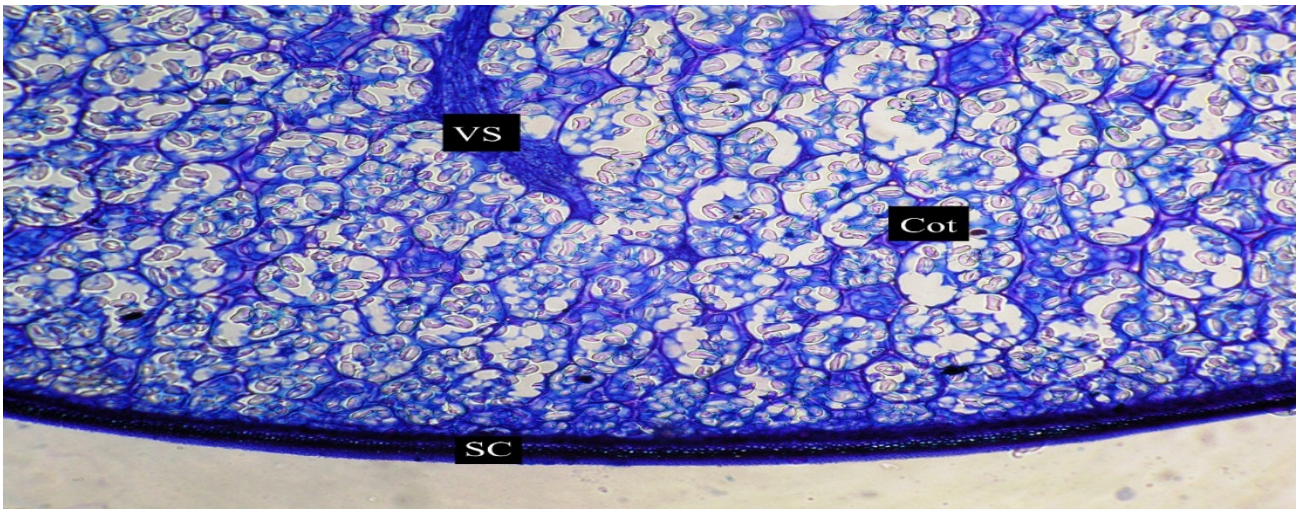
The seeds were elliptical with broadened middle part and narrow at the ends. The raphe was long and elliptical with pale white color (Fig. 2a). The seeds were 0.46 cm in diameter (Fig. 2b) with characteristic odor and mucilaginous taste.

Fig. 2: Macroscopic characteristics of *L. culinaris* seeds

Microscopic examination:

Anatomical structure of Seed

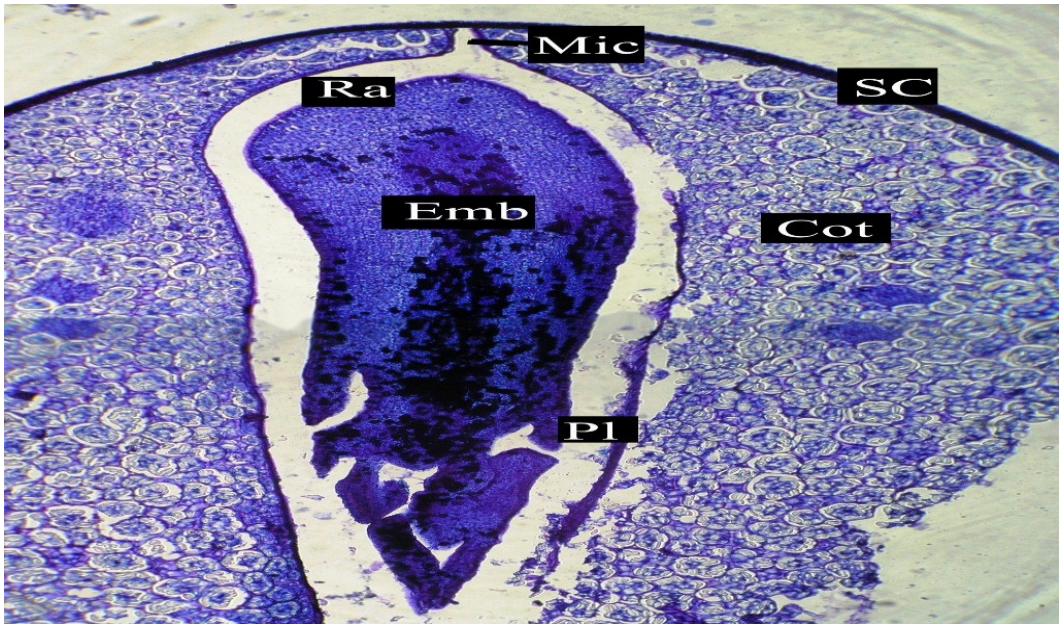
The seed was circular in outline, measuring 4.25 mm in diameter. It consisted of thin testa (seed coat) and wide massive cotyledonary tissue. The thick and prominent vascular strands ramified in the cotyledonary tissue (Fig. 3).



