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Authentication of *Carissa macrocarpa* Cultivated in Saudi Arabia; Botanical, Phytochemical and Genetic Study

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Abstract

This study presents an investigation of the botanical features, phytochemical screening (including determination of total phenolic and flavonoid contents) and DNA fingerprint profiling of *Carissa macrocarpa*. The current study revealed that *Carissa macrocarpa* is characterized chemically by the presence of various secondary metabolites such as flavonoids, saponins, triterpenoids/steroids, anthraquinones, tannins and carbohydrates at different levels in different extracts of different plant organs and the absence of cardiac glycosides and alkaloids. The plant under investigation is characterized by presence of high amounts of phenolic contents and moderate amounts of flavonoids. The plant is characterized microscopically by presence of big Ca oxalate clusters as well as forked apex and undulating wall fibers and absence of any kind of hair. Furthermore, the DNA of the plant was extracted and analyzed using six random decamer primers. A total of 55 random amplified polymorphic DNA (RAPD) markers were obtained. The results from DNA fragmentation pattern recommend the use of primers UBC-210, UBC-405, UBC-509, UBC-54, UBC-292and UBC-123 for the fingerprinting of DNA of *Carissa macrocarpa.* This study will give a complementary tool to confirm the identity of *Carissa macrocarpa*.

Key words: *Carissa macrocarpa*, Apocynaceae, botanical, phytochemical screening, DNA fingerprint.

1. INTRODUCTION

The use of adulterating materials and/or substituents will result in decrease of quality of herbal products with partially or complete loss of the therapeutic potentials in plants to which the activities are attributed compared to the genuine products. Hence, the authentication of crude materials for commercially available herbal products in market is considered very essential nowadays. Despite the presence of number of analytical tools for the detection of adulterants/substituents in the herbal drug industry, the most recent analytical tool is based on genomic identification at DNA level, which is not affected by the age or ecological factors on sample as well as it can be used at any stage of an herb's development. The study of the botanical, phytochemical as well as DNA fingerprint profiling will be of great help in authentication process [1].

Apocynaceae is a family of flowering plants that includes trees, shrubs, herbs, stem succulents and vines, which consists of about 250 genera and 2000 species distributed among five subfamilies: Rauvolfioideae, Apocynoideae, Periplocoideae, Secamonoideae, and Asclepiadoideae. *Carissa*, one of genera of Apocynaceae, consists of about 35 species distributed in tropics and subtropics of Africa, Asia and Australia. Its members are shrubs, climbers, or small trees, mostly spiny [2-6].

Carissa macrocarpa (*C. macrocarpa;* synonym: *Carissa grandiflora*) grows worldwide including Saudi Arabia. It is a small, evergreen, twiggy shrub with star like-shaped scented white to yellowish white flowers, Y-shaped spines and delicious red fruits. Its fruits are well reputed to its high contents of vitamin C, Ca and Mg [7-10].

A literature survey indicated that nothing could be traced concerning the anatomical features, phytochemical screening and genetic profiling of *C. macrocarpa*. Thus, the present work will include deep and detailed studies on the morphological and anatomical features, as well as DNA fingerprint of the plant under investigation to facilitate its identification both botanically and genetically together with classic phytochemical screening and determination of total phenolic and flavonoids contents.

2. MATERIALS AND METHODS

2.1. Plant Material

C. macrocarpa was collected from gardens of King Faisal University, Al-Ahsa region (September 2014**).** A voucher specimen of the plant is deposited in the Herbarium of Department of Pharmaceutical Sciences, College of Clinical Pharmacy, King Faisal University, Al-Ahsa, Saudi Arabia (01-14-Sept-CM).

2.2. Extraction and fractionation of different plant organs extracts

The powdered air dried plant material [leaves, stems and roots of *C. macrocarpa*, 500, 250 and 100 g respectively] were exhaustively extracted twice at room temperature (each for 3 days) using 5 L 70% methanol and applying cold maceration method to avoid destruction of active constituents. The solvent mixtures were distilled off under reduced pressure using rotary evaporator and then freeze-dried to yield the total dry extracts of leaves, stems and

roots, 100, 40 and 10 g respectively, which were stored in freezer for the next steps.

Exactly 50, 20 and 10 g of leaves, stems and roots total extracts respectively were suspended in distilled deionized water (200 ml) using a separating funnel and partitioned with n-hexane (6×500 ml). The resulting n-hexane phases were combined to be concentrated to the least amount using rotary evaporator and then dried to give 20, 5 and 1 g respectively then were stored in a deep freezer in well-closed container. The remaining aqueous part was subjected to partition with dichloromethane (4×500 ml). The obtained dichloromethane fractions were also combined and its amount was reduced to the minimal amount through using rotary evaporator and then freeze dried to give10, 3 and 2 g, respectively, and then all were put in fridge in a strong-tight container for later use. Similarly, the ethyl acetate as well as n-butanol extracts were also developed using the same above-mentioned procedure to give 5, 2 and 3 g respectively for ethyl acetate fraction and to give 4, 3 and 2 g respectively for n-butanol fraction. The remaining aqueous fraction was also freeze-dried to powder to give 5, 4 and 1 g respectively and kept for further use in the freezer in an air-tight container.

50, 20 and 10 g of the dry different extracts of leaf, stem and root, respectively was subjected to the phytochemical screening tests using referenced methods

2.3. Phytochemical Screening of different plant organs extracts

Chemical testes were carried out on the n-hexane, chloroform, ethyl acetate, butanol and aqueous extracts of different organs using standard procedures to identify the nature of its constituents [2, 11-12].

2.3.1. Test for flavonoids

Half gram of dried extracts of each organ was boiled with 10 ml of distilled water for 5 min and filtered while hot. Few drops of 20% sodium hydroxide solution were added to 1 ml of the cooled filtrate. A change to yellow color, which on addition of acid changed to colorless solution, depicts the presence of flavonoids.

2.3.2. Test for saponins

Half gram of dried extracts of each organ was separately boiled with 10 ml of distilled water in a bottle bath for 10 min. The mixture was filtered while hot and allowed to cool. Demonstration of frothing: 2.5 ml of filtrate was diluted to 10 ml with distilled water and shaken vigorously to form a stable persistent froth.

2.3.3. Test for steroids and/or triterpenoids

Half gram of dried extracts of each organ was separately boiled with 10 ml of distilled water in a bottle bath for 10 min. The mixture was filtered while hot and allowed to cool. Five milliliters of each extract was mixed in 2 ml of chloroform. Three milliliters of concentrated H_2SO_4 was then added to form a layer. A reddish brown precipitate coloration at the interface formed indicated the presence of steroids and/or triterpenoids.

2.3.4. Test for alkaloids

Half gram of dried extracts of each specimen was separately boiled with water and 10 ml hydrochloric acid on a water bath and filtered. The pH of the filtrate was adjusted with ammonia to about 6-7. A very small quantity of Dragendorff's reagent (potassium iodide 0.11 M, bismuth nitrate 0.6 M in acetic acid 3.5 M), the test tubes were observed for orange to brown turbidity.

2.3.5. Test for anthraquinones

Half gram of dried extracts of each organ was boiled with 2 ml of 10% hydrochloric acid for 5 min. The mixture was filtered while hot and filtrate was allowed to cool. The cooled filtrate was partitioned against equal volume of chloroform and the chloroform layer was transferred into a clean dry test tube using a clean pipette. Equal volume of 10% ammonia solution was added into the chloroform layer, shaken and allowed to separate. The separated aqueous layer was observed for any color change; delicate rose pink color showed the presence of an anthraquinone.

