Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
https://www.phytojournal.com
JPP 2023; 12(5): 422-427
Received: 07-08-2023
Accepted: 08-09-2023

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Phenolic principles and pharmacognostic studies of fruits of *Aegle marmelos*

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Abstract
Fruits of *Aegle marmelos*, a well-known medicinal plant used against gastric problems, has pharmacognostic data which are almost nil or insufficient. Fresh fruits of *Aegle marmelos* and its powder were subjected to microscopy studies using standard procedures. Phenolic acids were identified using 2-dimensional paper chromatography, while flavonoids were identified by spectral studies after separation using paper chromatography. The pulp and rind of fruits contain flavonols like quercetin and isorhamnetin. The rind also contained additional flavonols kaempferol and 4'-methoxy kaempferol. Vanillic and *o*-coumaric acids have been identified for the first time from pulp. Rind also have been found to contain syringic, vanillic, melilotic and *o*-coumaric acids. Condensed tannins were present in higher amounts in the pulp than in the rind. The amount of mucilage (37%) in pulp also is found to be much higher than reported. Pharmacognostically pulp is very distinguishable with typical sclereids, broad spirally thickened tracheids with oblique end walls and mucilaginous slime cells. The rind is essentially characteristic of the plant with heavily lignified sclereids of varying shapes and sizes, thick walled colourless cells containing chromoplasts and oil ducts. The absence typical parenchyma, chloroplasts, hairs, glands, starch, sphaeraphides etc are other features of identification. The present work puts forward key microscopic characters present in *Aegle marm*elos fruits, as well as establishes the difference in flavonoids and phenolic acids present in pulp and rind of the fruit.

Keywords: *Aegle marmelos*, fruits, flavonols, phenolic acids, pharmacognosy, sclereids, slime cells

Introduction
Aegle marmelos (Linn.) Corr. (Sanskrit-Bael or Bilva) is a well-known medicinal plant of India, all parts of which are used in medicine. The roots form one of the components of "Dasamoola". Leaves are considered efficacious in diabetes and fever. Root bark is found to inhibit palpitation of heart. Fruits are proved to be excellent remedy for diarrhea and irritable bowel syndrome [1]. The fruit is globoid and yellowish orange in colour, when ripe, with a sweet aroma. It has a woody rind and a yellowish pink mucilaginous pulp. The pulp is medicinally important. The pulp diluted with water gives a delicious drink and are used to prepare squash, jam and fruit nectar.

Traditionally the unripe fruits of Bael are used to assist digestion and relieve stomach irritation. They are bitter in taste and astringent in nature. The ripe fruits are considered to be more effective than the raw fruits since they prevent chronic and sub-acute dysentery. The fruit pulp stimulates the mucous membranes of the intestines and helps control diarrhea [2].

Methanolic extracts of fruit is found to act against SKBR3 human breast adenocarcinoma cells. Aqueous homogenate of fruit is proved to prevent CCl_4 induced hepatotoxicity in rat and mice models. The fruit is also found to possess number of activities such as reducing burning sensation in eyes, prevention of diabetes and cardiac dysfunction [3]. Marmalide present in all parts of plant showed great antiviral activity against human coxsackie virus [1, 4]. Aqueous extract of the fruit is found to possess high radical scavenging activity as against DPPH and nitric oxide free radicals [5, 6].

Fruit pulp of *A. marmelos* indicate presence of tannins, phenolic acids, flavonoids, alkaloids, terpenoids, coumarins and pectins. Coumarins present are aegeline, aegelenine, *o*-methyl halfordinol, o-isopentenyl halfordinol, marmelin, marmelosin, psoralenluvangetin, aurapten and alloimperatorin [7-12].

Volatile principles in fruit include p-cymene, dehydro-p-cymene, citronellal, hexanal, isoamyl acetate, β-phellandrene, limonene, carvone, linalool; 3,5-octadiene2-one, α-cubebene, β-cubebene, trans-p-mentha-2,8-dienol, citronellal, cineole, citral, cuminaldehyde, β-caryophyllene, hexadecane, pulegone, acetoin, verbenone, carvyl acetate, β-ionone, dihydro-β-ionone, caryophyllene oxide, α-humulene, humulene oxide, hexadecanoic acid (E)-2-octenal, (E,E)-2,4-heptadienal and (E)-6,10-dimethyl-5, 9-undecadien-2-one [9].