Cot: Cotyledon; SC: Seed coat; VS: Vascular strand

Fig. 3: Paracotyledonary section of *L. culinaris* seed at 10X

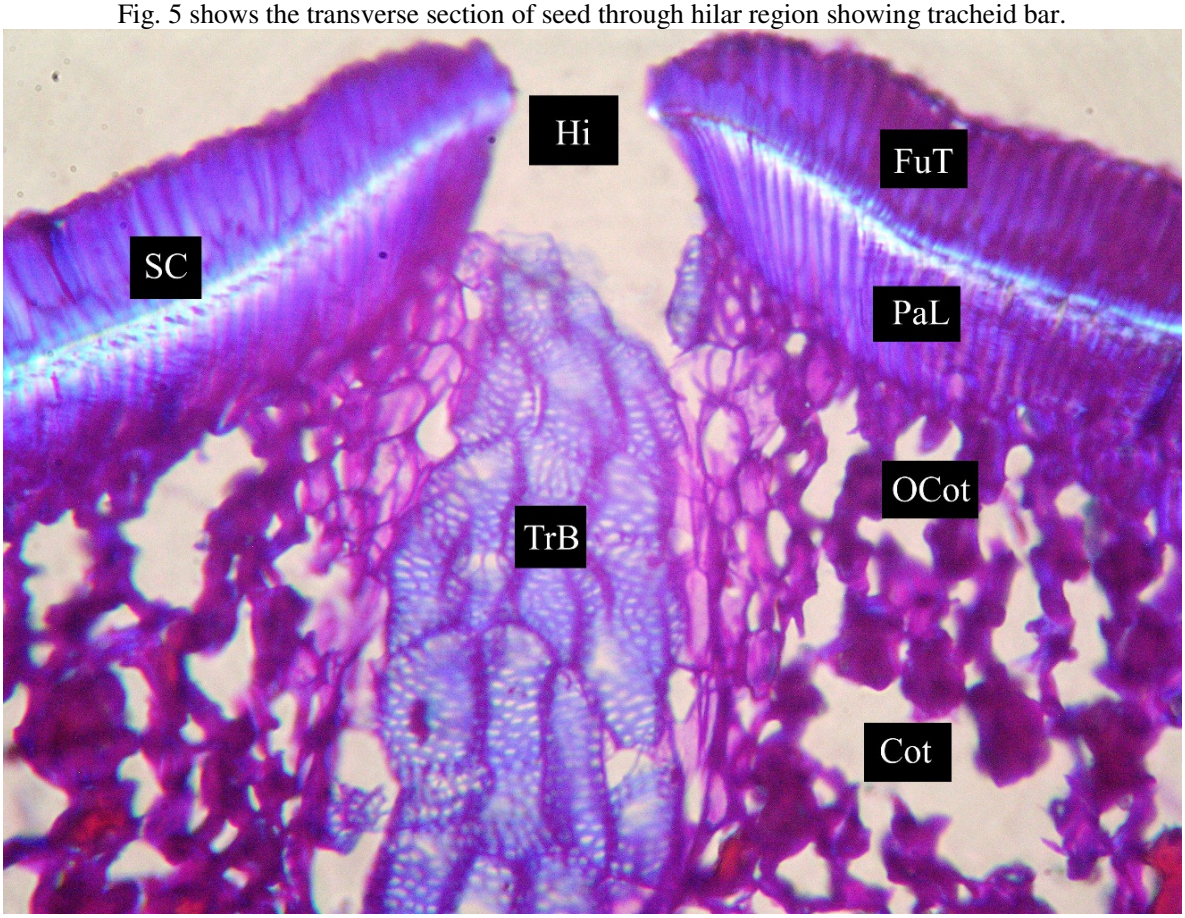
In the median longitudinal sections of the seed, the embryo was seen in between the two cotyledons. The embryo was cylindrical and vertically oblong (Fig. 4). It consisted of thick conical radicle which was directed towards the micropylar end. The radicle was 300 µm thick. The lower end of the embryo had the plumule shoot apex. It consisted of the shoot apex and juvenile leaves. The entire length of the embryo was 2 mm.