2.3.6. Test for tannins

Half gram of each dried extracts of each organ was separately boiled with 20 ml distilled water for 5 min in a water bath and was filtered while hot. One milliliter of cool filtrate was distilled to 5 ml with distilled water and a few drops (2-3) of 10% ferric chloride were observed for any formation of precipitates and any color change. A bluish-black or brownish-green precipitate indicated the presence of tannins.

2.3.7. Test for cardiac glycosides

Half gram of dried extracts of each organ was separately boiled with 10 ml of distilled water in a bottle bath for 10 min. The mixture was filtered while hot and allowed to cool. Five milliliters of each extract was treated with 2 ml of glacial acetic acid containing one drop of 10% ferric chloride solution. This was underplayed with 1 ml of concentrated H_2SO_4. A brown ring at the interface indicated the deoxy-sugar characteristics of cardenolides. A violet ring may appear below the ring while in the acetic acid layer, a greenish ring may be formed.

2.3.8. Test for carbohydrates

Half gram of various extracts were dissolved separately in 4 ml of distilled water and filtered. The filtrate was treated with 2-3 drops of alcoholic alpha-naphthol and 2 ml of concentrated H_2SO_4 was added along the sides of the test tube. Appearance of brownish violet ring at the junction of the two liquids indicates the presence of carbohydrates.

2.4. Determination of total phenolic contents

The total phenolic content was measured using the Folin-Ciocalteu index method [13]. Stock solutions (1 mg/ml) of different extracts were prepared in methanol. Six ml of water and 0.5 ml of Folin-Ciocalteu reagent were successively added to 0.1 ml of stock solution of each extracts. In addition, 1.5 ml of a 20% sodium carbonate solution and water were added to obtain 10 ml. A reaction took place within 120 min. at room temperature. Absorbance was measured at 760 nm. Calibration was done using gallic acid serial dilution as a standard (0.5, 0.4, 0.3, 0.2 and 0.1 mg/ml in distilled water, $y = 0.0156x + 0.0004$, $r^2 = 0.9697$). The concentration of phenolic components was expressed as the equivalent to milligrams of gallic acid per gram of dry extract (mg GAE/g).

2.5. Determination of total flavonoids

The total flavonoid content was determined according to Heimler et al. [14]. Ten mg of extracts were diluted in 100 ml of acetone/water (1:1 v/v). A solution of 0.25 ml of the

suitably diluted sample was added to 0.75 µl of a $NaNO_2$ (5% w/v) solution, as well as 0.15 ml of a freshly prepared $AlCl_3$ (10% w/v) solution, and 0.5 ml of 1 M NaOH solution. The final volume was adjusted to 10 ml with deionized water. The mixture was allowed to stand for 5 min and the absorption measured at 510 nm against the same mixture without the sample. Calibration was done using quercetin as standard, for which a calibration curve was obtained with solutions of 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml ($y = 0.0009x + 0.0227$, $r^2 = 0.9771$). The results were expressed as the equivalent to milligrams of quercetin per gram of dry extract (mg QE/g).

2.6. Botanical study

Anatomical investigations were carried on cross sections prepared previously using microtome (SLEE, Germany) of both old and young stems and leaves as well as spins and roots. The photographs were captured for both macro-morphology and micro morphological features using digital camera (Canon IXY, 510-1S, Japan) and Olympus BX41 System Microscope connected to DP-25camera, Japan, respectively.

2.7. DNA fingerprint profiling

2.7.1. DNA extraction

Genomic DNA of *C. macrocarpa* was efficiently extracted from 5 g of leaves tissue in 1.5 ml micro centrifuge tube by applying a little modified standard DNA extraction technique [15].

2.7.2. Oligonucleotide primers

6 random decamer oligonucleotide universal primers were used to amplify *C. macrocarpa* genomic DNA which are UBC-210, UBC-405, UBC-509, UBC-54, UBC-292and UBC-123.

2.7.3. Polymerase chain reaction (PCR)

PCR amplification was conducted using 25µl of reaction media containing 1% Triton 10-X buffer [100 mM Tris–HCl (pH= 8.3), together with 500 mM KCl, 0.01% (w/v) gelatin], 2.0 µl $MgCl_2$ (25 mM), 2.5 µl of dNTP (2 mM), 3 µl primer was inoculated to reaction media, 0.3 µl of Taq polymerase (Promega, USA), and 2.5 µl of genomic DNA material of sample under study, and then was completed to the required volume with distilled water. The thermocycler was previously programmed to work for 1 cycle of 5 min initial strand separation at almost fixed temperature of 94°C and next for 40 cycles each to work for 1 min at the same temperature 94°C to cause denaturation, 1 min primer annealing at 36°C, a 7 min primer elongation at 72°C, finally followed by 1 cycle of final primer extension at a temperature of 72°C to a period of 10 min.

2.7.4. Gel electrophoresis and staining

PCR products were discriminated in 1.5% agarose gel through electrophoresis in TE buffer (10 mM Tris-HC1, 1.0 mM EDTA, pH =8.0) with a constant power supply of 95 volts for about 180 min. Staining of products were carried out with 0.5 µg/ml ethidium bromide and then visualized and photos were captured under UV supported light conditions [16-18].

3. Results And Discussion

3.1. Phytochemical screening of different plant organs extracts

The preliminary phytochemical screening of extracts of different plant organs of *C. macrocarpa* showed the presence of various secondary metabolites such as flavonoids, saponins, triterpenoids/steroids, anthraquinones, tannins and carbohydrates at different levels in different fractions of plant organs and the absence of cardiac glycosides and alkaloids as shown in Table 1.

Table 1: Preliminary phytochemical screening of different plant organs extracts.

Chemical test/Plant organ	Leaves					Stems					Roots				
	nH	DC	EA	BT	Aq.	nH	DC	EA	BT	Aq.	nH	DC	EA	BT	Aq.
-Flavonoids Alkaline solution test	-	+	+	+	+	-	+	+	+	+	-	-	±	±	-
-Saponins Foam test	-	+	+	+	+	-	+	+	+	+	-	-	+	+	-
-Triterpenoids/steroids Liebermann-Burchard test	-	+	+	+	+	-	+	+	+	+	-	-	+	+	-
-Alkaloids Dragendorff's reagent	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-Anthraquinones Borntrager's test	-	+	+	+	+	-	+	+	+	+	-	-	-	-	-
-Tannins 10% $FeCl_3$	-	-	+	+	-	-	-	+	+	-	-	-	±	-	-
-Cardiac glycosides Keller Killiani test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-Carbohydrates Molisch's test	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+

nH, n-Hexane fraction; DC, Dichloromethane fraction; EA, Ethyl acetate fraction; BT, Butanol fraction; Aq., Aqueous fraction. + (present); - (absent); ± (traces).