The fruits contain a group of coumarins, volatile oil, gum (2%) and tannins (7-9% in pulp and 18-22% in rind). The principal coumarins are furano-derivatives marmelosin and allo-imparatorin. β-sitosterol is also reported to be present. Tannin is more concentrated in the rind (18-22%). Seeds yield a fixed oil (35%) with oleic (30%) and linoleic acids (36%) as the major fatty acids [12]. The gum is an acidic polysaccharide with galactose (71%) arabinose (12.5%), rhamnose (7.5%) and galacturonic acid (7%) [13]. In the present work, the fruit is studied for its phytochemical and pharmacognostic characters. The rind and pulp have been studied individually since there is no data on the pharmacognosy of the fruit.

Materials and Methods

The fruits were collected from the Botanical Garden, The Maharaja Sayajirao University of Baroda, Vadodara. The pulp and rind were separated, dried at 60 ºC, and powdered. They were then extracted with methanol and analyzed for flavonoids, tannins and phenolic acids using standard procedures [14, 15].

Flavonoids

Phytochemical analysis of flavonoids, phenolic acids and sterols were performed using methanolic extract of *A. marmalos* fruit and rind powder for which 50g each were subjected to extraction using a Soxhlet's apparatus with methanol. The methanol from the extract was removed under reduced pressure and about 100 ml water was added. The water soluble glycosides were filtered out and the filtrate was hydrolysed in a water-bath for one hour using 7% HCl. Precipitates of phlobaphenes (condensed tannins) in the hydrolysate were filtered out and estimated gravimetrically. The hydrolysate was cooled and aglycones were extracted using diethyl ether, which was then used for further analysis. The ether fraction was banded on Whatman No.1 chromatographic paper and developed in 30% acetic acid solvent system. The developed paper was observed under UV 366 nm light and separated bands of flavonoids were marked out. These marked bands were cut out from the paper and eluted using spectroscopic grade methanol. The UV spectra of each eluted band was recorded on Shimadzu UV1800 spectrophotometer.

Phenolic acids

For identification of phenolic acids, the ether extract was spotted on Whatman No.1 chromatographic paper and subjected to two-dimensional ascending paper chromate-graphy. The paper was first developed in toluene: acetic acid: water (6:7:3 v/v/v, upper organic layer) in the first direction and sodium formate: formic acid: water (10:1:200 w/v/v) in the second direction. Stock solution of derivatizing agent *p*-nitroaniline was prepared by dissolving 700mg *p*-nitroaniline crystals in about 10 mL conc. HCl and diluted up to 100mL. The final diazotizing spray reagent was prepared by mixing 5mL of 1% $NaNO_2$ solution and 5mL of *p*-nitroaniline stock solution and making up the volume to 100mL using ice-cold water. The 2-D chromatography paper developed using the two solvent system was sprayed with the prepared diazotizing reagent, followed by a spray of 10% Na_2CO_3 solution. The various phenolic acids separated on the paper were identified using the colors they developed at specific R_f values.

Steroids

For analysis of steroids the ether fraction was spotted on a $60F_{254}$ silica percolated TLC plate and developed using solvent system chloroform: carbon tetrachloride: Acetone (2:2:1 v/v/v). The plate was sprayed with 5% sulfuric acid solution and heated to 85-90 °C for 15 minutes. Characteristic bands of steroids appeared on heating the plate.

Quinones

Quinones were analyzed using the benzene extract of the fruit pulp, prepared by subjecting 15g each of fruit and rind powders to extraction by toluene using a Soxhlet's apparatus. The solvent was removed under reduced pressure and the residue was dissolved in diethyl ether, and repeatedly shaken with 2N Na_2CO_3 solution in a separating funnel. The upper diethyl ether layer is referred to as neutral fraction. The aqueous layer was separated and treated with ice-cold 2N HCl dropwise till the precipitate formed settled down. This acidified solution was treated with another aliquot of diethyl ether in a separating funnel. The lower aqueous layer was discarded and the upper ether layer was taken. This is referred to as acidic fraction. Both acidic and neutral fractions were spotted on a $60F_{254}$ silica precoated TLC plate and developed in solvent system petroleum ether-toluene (9:1 v/v). The developed plate was sprayed using 2% magnesium acetate solution and heated to get characteristic bands of quinones.