Cot: Cotyledon; Emb: Embryo; Mic: Micropyle; Pl: Plumule; Ra: Radicle; SC: Seed coat

Fig. 4: Longitudinal section of seed showing the embryo in between the cotyledons at 10X

Fig. 5 shows the transverse section of seed through hilar region showing tracheid bar.



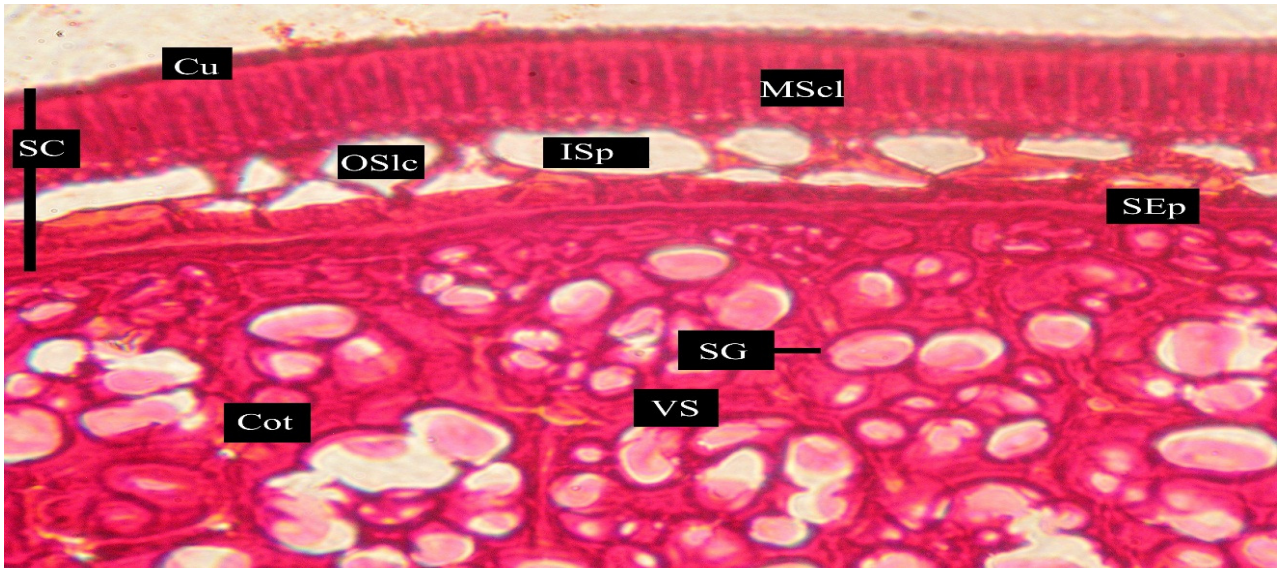
FuT: Funicular tissue; Cot: Cotyledon; Hi: Hilum; PaL: Palisade layer; SC: Seed coat; TrB: Tacheid bar; OCot: Outer cotyledonary cells