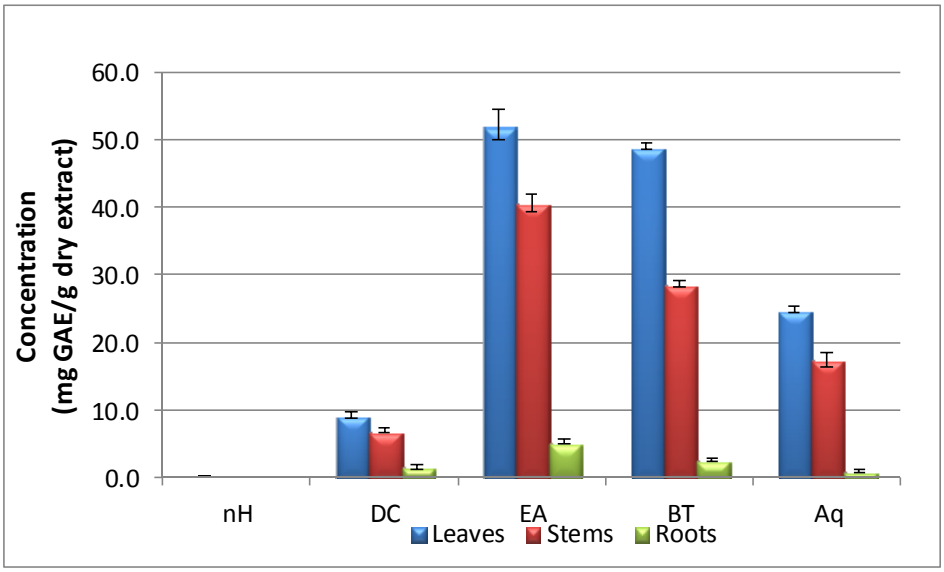

Fig. 1: Total phenolic contents of different extracts of *Carissa macrocarpa.* nH; n-Hexane fraction, DC; Dichloromethane fraction, EA; Ethyl acetate fraction, BT; Butanol fraction, Aq.; Aqueous fraction, GAE; gallic acid equivalent. Data are the means ± standard deviation of three replicate.

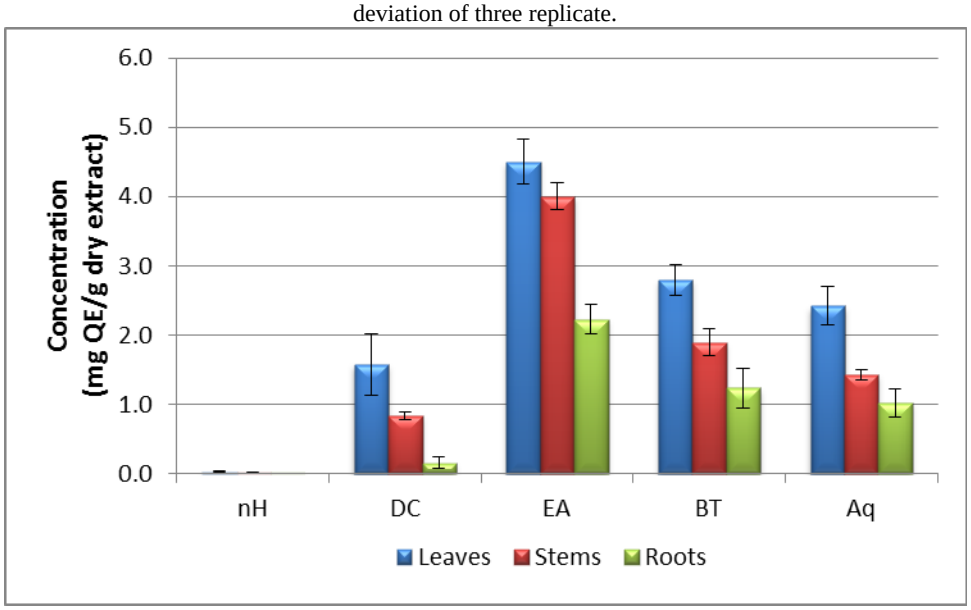

Fig. 2: Total flavonoid contents of different extracts of *Carissa macrocarpa.* nH; n-Hexane fraction, DC; Dichloromethane fraction, EA; Ethyl acetate fraction, BT; Butanol fraction, Aq.; Aqueous fraction, QE; quercetin equivalent. Data are the means ± standard deviation of three replicate.

3.2. Determination of total phenolic contents

Determination of total phenolic contents showed that the quantity of total phenolic components varies from 0.028±0.003 to 52.296±2.155 mg GAE/g of dry extract (Fig. 1). Ethyl acetate fraction contains the highest percentage of total phenolic components, followed by butanol fraction then the aqueous fraction. Di-chloromethane fraction contains the least amount while n. hexane extract hardly contains any phenolic contents.

3.3. Determination of total flavonoids

Determination of total flavonoids contents depicted that the amount of total flavonoid constituents differed from organ to organ and varies from 0.002±0.001 to 4.499±0.325 mg QE/g of dry extract (Fig. 2). Ethyl acetate fraction is the richest fraction in flavonoid components, next to it the butanol fraction then the aqueous fraction. Similarly, to phenolic contents, the Di-chloromethane fraction contains the least amount compared to other fractions, while n-hexane fraction hardly contains any flavonoid constituents.

3.4. Botanical study

3.4.1. Macromorphology

Macromorphology of *C. macrocarpa* (Fig. 3A and 3B) is a perennial shrub, widely grown in Saudi Arabia gardens and streets. The shrub has a thick strong erect stem and large spreading branches carrying simple leaves. It attains a height of 15–100 cm. Flowers are white, very small, stellate shaped and very pleasant odor appearing for a short flowering period at the beginning of March till the end of April. The fruit is an edible drupe, fleshy with one seed, green in color and becomes brownish red once it ripens; it appears by the end of April and fully ripened in May.

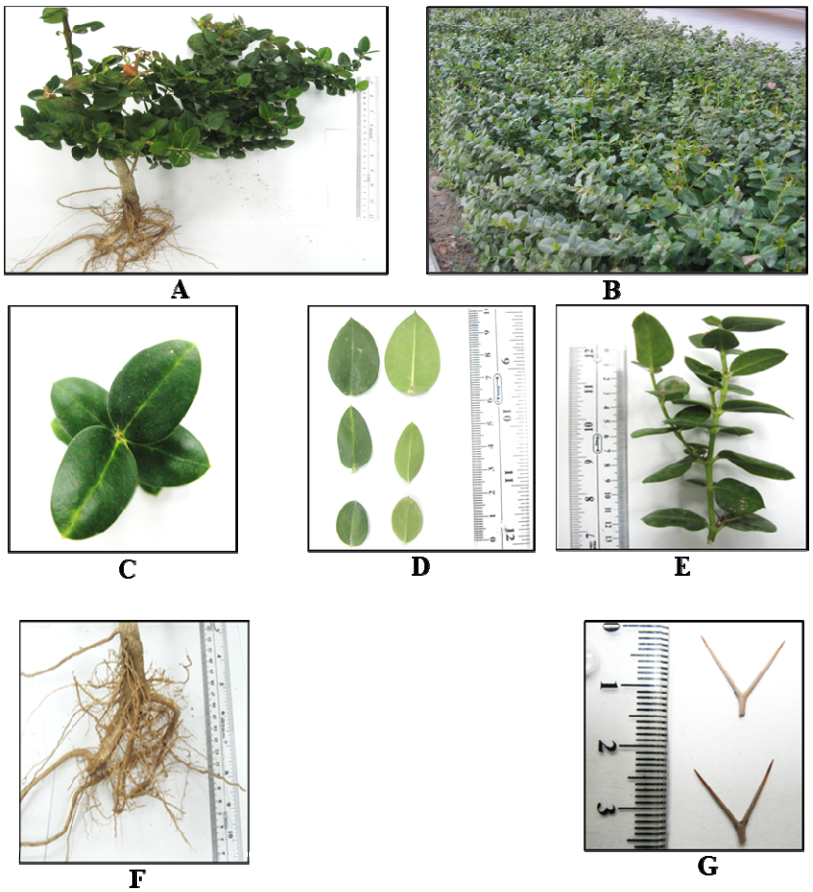

Fig. 3: Photographs of *Carissa macrocarpa* (A) *Carissa macrocarpa* shrubs, (B) Plant in gardens of KFU,(C) Leaves showing opposite decussate phyllotaxis, (D) Leaves of *Carissa macrocarpa*, (E) Part of *Carissa macrocarpa* showing leaves ,stems and spins together, (F) Roots of *Carissa macrocarpa*, (G) Spins of *Carissa macrocarpa.*