Coumarins

Methanolic extract of the fruit and rind were spotted on $60F_{254}$ silica pre-coated TLC plate and developed in solvent system toluene-diethyl ether (1:1 v/v) saturated by 10% acetic acid. The plate was then sprayed using 10% ethanolic KOH to observe bright fluorescent bands of coumarins when observed under UV light.

Mucilage

Mucilage content was estimated gravimetrically by boiling 10 g of fruit pulp in 500 mL water for about an hour. The solution was filtered and the filtrate was concentrated to about 50 mL. About 500 mL of methanol was added which caused the precipitation of the mucilage. The precipitates were filtered and dried in hot air oven at 110ºC, and weighed to calculate mucilage content.

Micro morphological and anatomical studies were carried out using fresh fruit material. Thin sections were taken by hand, stained with safranin and mounted in glycerine. They were then observed under microscope and photographs were taken. The size (dimensions) of various cells were measured using stage and ocular micrometers. The quantitative data are based on the average of 20 readings. The powder of dried rind and pulp were taken in microscopic slides, stained with safranin and mounted in glycerine. The constituent cells observed under various magnifications and the cellular dimensions marked by ocular and stage micrometers and identified [16].

Results

Phyto-chemistry

The fruit pulp is found to contain flavonoids, phenolic acids, tannins, coumarins and mucilage. The flavonoids isolated using paper chromatography and spectral analysis were identified were quercetin and isorhamnetin. Vanillic and *trans*-o-coumaric acid were the phenolic acids present post separation using 2-D paper chromatography and derivatization using diazotization reagents, latter in large amounts. The tannins present are of condensed tannins as evidenced by presence of large amount of phlobaphenes and anthocyanins. Phlobaphenes estimated gravimetrically post acid hydrolysis amounted to 37%. The mucilage precipitated by addition of methanol to the aqueous extract was quantified to be 24% by gravimetry. Also present in pulp are coumarins

and β-sitosterol. The fruit rind contained flavonols, quercetin, isorhamnetin, kaempferol and 4'-methoxy kaempferol. The phenolic acids identified were vanillic, syringic, melilotic and *o*-coumaric acids. Tannin content in the form of phlobaphenes was calculated to be 3.75% which is quite low as compared to pulp, and mucilage content was found to be 19%. In addition, TLC analysis showed the presence of quinone glycosides in the rind.

Pharmacognosy

The rind is very hard and is about 1.5 to 2 mm in thickness. It is differentiated into two regions, an outer thin yellow region delimited by epidermis on the outside and the inner dark orange layer which encloses the pulp inside (Fig.1A). The outer layer which is about 400 µm in thick, contains many oil ducts. This layer consists of sclerenchyma like thick walled cells, but their walls are not stained by safranin. Therefore, the walls would be made of cutin or other lipids. The wall is smooth and the cells are closely packed with no intercellular spaces. The shape of cells is hexagonal, square or rectangular. The hexagonal cells are approximately 30 µm broad and 40 µm in height. Most of the cells contain numerous yellow chromoplasts. The oil ducts are schizolysigenous in nature containing many broken cells (Fig.1B). They vary in size. One of the large oval glands has been found to have an approximate breadth of 270 µm and height of 180µm. Epidermis consists of square or rectangular cells having 15-17 µm breadth and 12-14 µm height. The cuticle is very heavy having the same breath as of the epidermis (15-17 µm), (Fig 1C).

The inner layer of rind is about 3 to 4 times thicker than the outer layer and consists of closely packed heavily pitted lignified sclereids. Three types of sclereids are recognized based on size and shape. The majority are hexagonal sclereids having a diameter of about 50 µm with thick walls (13 µm thick) and a lumen of 23-25 µm (Fig.1D). They are arranged as a continuous layer extending from the colourless outer region to the pulp. Transversing this are many benches of parallel arranged long sclereids (about 170 µm or more in length and about 25-30 µm in breadth and a little or no lumen inside) which are arranged radially as partitions separating the hexagonal sclereids. This bunch of sclereids extends a little inside to outer colourless region and inner pulp region and goes deep down to parenchymatous layer. In both the protruding regions, sclereids of intermediate sizes are observed. Inner to the sclereid layer is the layer of parenchyma which joins with the pulp tissues, which are of the same size as sclereids of rind (50 µm). The walls of these cells are thick with mucilage, closely packed with no intercellular spaces (Fig.1E).