Fig. 5: Tracheid bar

Anatomical Structure of Seed Coat

The seed coat and the cells of the cotyledon were distinctly visible. The seed coat (testa) measured 50 µm in thickness. The seed coat became thick at hilar region and consisted of funicular tissue and palisade layer (Fig. 5). Seed coat consisted of outer thin smooth cuticle on the surface of the seed coat. Inner to the cuticle was a thick layer of macrosclereids which were vertically elongated narrow columnar cells with uniform height and thickness. The cell walls were lignified (Fig. 6). The macrosclereids were 30 µm in height.

The second layer of the seed coat consisted of lagenosclereids or osteosclereids. The cells were hour-glass shaped with wide upper and lower ends. The middle part was found to be constricted. In between the osteosclereids, there was a wide space (Fig. 6). The osteosclereid layer was 20 µm thick. The innermost layer of the seed coat was parenchyma which was smooth and thick.



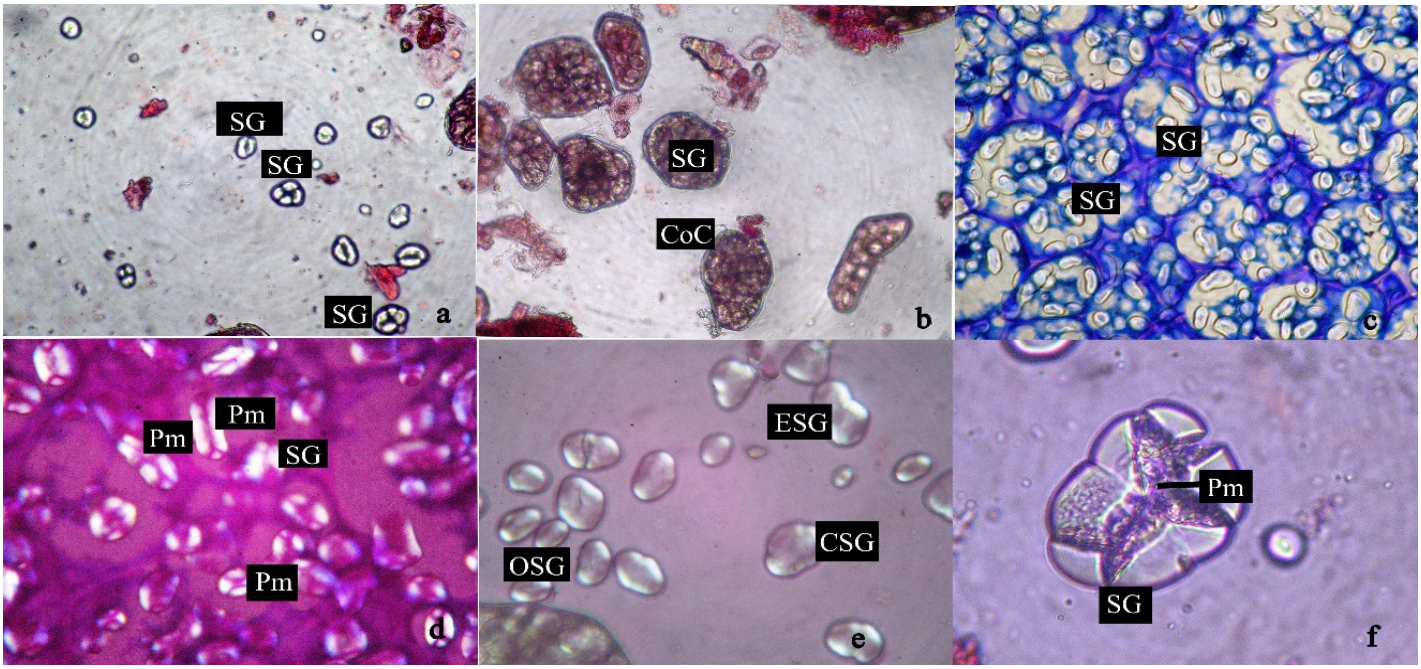
Cot: Cotyledonary cells; Cu: Cuticle; VS: Vascular strand; SEp: Smooth epidermis; ISp: Intracellular spaces; Osteosclereids; MScl: Macrosclereids; SG: Starch grains; SC: Seed coat