3.4.1.1. The leaf

The leaves are simple, cauline insertion, opposite decussate phyllotaxis, ex-stipulate (Fig. 3C and 3D) and carried on short petiolate. The leaf venation is pinnate reticulate venation. Leaves are green in color. Lower surface is lighter than the upper surface. The leaf blade is green, ovate to elliptical in outline having an acuminate hard apex. The base of the leaf is symmetric. Both the lower and upper surfaces are glabrous. The midrib is prominent on the lower side and slightly on the upper side. The leaf measures 1-5 cm in length and 1-2.5 cm in width. The leaves show a leathery texture giving white latex on breaking. The petiole is cylindrical, short, pale green in color and grooved on the upper surface. The petiole measures 0.3-0.5 cm in length and about 0.1-0.3 cm in diameter. The leaf is odorless and possesses a bitter astringent taste.

3.4.1.2. The stem

The young stem is green in color while the old stem is greenish brown in color, cylindrical, hard and with a rough surface longitudinally striated (Fig. 3E). The branching is monopodial type. Old and young stems are slightly hard to break, both of different age stems are breaking giving a fibrous ends and giving white latex on breaking. Both young and old stems possess slightly bitter astringent taste and odorless.

3.4.1.3. The root

The root is yellow to yellowish brown in color. It shows lateral branching (Fig. 3A and 3F), where the main root is tough and harder than the smaller lateral ones. The surface is usually rough showing numerous wrinkles and longitudinal fissures. The cork is easily separated from the inner tissues exposing a yellow interior. The fracture of the root is fibrous on the inner part and smooth on the outer part. The main root measures 0.5-1 cm in diameter, while the lateral branch measures 0.1-3 cm in diameter and extends10-30 cm laterally below the soil. The root has no odor and acrid taste.

3.4.1.4. Spine

Spines are Y-shaped with green to greenish brown color (Fig. 3G). The spines are kind for modified leaves for plant protection. The spine is very hard and sharp. It measures 0.5-2 cm in length and about 0.1-0.3 cm in diameter.

3.4.2. Micromorphology

3.4.2.1. The young leaf

3.4.2.1.1. The leaf lamina

A transverse cross section throughout the young leaf (Fig. 4A and 4B) shows both upper and lower epidermises, enclosing a dorsi-ventral type mesophyll. The vascular bundles are of collateral type surrounded with parenchymatous pericyclic tissue.

The epidermis; the upper and lower epidermis (Fig. 4E and 4F) are nearly similar. They show slightly axially elongated or polygonal with isodiametric wall cells with straight anticlinal walls for the upper epidermis and slightly wavy anticlinal walls for the lower epidermis. The cuticle that covers both upper and lower epidermises is thick smooth. The lower epidermal cells show mostly paracytic and few anomocytic stomata while upper epidermis is devoted of any kind of stomata.

The mesophyll; the palisade tissue (Fig. 4 C) consists of 1-2 rows of columnar closely packed cells, having straight anticlinal walls and filled with green plastids. The palisade cells are extended continuously under the upper surface crossing the midrib tissue. The spongy tissue is formed of irregularly shaped parenchyma cells some cells contain big Ca Oxalate clusters and showing some secretory ducts expecting that they are responsible for production of the white milky exudate that comes out upon breaking the leaf. Small vascular bundles are embedded within the spongy tissue.

3.4.2.1.2. The midrib

The cortical tissue; the cortical tissue of the midrib (Fig. 4A and 4B) consists of rows of 2-3 in number of irregularly shaped thin-walled parenchyma cells beneath the palisade on the upper surface. Small thick walled parenchyma cells are present above the vascular bundle. Some of The parenchyma cells showing yellowish content. Presence of rows of 2-3 in number of irregularly shaped thin-walled parenchyma cells above the lower epidermis.

The pericycle; it is composed of slightly thick walled rounded parenchyma cells (Fig. 4 B).

The vascular tissue; (Fig. 4A and 4B) it is composed of a collateral vascular bundle oval in shape. The xylem vessels are of both spiral and annular lignified thickenings. The cambium is formed of 1-2 rows of radially arranged small cellulosic thin-walled cells. The phloem tissue is very soft and little compressed composed of sieve elements and phloem parenchyma.

3.4.2.2. Powdered leaf

The powder of leaf is light green in color, odorless and slightly astringent bitter taste. Powder of leaf is characterized by the presence of the following elements:

1. Fragments of the upper and lower epidermises (Fig. 4E).
2. Many big clusters of Ca oxalate (Fig. 4G).
3. Fragments of palisade cells which are columnar, thin-walled cells containing green matters (Fig. 4I).