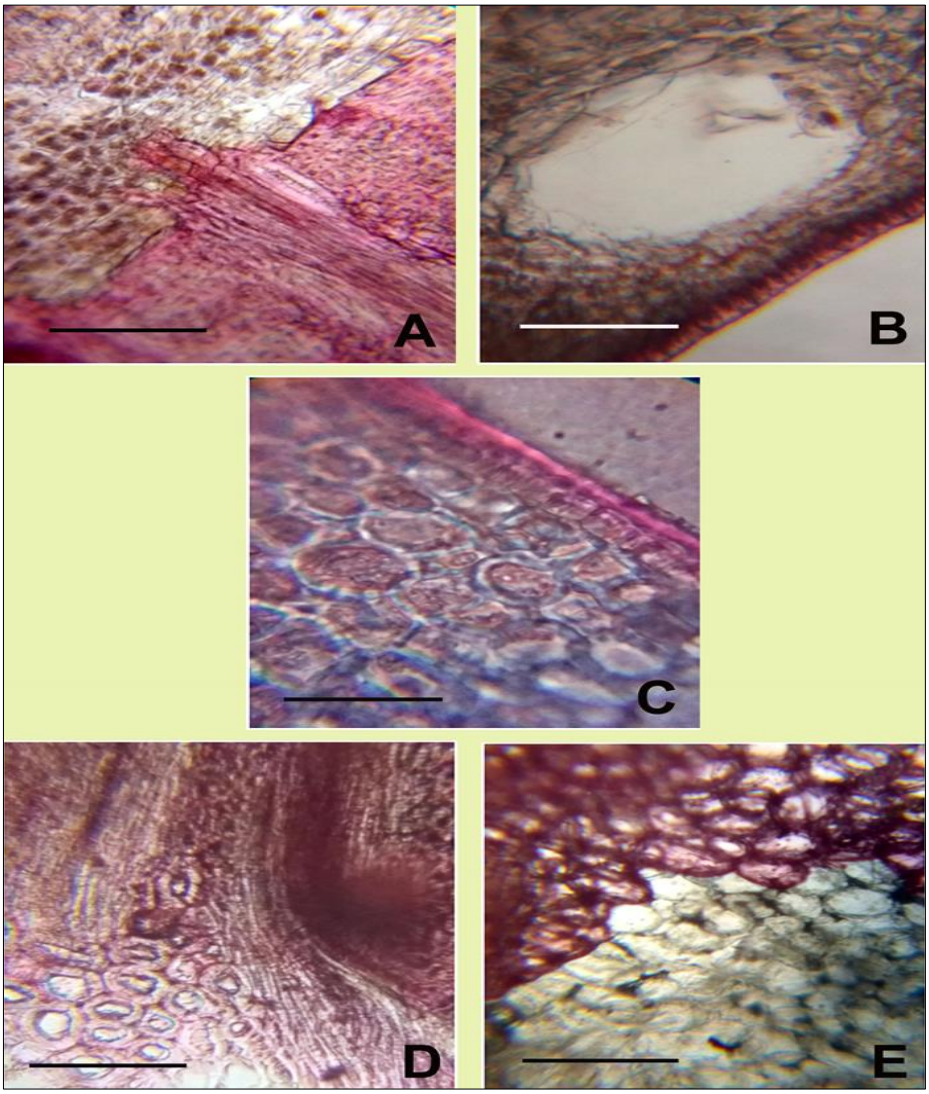

Fig 1: (A) Outer colorless region and inner lignified region of the rind. (B) Schizolysigeneous oil duct. (C) Outer region with chromoplasts and thick cuticle. (D) Sclereids of varying shapes and sizes in inner rind. (E) The region where rind (thick walled, red in colour) joins the pulp (white-thin walled). Scale bar: 100 µm