Fig. 6: Seed coat at 10X

Powder microscopy

Starch grains : Large, polyhedral parenchyma cells were seen in abundance (Fig. 7a). The cotyledonary cells possessed densely loaded starch grains (Fig. 7b). The starch grains were randomly arranged in the cells (fig. 7c). The grains varied in shape and size as some were circular with (+) shaped polarimark, while others were cylindrical with ƛ shaped polarimark (Fig. 7d). Ovate and spindle shaped (elongated) grains are also observed. The circular grains were 30 µm in size and the ovate grains were 10×20 µm in size (Fig. 7e).

Polarimark : When the starch grains are viewed under polarized light microscope, the starch grains exhibited different patterns of dark lines called polarimarks (Fig. 7f). The polarimarks were observed due to their birefringent property which, under polarized light, appeared bright against dark background. Starch grains bearing different patterns of polarimarks were seen in the powder.



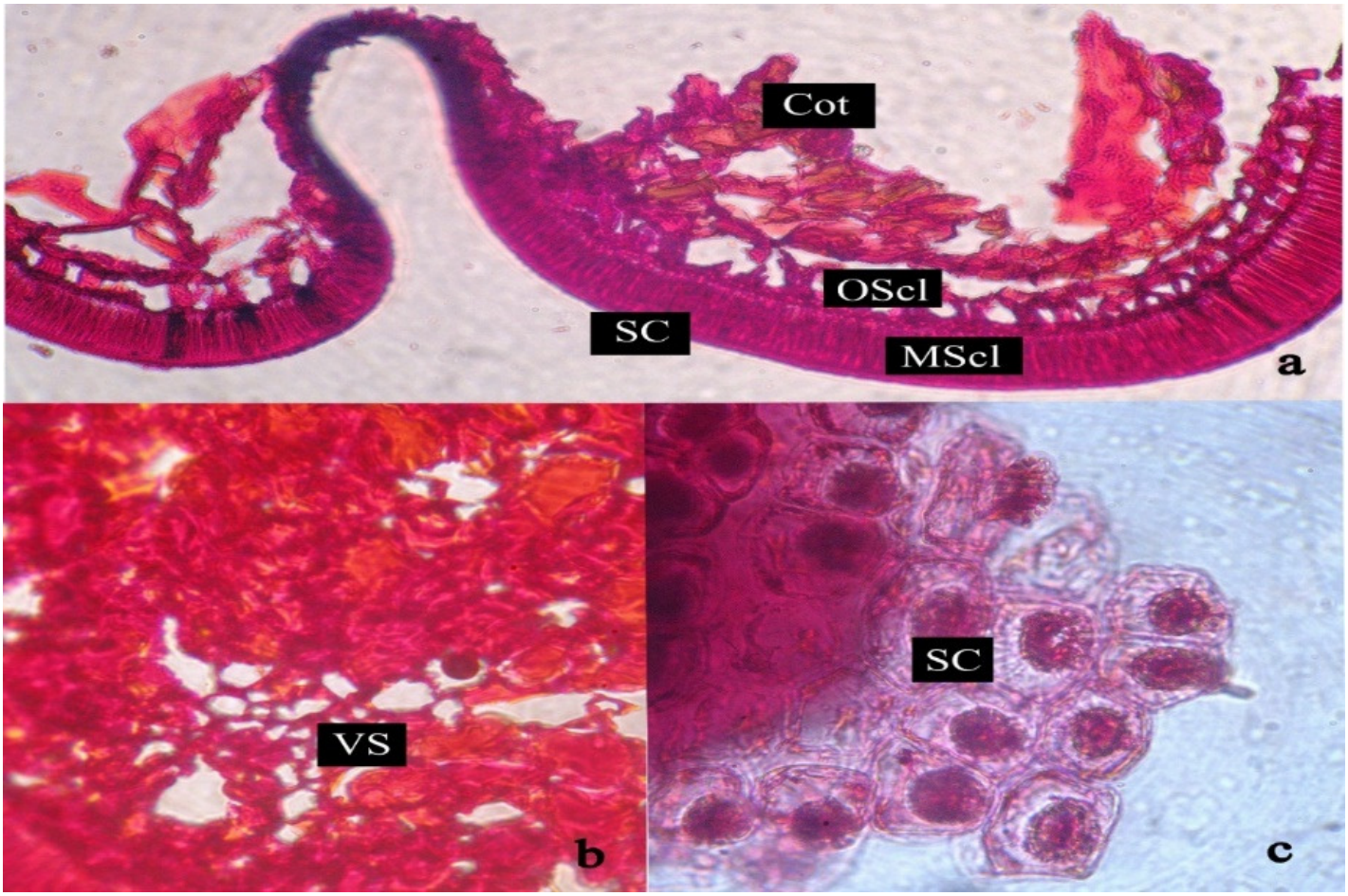
SG: Starch grains; CoC: Cotyledonary cells; Pm: Polarimarks; ESG: Elongated starch grains; OSG: Ovate starch grains; CSG: Circular starch grains