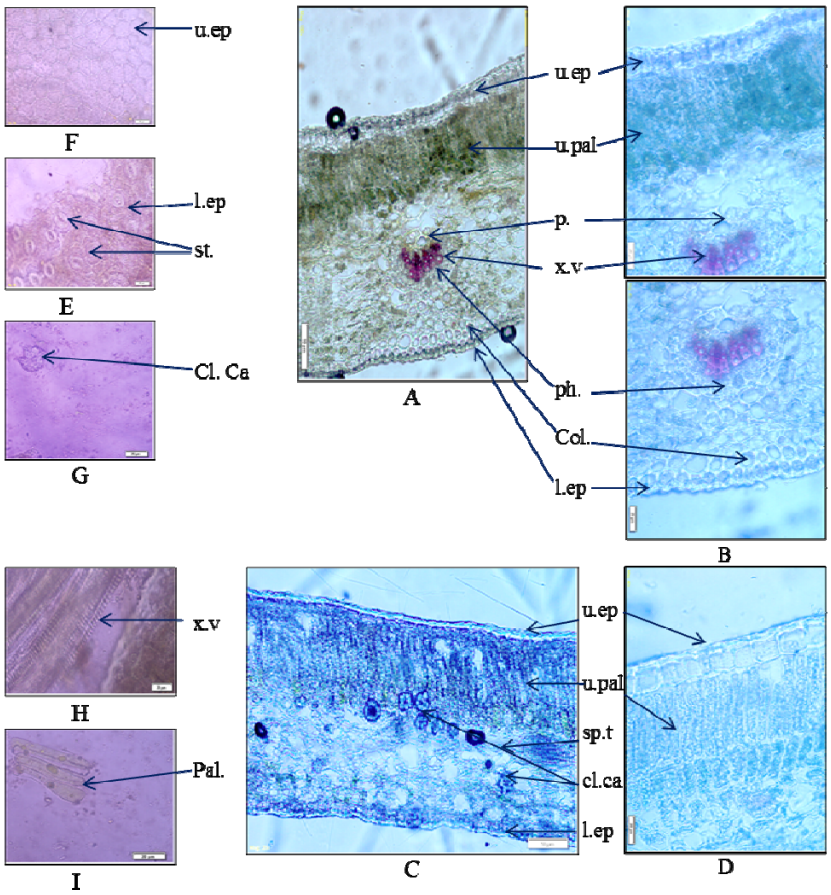

Fig. 4: Micromorphology of the young leaf *Carissa macrocarpa* (A) low power view of the lamina (×100). (B) High power view of the lamina (×200). (C) Low power view of the lamina (×200). (D) High power view of the midrib (×400). (E) The upper epidermis (×400). (F) The lower epidermis (×400). (G) Ca Oxalate clusters (×300). (H) Xylem vessels (×400). (I) palisade cells (×400).col., Collenchyma; cl.ca., cluster of calcium oxalate; l.ep., lower epidermis; p., pericyclic; ph., phloem; sp.t., spongy tissue; st., stomata; u.ep., upper epidermis; u.pal., upper palisade; x.v., xylem vessel.

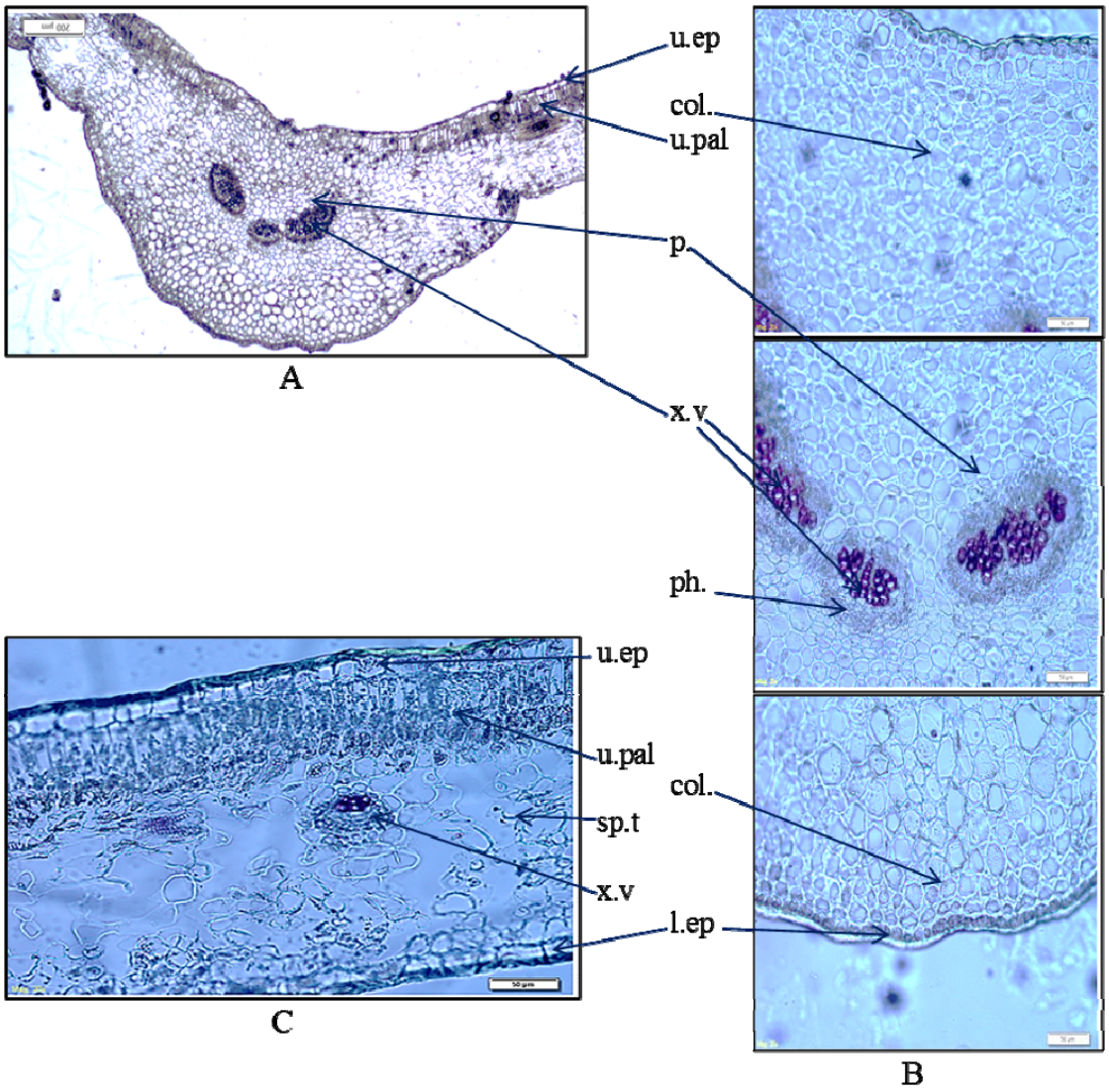

Fig. 5: Micromorphology of the old leaf *Carissa macrocarpa* (A) low power view of the lamina and midrib (×40). (B) High power view of the midrib (×200). (C) Low power view of the midrib (×200). col., Collenchyma; l.ep., lower epidermis; p., pericycle; ph., phloem; sp.t., spongy tissue; u.ep., upper epidermis; u.pal., upper palisade; x.v., xylem vessel.

3.4.2.3. The old leaf

The structure of the old leaf (Fig. 5A, 5B and 5C) is more or less similar to that of the young stem, with the following differences:

3.4.2.3.1. The mesophyll of lamina; the spongy tissue is wider than that of that of old and formed of more irregularly shaped parenchyma. The vascular bundles are embedded within the spongy tissue are of more than that in the young one in number and size.

3.4.2.3.2. The midrib; the cortical elements of the midrib composed of 10-12 rows of round to irregularly shaped thick-walled parenchyma cells beneath the upper surface and absence of palisade cells. The collateral vascular bundle is dissected longitudinally into three strands. Each vascular strand is oval in shape. The presence of 2-3 rows of thick-walled parenchyma above the lower epidermis followed by 9-10 rows of round to irregularly shaped thick-walled parenchyma cells.

3.4.2.4. The young stem

A transverse section throughout young stem (Fig. 6A, 6B and 6C) shows circular outline. It is shows from outside to inside an epidermis, followed by cortex, which is formed of collenchymatous and parenchymatous tissues. The pericycle is parenchymatous showing separated groups of non-lignified weak fibers. The vascular tissue is wide forming a continuous ring. The pith is wide consisting of parenchymatous cells.

3.4.2.4.1. The epidermis; the epidermis (Fig. 6C) is composed of a single row of polygonal cells, having straight anticlinal walls and covered with thick smooth cuticle. The epidermal cells show no stomata and glabrous.

3.4.2.4.2. The cortex; the cortex (Fig. 6B and 6C) is composed of 3-4 rows of rounded collenchymatous cells followed by 1-2 rows of rounded to irregular shaped parenchyma cells. Some cells containing few starch granules (stained blue with iodine solution in T.S.) as well as some cortical cells show brownish to yellowish brown

content and some contain Ca oxalate clusters. The endodermis is not distinct.