In the center portion of the fruit pulp, the axis consists of a number of vascular bundles arranged in the form of a stele. Most commonly the stele consists of two umbrella shaped (somewhat T shaped) large vascular bundles (1200-1400 µm diameter at the broadest outer end and about half in height 600- 700 µm) arranged in a line with the tail ends pointed to each other (Fig. 2A). Each vascular bundle consists of 10-15 rows of panels containing tracheids separated by medullary rays containing rectangular or square calcium oxalate crystals. The region of tracheids is flanked on either side obconical panels consisting of closely vertically packed xylem fibers (Fig. 2B). These two large vascular bundles are connected with a central patch of large sclereids similar to that of rind. On the outer side of T-shaped bundle are three smaller vascular bundles, appearing like three leaf traces. These vascular bundles are having a peculiar structure. They have 2-5 xylem tracheids either arranged in radial rows or as a group towards the center. This is surrounded by an incomplete broad ring of xylem fibers and the ring is open where it meets tracheids (Fig 2C). The fibers are long (up to 400 µm), thick walled and have a narrow lumen. This patch is thickest up to 10 layers on outside (towards periphery) 6-7 layers on both the sides and 1-2 layers towards center of axis. This fibrous ring is surrounded by a larger ring of phloem cells which is also is open at the side of xylem tracheids. Outer to phloem are a few pericyclic sclereids in a layer.

The fruit pulp is sticky with mucilage. It is yellowish in colour and contained many seeds suspended in pulp. A large number of vascular bundle originate from the axis of fruit and extend to the pulp and traverse the pulp at all regions and appear as threads. Xylem is composed of linear rows of tracheids separated by xylem parenchyma. These tracheids have annular or close spiral thickenings. Each tracheid is 34 to 37µm broad and 110 to 130 µm in length with oblique or near transverse end walls (Fig. 2D). Surrounding the vascular bundles are slime parenchyma which are rectangular reaching a length of 160- 175 µm and a breadth of 35 to 45 µm (Fig 2E).

Powder study

Both pulp and rind have been studied separately. The pulp powder is very soft and contained central vascular bundles with broad tracheids with many annular/spiral thickenings having oblique end walls and phloem parenchyma with square or rectangular crystals (Fig 3A, 3B & 3C), small vascular bundles running criss-cross to the pulp with one or two tracheids surrounded by phloem, (Fig.3D), sclereids seen in the center of axis (Fig.3E) and mucilaginous slime cells (Fig 3F).

The powder of rind is simple in structure with lignified sclereids of varying types of the inner region (Fig. 3G & 3H), colorless thick-walled cells with chromoplasts of outer region (Fig 3I) and oil glands (Fig 3J).