Fig. 7 a: Free starch grains; b: Starch grains within cotyledonary cells; c: Cells of the cotyledon bearing starch grains; d: Starch grains under polarized light; e: Starch grains mounted without staining; f: A single starch grain with many wide polarimarks

Fragments of seed coat : Broken fragments of seed coat were frequently seen in the powder. The seed coat fragments exhibited outer layer of macro or columnar sclereids, hourglass shaped osteosclereids and inner parenchyma layer (fig. 8a).

Cotyledonary tissue : Cotyledonary tissue possessed small group of xylem elements/ vascular strands (fig. 8b). Xylem elements were polygonal in outline with thick secondary walls.

Fragments of epidermal peeling of the seed coat : The peeling appeared in the surface view. The cells were angular and thin walled and had dense, darkly stained spherical bodies. These dark bodies occupied more or less the entire lumen of the cells (fig. 8c).



SC: Seed coat; OScl: Osteosclereids; MScl: Macrosclereids; VS: Vascular strands; Cot: Cotyledonary cells

Fig. 8 a: **A broken piece of seed coat seen in the powder; b: Vascular strands in the cotyledon; c: Seed coat cells as seen in surface view**

Discussion

Authentication of medicinal plants basically refers to the confirmation of plant's identity where botanical microscopy plays an essential role. In addition to plant/ plant part authentication, it contributes in ensuring the identity, quality and purity of the medicinal part (Lachumy and Sasidharan, 2012; Upton, 2010).

Years ago, the plants were authenticated by qualitative evaluation such as macroscopic studies, based upon the study of morphological and organoleptic characters of the plant materials including color, texture and odor. Moreover, macroscopic techniques are generally used to identify or differentiate the plant/ plant material among various species which are morphologically similar, but still can be distinguished. However, this was later considered as an unreliable technique of identification due to various complications such as adulteration, broad genus spectrum. This led the role of microscopic studies to come into play. Microscopic studies may be considered as a reliable, easy, cheap, quick and convenient method of identification as well as authentication of a plant (Kumar and Lalitha, 2017; Lachumy and Sasidharan, 2012; Smillie and Khan, 2010). World Health Organization (WHO) also considers macroscopic and microscopic description of a plant material as the initial step for its authentication (W.H.O, 1998). Microscopy utilizes various types of microscopes to determine the presence of various characteristics such trichomes, starch grains, calcium oxalate crystals, *etc*. (Smillie and Khan, 2010).