3.4.2.4.3. The pericycle; the pericycle (Fig. 6B and 6C) is formed of oval parenchymatous cells with scattered groups of non-lignified fiber. The fibers are polygonal with thick non-lignified walls and narrow lumen.

3.4.2.4.4. The tissue of vascular elements; it is composed of a totally complete ring of collateral vascular bundle (Fig. 6B and 6C). The phloem consists of thin-walled phloem parenchyma, sieve tubes and companion cells. The xylem is formed of lignified radially arranged elements. The vessels show spiral and pitted thickenings. Wood fibers are present in groups; they are fusiform, with narrow lumen and acute apices and some of forked apices.

3.4.2.4.5. The pith; the pith (Fig. 6A and 6B) is formed of rounded to irregularly shaped parenchyma cells.

3.4.2.5. The powder stem

The powder is greenish yellow in color having a characteristic odor and a slightly astringent and bitter taste. Microscopically, it is characterized by the presence of the following:

1-Fragments of polygonal slightly elongated parenchmatous cells having straight anticlinal walls containing starch granules and brown contents (Fig. 6H and 6G).

2- Parts of lignified xylem vessels showing spiral thickening (Fig. 6F).

3- Fragments of wood and pericyclic fibers showing narrow lumen and acute and forked apices (Fig. 6E and 6D).

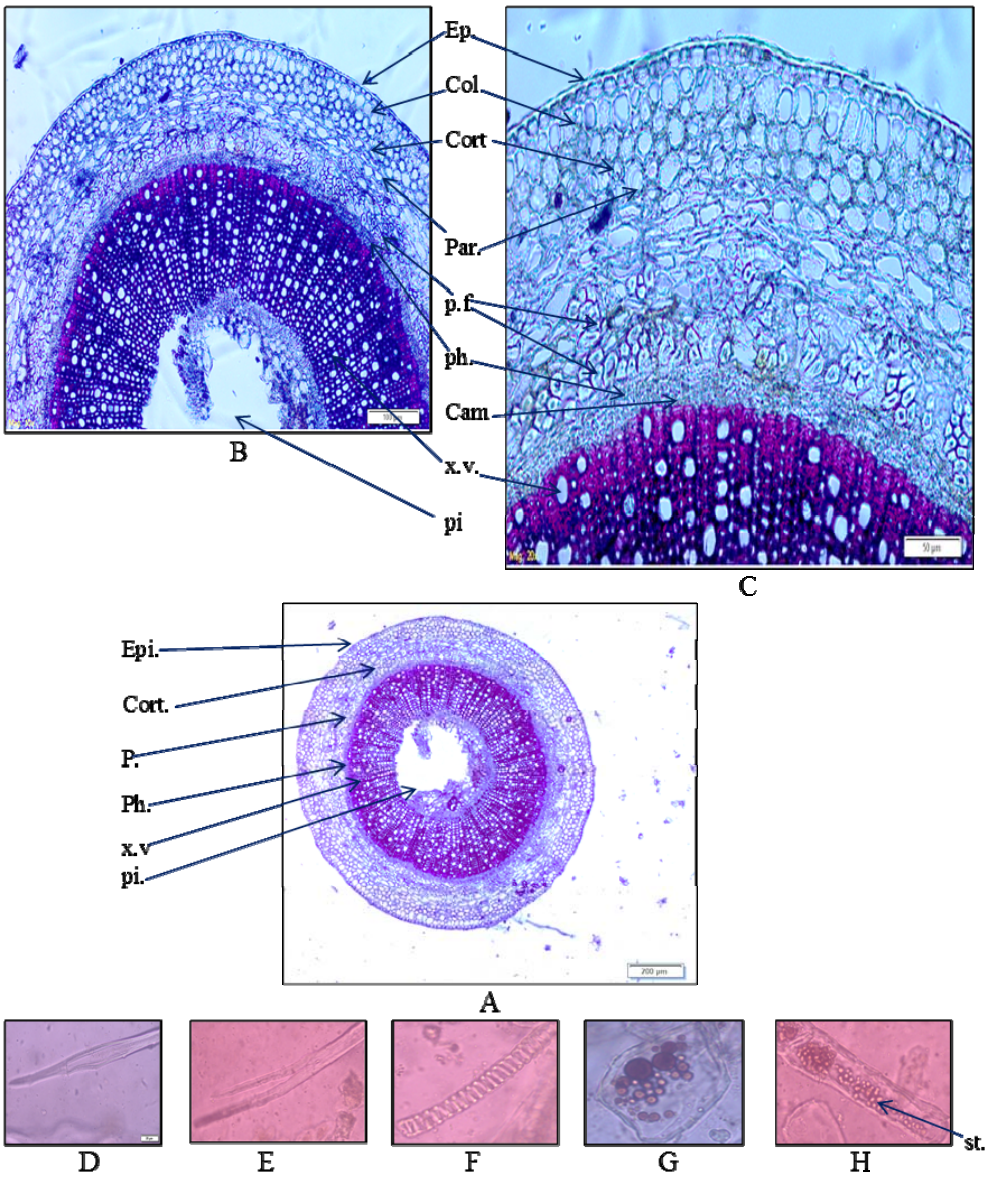

Fig. 6: Micromorphology of the young stem *Carissa macrocarpa* (A) low power view of the young stem (×40). (B) High power view of the young stem (×200). (C) High power view of the young stem (×400). (D) Pericyclic fiber (×400). (E) Wood fiber (×400). (F) Xylem vessel (×400). (G) Parenchyma filled with brown contents. (×400). (H) Parenchyma filled with starch granules (×400). cam., Cambium; cort., cortex; ep., epidermis; p.f., pericyclic fibres; ph., phloem; pi., pith; st., starch granules; x.v., xylem vessel.