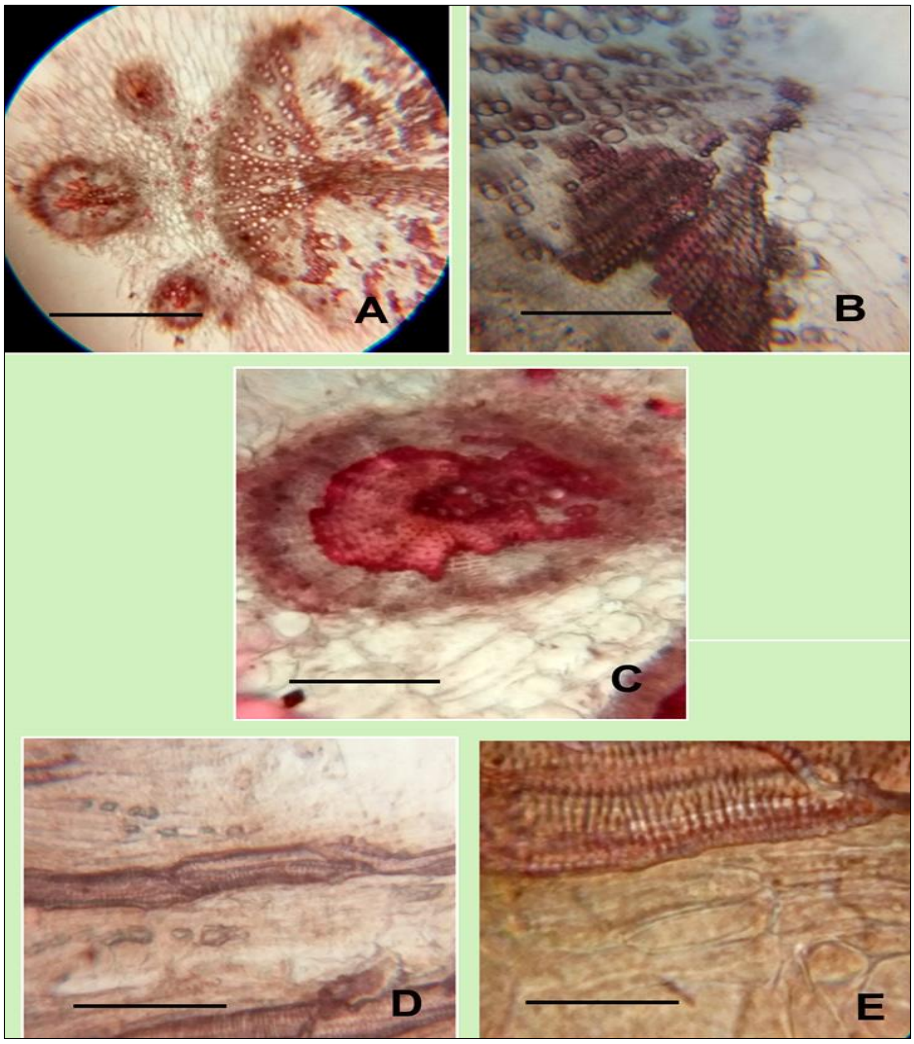

Fig 2: (A) Umbrella shaped vascular bundle with three peripheral smaller vascular bundles. (B) Two panels of xylem fibers on either side of tracheids. (C) Outer bundles with tracheids surrounded by xylem fibers, circular phloem and pericyclic fibers. (D) Tracheids of vascular bindles of pulp. (E) Tracheids of vascular bindles of slime parenchyma surrounding. Scale bar: 100µm