Microscopic technique has been now not only used for authentication, but also for standardizations of the herbal products. This method has been considered important and has been included for identification of medicinal plants in various Pharmacopoeias, as well as in other reference books such as Chinese Materia Medica, New compendium of Chinese Materia Medica, British Herbal Pharmacopoeia, American Herbal Pharmacopoeia, Japanese Pharmacopoeia, Korea Herbal Pharmacopoeia, and Indian Ayurvedic Pharmacopoeia (Zhao *et al*., 2005). Previous studies also support the role of macroscopic and microscopic studies in plant identification and authentication (Murti, Yogi, and Pathak, 2010; Singh, Saharan, and Bhandari, 2014; Smillie and Khan, 2010).

Microscopic techniques have been previously used to study the differences in the seed coat pattern of various papilionoid seeds, thereby assisting in distinguishing various seeds belonging to one sub-family (Lersten and Gunn, 1982). The present study was conducted to perform detailed microscopy on lentil seeds. Lentils, being one of the oldest cultivated legumes, are rich in protein and low in fat content. In addition to high nutritional value, studies report the presence of carbohydrates, saponins, polyphenols, as well as phytosterols in lentil seeds (Vohra and Gupta, 2012). According to Wolf, 1977, legumes consist of three major parts, *i.e.*, coat, cotyledons, and hypocotyl. Lentils seeds contain 8.1% of seed coat, 90% of cotyledon and 2.0% of hypocotyl (Wolf, 1977). Although, lentils seeds are gaining dramatic popularity, but information related to their its standardization is still not sufficiently elaborated (A.P.I,

2007; Vohra and Gupta, 2012). Moreover, the small size of the seed constitutes as one of the most complicated technical barriers to their anatomical study.

The present study used bright-field light and polarized light microscopy to study the anatomical structure of the lentil seeds. The bright-field illumination is based upon the differences in the tendency of various structures to absorb light, their refractive index and color. It has been considered as one of the primary and widely used techniques. It is more commonly used to examine specimens stained with visible light absorbing dyes, while its use gets restricted when examining unstained specimens. Polarized light microscopy is mainly used to understand the birefringent property of microscopic structures such as plant cell wall, and starch grains. It enhances the contrast and improves the quality of the images of microscopic specimens with birefringent property ("Education in Microscopy and Digital Imaging,").

Toluidine blue is a polychromatic dye used to determine the presence of various microscopic structures in plant cells. It imparts different color to different structures, such as pink color to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies (Evans, 2002).

The present study reports the presence of layers (outer to inner side) of macrosclereids, lagenosclereids or osteosclereids and parenchyma cells in seed coat. The seed coat broken open to hilum followed by tracheid-bar. The seed consisted of embryo, oval/ elongated/ circular starch grains packed randomly in cells of cotyledon. The powder microscopy revealed the presence of starch grains, fragments of seed coat, cotyledon tissue, and fragments of epidermal peeling of the seed coat.

The hard seed coat of seeds is attributed by the development of secondary wall (columnar macrosclereids) in the epidermis and osteosclereids in the layers or layers beneath the epidermis. Sclereids are lignified walled- short sclerenchyma cells which provide mechanical strength to the seeds. The two types of sclereids, macrosclereids and osteosclereids, are also present in lentil seed coat. Macrosclereids are elongated/ rod shaped sclerenchyma cells while osteosclereids are columnar cells ("Sclerenchyma,").

Previous studies report the presence of characteristic palisade, sub-epidermal and parenchyma layers in seed coats of legume seeds. Our study also reveals the presence of all these micro-components in lentil seed coat. Tracheid bars are only observed in the legume seeds belonging to Papillionaceae family. It consists of large strips consisting of pitted and lignified tracheid like cells (Lersten, 1982). Lentil seeds, being a papilionoid legume, also consist of tracheid bar. The examination of the interior of legume seeds by scanning electron microscope reveals tightly packed storage cells in the cotyledons (Swanson, Hughes, and Rasmussen, 1985), which are also observed on lentils seeds. Hughes and Swanson, 1986 reported closed or slightly open micropyle (Hughes and Swanson, 1986). Micropyle, slightly open, was also observed in the lentil seeds in the present study.

To the best of our knowledge, the present study is the first comprehensive microscopic study conducted on lentil seeds which may be used for identification as well as standardization of the plant material.

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