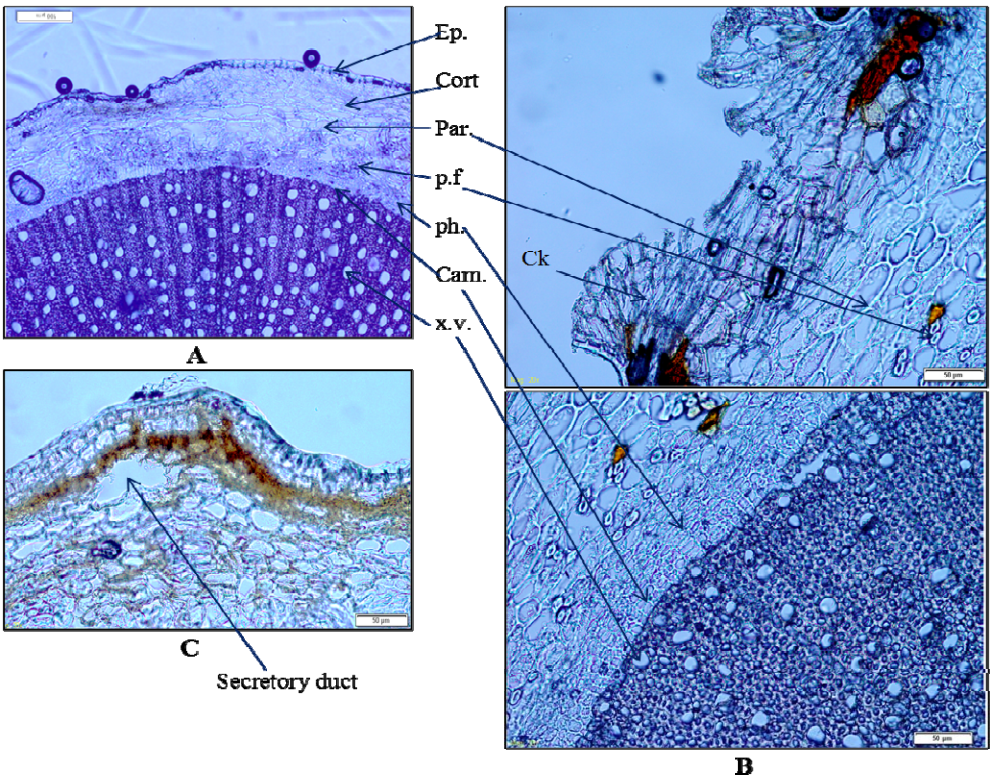

Fig. 7: Micromorphology of the old stem *Carissa macrocarpa* (A) High power view of the old stem (×100). (B) High power view of the old stem branch (×200). (C) High power view of the old stem showing secretory duct (×200).cam., Cambium; ck., cork; cort., cortex; ep., epidermis; p.f., pericyclic fibres; ph., phloem; ph.f., pi., pith; v., xylem vessel.

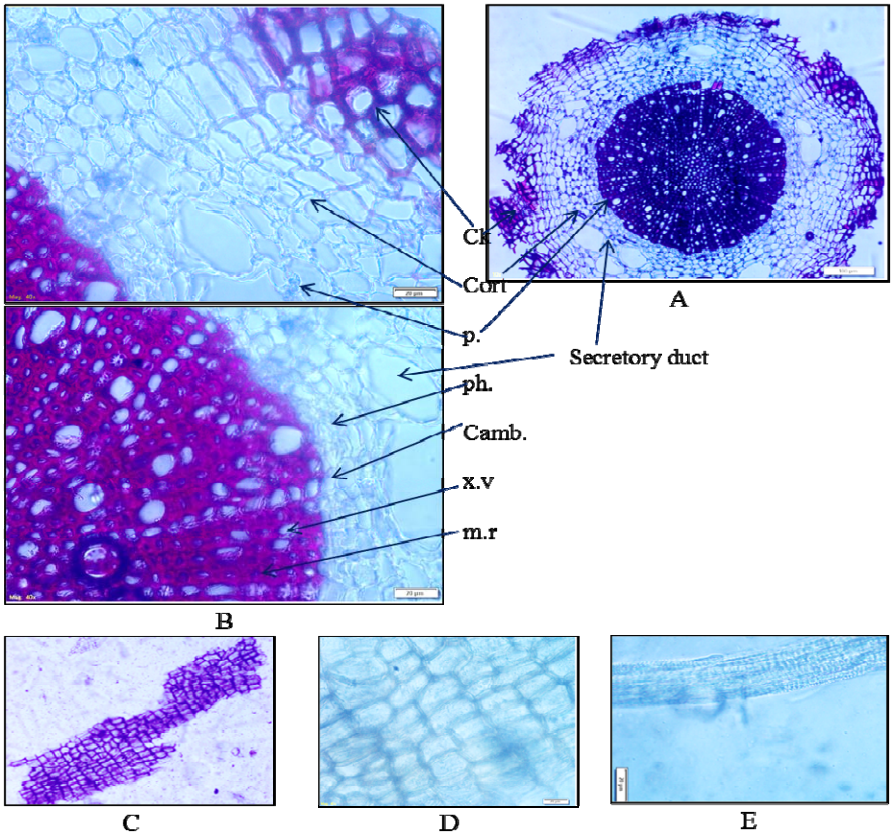

Fig. 8: Micromorphology of the root of *Carissa macrocarpa* (A) Low power view of the T.S. in the root (×100). (B) Detailed sector of the root (×200). (C) Cork cells side view (×100). (D) Cork cells top view (×400). (E) Xylem vessels (×400). camb. , cambium; ck., cork cells; cor., cortex; ph., phloem; x.v., xylem vessel.

3.4.2.6. The old stem

The structure of the old stem (Fig. 7A and 7B) is almost similar to that of the young stem, with the following differences:

1. In addition to usual epidermal cells, it is characterized by presence of some cork cells (Fig. 7B) which are radially arranged in several rows that may reach 5-6 rows and some are ruptured. They are polygonal, tangentially elongated cells with lignified walls. A narrow normal cortical tissue like that of young stem follows the cork.
2. There is a distinct layer of parenchymatous cells under the epidermis containing brownish to yellowish brown content. As well as there are some schizolysogenous ducts (Fig. 7C) in the cortical tissue from which the white milky exudate comes out upon breaking the stem.
3. The vascular tissue is wider and of the same type (collateral) while the pith is, narrower compared to that of young stem.

3.4.2.7. The root

A transverse section (Fig. 8A and 8B) in the root is almost circular in outline. It is composed of an outer cork, followed by the secondary cortex, which is formed of parenchyma cells. The pericycle is formed of paernchymatous cells. The endodermis is indistinct. The vascular system is relatively wide forming a continuous ring at the middle zone of the root.

3.4.2.7.1. The cork; the cork cells (Fig. 8A, 8B and 8C) are tangentially elongated tabular cells with lignified walls. They are radially arranged in 6-9 rows where some rows are collapsed and some are ruptured.

3.4.2.7.2. The cortex; it consists of 4–5 rows of oval to elongated thin walled parenchyma cells (Fig. 8A and 8B) containing scattered starch granules which are simple or compound (2-3), rounded, without visible hila or striations.it contains large ducts from which the white milky exudate come out upon breaking the root. The endodermis is not distinct.

3.4.2.7.3. The pericycle; it is formed polygonal isodiametric or elongated thin-walled parenchyma cells. (Fig. 8A and 8B)

3.4.2.7.4. The vascular tissue; the vascular tissue (Fig. 8A and 8B) is very wide and cross-sectioned by uni-seriate medullary rays. The phloem is formed mainly of thin-walled phloem parenchyma, sieve tubes, and companion cells. The cambium is formed of 2 rows of thin-walled tangentially elongated cellulosic cambiform cells. The xylem is formed of lignified radially arranged elements. The vessels are mostly spiral. Wood fibers are fusiform, with straight or undulating lignified walls, narrow lumen and acute apices.

3.4.2.8. The powdered root

The powder (Fig. 8C, 8D and 8E) is yellow in color having a characteristic odor and a pungent taste. Microscopically, it is characterized by the presence of the following; Small parts of cork cells which are polygonal cells with lignified walls. Fragments of lignified xylem vessels, which are mostly of spiral thickening.