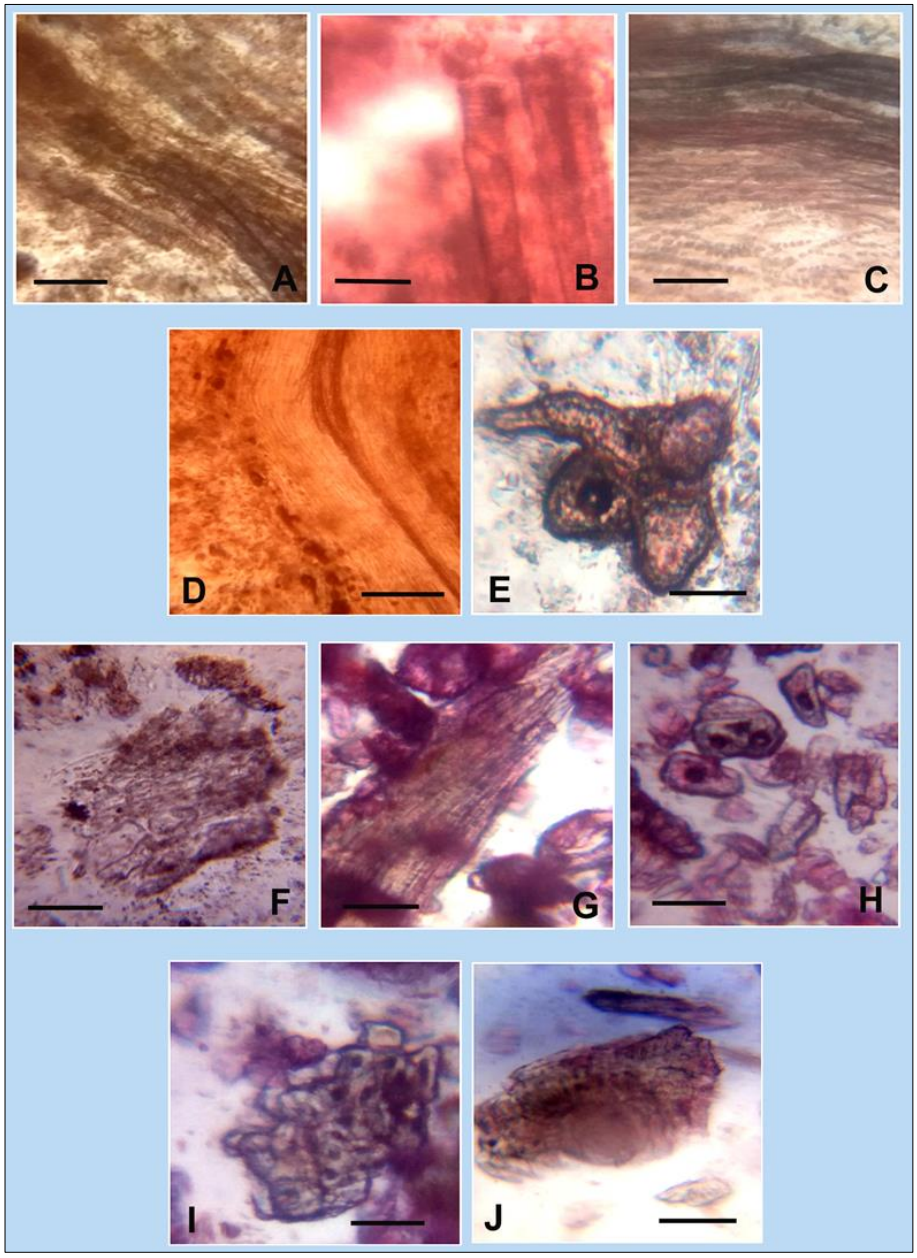

Fig 3: (A, B) Annular tracheids with oblique end walls. (C) Phloem parenchyma containing rectangular crystals. (D) Small vascular bundles running criss-cross in the pulp. (E) Sclereids in the center of axis. (F) Mucilaginous slime cells. (G, H) Lignified sclereids of varying types of the inner rind region. (I) Colorless thick-walled cells with chromoplasts of outer rind. (J) Rind with oil glands. Scale bar: 50 µm

Discussion

The present study unearths a number of phytochemical as well as a complete set of pharmacognostic characters. The presence of flavonols, quercetin and isorhamnetin and phenolic acids like vanillic and *trans*-o-coumaric acid are reported for the first time from pulp. Even the occurrence of four flavonols, quercetin, isorhamnetin, kaempferol and 4'methoxykaempferol and a number of phenolic acids such as vanillic, syringic, melilotic and *o*-coumaric acids as well as location of quinones from rind are new reports. The amount of mucilage and tannins are different from those reported earlier. Contrary to previous report of more tannins in rind (18-22%) and less in pulp (7-9%), we quantified more tannins in pulp (37%) and very less in rind (3.75). As against the earlier reports of 2% gum in fruits we quantified gum/mucilage in pulp to be 24% and of rind as 19%.

As the data on pharmacognosy of *Aegle marmelos* fruits are very insufficient, the present study unveils a whole range of such characters like, typical sclereids, broad spirally thickened tracheids with oblique end walls and mucilaginous slime cells from pulp. The rind is essentially typical of the plant with heavily lignified sclereids of varying shapes and sizes, thick walled colorless cells containing chromoplasts and oil ducts. The absence typical parenchyma, chloroplasts, hairs, glands, starch or cluster crystals etc. are other features of identification.

Conclusion

The study reveals a whole gamut of both phytochemical as well as pharmacognostic characters which were unavailable so far. Flavonols quercetin and isorhamnetin, phenolic acids vanillic and *o*-coumaric acids were found common in both pulp and rind of the fruit. Kaempferol and its methoxy derivative were the additional flavonols, while vanillic and o-coumaric acids were the additional phenolic acids identified in the rind. Tannins were observed to be higher in pulp than in the rind. The amount of mucilage (37%) in pulp also is found to be much higher in the pulp. Pharmacognostically pulp is very distinguishable with typical sclereids, broad spirally thickened tracheids with oblique end walls and mucilaginous

slime cells. The rind is essentially characteristic of the plant with heavily lignified sclereids of varying shapes and sizes, thick walled colourless cells containing chromoplasts and oil ducts. The absence typical parenchyma, chloroplasts, hairs, glands, starch and sphaeraphides are other features of identification.

Source of Funding: None

Acknowledgments: None

Approval under ethical considerations: Not applicable

Author contributions

Mammen Daniel contributed to data collection, data analyses, data interpretation, and drafting the manuscript; Denni Mammen contributed to data collection and drafting the manuscript. Both authors approved and read the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

Ethical Considerations

All authors have considered all ethics in research for the entire work.

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