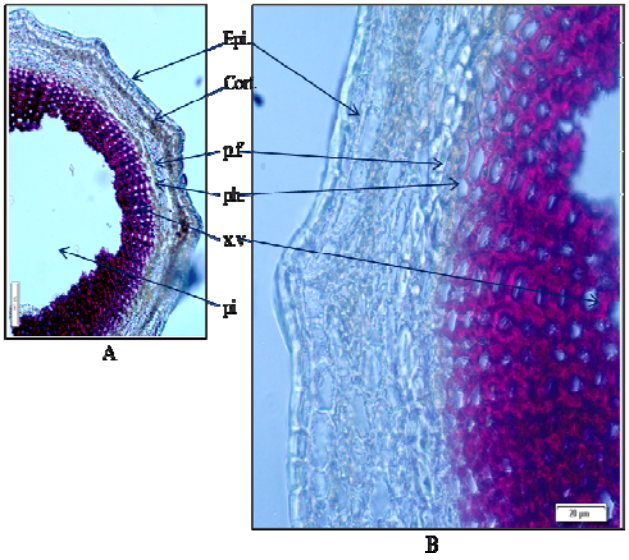

Fig. 9: Micromorphology of the root of *Carissa macrocarpa* (A) Low power view of the T.S. in the root (×200). (B) Detailed sector of the root (×400). ep., epidermis cor., cortex; p.f., pericyclic fibres; ph., phloem; x.v., xylem vessel; pi., pith.

3.4.2.9. The Spine

A transverse section in the spine (Fig. 9A and 9B) shows circular outline with some projections at intervals to look like stellate shape outline. It is clearly consists of an epidermis to outside, then the cortex which is composed of parenchymatous tissue. The pericycle tissue is parenchymatous showing scattered groups of non-lignified fibers. The vascular tissue is wide forming a continuous ring. The pith is wide hollow hole.

3.4.2.9.1. The epidermis; the epidermis (Fig.9A and 9B) is composed of one row of polygonal cells, having straight anticlinal walls and its covering cuticle is thick smooth. The epidermal cells are glabrous and show no stomata.

3.4.2.9.2. The cortex; the cortex (Fig.9A and 9B) is formed of 3-4 rows of rounded to irregular shaped parenchyma cells. The first layer close to the epidermis is empty from any contents followed by continuously row layer of parenchymatous cells containing brownish to yellowish brown content.

3.4.2.9.3. The pericycle; the pericycle (Fig.9A and 9B) is formed of collapsed parenchymatous cells with scattered groups of non-lignified fiber.

3.4.2.9.4. The vascular elements; it is composed a clear complete ring of collateral vascular bundle (Fig.9A and 9B). The phloem consists of collapsed undistinguishable elements. The xylem is formed of lignified radially arranged elements in columns of 4-5 vessels.

3.4.2.9.5. The pith; the pith (Fig. 9A and 9B) is formed of hollow central area.

Microscopical measurements of the different organs of *C. macrocarpa* are presented in Table 2

Table 2: Microscopical measurements of the different organs of *Carissa macrocarpa* (in microns).

Item	L			W			H			D		
Leaf												
Upper epidermis	22	28	29	6	8	13	6	10	13	-	-	-
Cortex parenchyma	-	-	-	-	-	-	-	-	-	15	19	25
Collenchyma	-	-	-	-	-	-	-	-	-	9	14	17
Lower epidermis	14	18	30	7	9	11	6	8	11	-	-	-
Xylem vessels	-	-	-	-	-	-	-	-	-	2	4	6
Stem												
Epidermis	-	-	-	10	12	14	7	8	10	-	-	-
Cortex parenchyma	-	-	-	-	-	-	-	-	-	13	16	20
Secretory duct	-	-	-	-	-	-	-	-	-	13	22	25
Fiber	-	-	-	-	-	-	-	-	-	4	5	7
Xylem vessels	-	-	-	-	-	-	-	-	-	4	8	12
Root												
Cork cells	21	25	29	13	14	18	8	9	10	-	-	-
Cortex parenchyma	-	-	-	-	-	-	-	-	-	12	16	23
Secretory duct	-	-	-	-	-	-	-	-	-	35	40	70
Xylem vessels	-	-	-	-	-	-	-	-	-	5	10	15
Spine												
Epidermis	-	-	-	8	9	10	5	7	8	-	-	-
fiber	-	-	-	-	-	-	-	-	-	5	6	10
Xylem vessels	-	-	-	-	-	-	-	-	-	4	5	6
Ca Oxalate clusters	-	-	-	-	-	-	-	-	-	21	25	29
Starch granules	-	-	-	-	-	-	-	-	-	1	2	3
Reddish brown droplets	-	-	-	-	-	-	-	-	-	1	3	5

L, length; W, width; H, height; D, diameter.

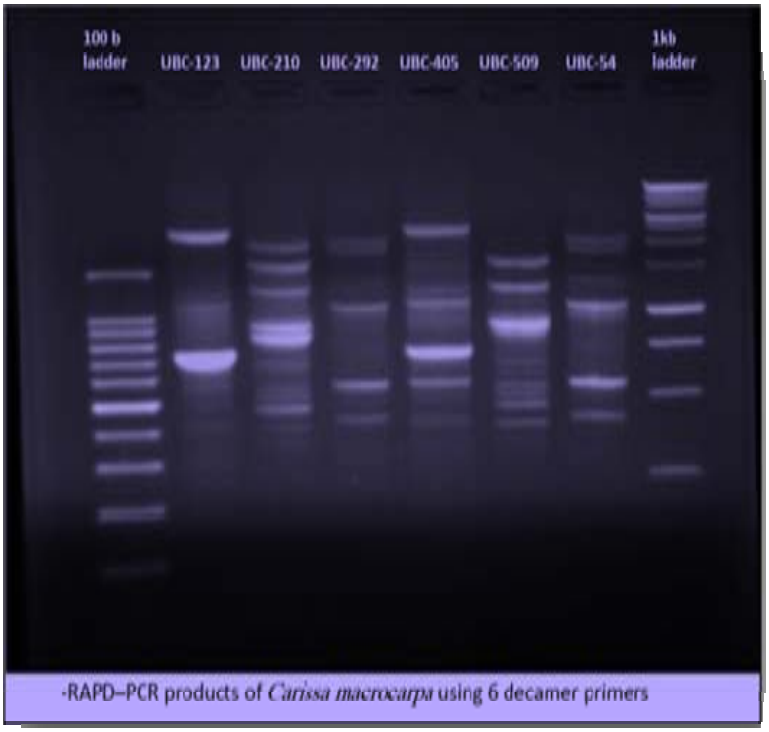

Fig. 10: RAPD-PCR products of *Carissa macrocarpa*

3-5-DNA fingerprinting

In the current study, the extracted genomic DNA of *C. macrocarpa* was amplified using 6 decamer primers to detect the possible amplification of its DNA. All of the 6 primers successfully directed the amplification of a genome-specific fingerprint of DNA material. The resulting

band profiles produced by the primers used in the RAPD analysis are represented in Fig. 10. The used 6 primers generated a total of 55 fragments. The 6 primers gave multiple band profiles with the biggest number of amplified DNA base pairs with UBC-210 which was 11 bands which is considered as the most effective one in selective amplification followed by UBC-405 which produced 10 bands then UBC-509, UBC-54 and UBC-292 each produced 9 bands. On the other hand, the least number of bands was 7, produced by UBC-123.

4. CONCLUSION

C. macrocarpa is characterized chemically by the presence of various secondary metabolites at different levels in different extracts of plant organs. This plant is rich in phenolic contents. In addition, it is characterized microscopically by presence of big Ca oxalate clusters as well as fibers with forked apex and undulating wall and absence of any kind of hair. The results from DNA amplification pattern recommend the use of primers UBC-210, UBC-405, UBC-509, UBC-54, UBC-292and UBC-123 for the selective discrimination of genomic DNA. The present study will be beneficial and supportive in authentication of *C. macrocarpa*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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