Anatomical investigation and GC-MS analysis of "Coco de Mer", *Lodoicea maldivica* (J. F. Gmel.) Pers. (Arecaceae)

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Lodoicea maldivica (J. F. Gmel.) Pers. (Arecaceae), 'Coco de Mer', is a palm, growing as endemic in the Seychelles islands. Its fruit weighs up to 20 kg and is characterized by a fleshy and fibrous envelope surrounding the nutlike portion. The present work combines a morpho-anatomical and a phytochemical analysis of the fruit exocarp and mesocarp.

The exocarp is composed by a layer of palisade cells. The mesocarp is characterized by vascular bundles and by sclereids. In the aerenchyma, the internal zone of the mesocarp, cells aggregates were positive to phenols, while idioblasts were positive to terpenes.

We performed a GC-MS analysis with a semi-quantitative relative amount calculation of the recorded compunds.

The GC-MS essential oil profile revealed the dominance of acyclic sesquiterpenoids (53.95%), followed by bicyclic sesquiterpenoids (31.69%), monoterpenes (11.89%) and monocyclic sesquiterpenoids (2.44%).

The terpens detected in higher amounts, β-caryophyllene and bicyclogermacrene, are known for activity against insect larves, but have been proposed as anti-viral candidates with against SARS-CoV-2. The third compound in amount, aromadendrene, is active against bacteria and, again, known to possess insecticidal properties.

Keywords: Coco de Mer • *Lodoicea malvidica*• Anatomy • Essential oil • GC-MS

Abbreviations: EI = Electron Ionization; GC-MS =; PAS = Periodic Acid Schiff reaction; PDMS = Polydimethylsiloxane

Introduction

Lodoicea maldivica (J. F. Gmel.) Pers. (Arecaceae), known as 'Coco de Mer' (Fig. 1A) is one of the six palm species, naturally occurring in two small islands of the Seychelles archipelago, i.e. Praslin (37 km2) and Curieuse (2.73 km2) in the Western Indian Ocean [1]. Until the 19th century, *L. maldivica* trees covered most of the surface of the isles [2],[3]; afterwards, the overexploitation of these forests by humans [4][5][6] have led to soil erosion, invasion by alien species and biodiversity loss. Since 2011 *L. maldivica* is recognized as endangered species by the International Union for Conservation of Nature (IUCN) [7], therefore the tree cutting for timber and the touristic demand of nuts are deemed illegal, to promote the regeneration of this endemic species. The largest forests of *L. maldivica* are located in protected areas (Vallée de Mai, Fond Peper within Praslin National Park, and Ravin de Fond Ferdinand Nature Reserve) [8][9][10][11] in the south of Praslin [12].

'Coco de Mer' is a dioecious plant. The male inflorescences are catkins 1.2-1.8 m long and 8-10 cm wide [13], consisting of 60-70 flowers embedded into leathery bracts [14]. The female plants (Fig. 1A) bear a zigzag rachilla with 5 up to 13 flowers which are the biggest (diameter of about 5 cm) among palms [12]. The mechanism of pollination in 'Coco de Mer' is still unclear [13] suggesting that it is carried out by animals attracted by the honey smell of both male and female flowers. Besides, rain and wind have an important role in pollination [11]. 'Coco de Mer' fruit is characterized by a fleshy and fibrous envelope surrounding the nutlike portion, which is generally two-lobed [15]. It is due to this peculiar morphology that *L. maldivica* is also called as "Double coconut". The fruit (Fig. 1B) is the largest in the plant kingdom [16], weighing 20 kg with a pyrene of a mass of

as much as 5 kg [3][17]. The fruit takes 7 years to reach complete maturity [11][13]. Unlike the Coconut that germinates even after floating 110 days in the seawater [18], 'Coco de Mer' nuts are generally too dense to float. Few nuts of *L. maldivica* can have enough air spaces (in aerenchyma) to float, but the seed can't survive for long journeys [19], thus it seems improbable that their diffusion occurs by sea and, as a matter of fact, the plant did not spread to other seasides outside the Seychelles Islands.

Figure 1. General aspect of the plant. (A) Female specimen of *L. malvidica* (courtesy of Chiara Falsini). (B) Fruit of *L. malvidica* (courtesy of Chiara Nepi, Herbarium Centrale Italicum, Museum of Natural Science of the University of Florence).

Since ancient times, various myths and legends have been associated with 'Coco de Mer'. The shells of Coco de Mer fruit were found floating in the Indian Ocean for centuries before their origin was discovered (whence the apparently wrong species name, depending of observations of the fruit on the Maldive Islands). For this reason, it was considered having magic properties [19]. 'Coco de Mer' fruits were used in many traditional beverages since they were considered by Seychelles natives energizing and aphrodisiac, thus to improve the performance of their everyday life [20]. Moreover, the fruit has long been used within Chinese Traditional Medicine for treating throat and chest complaints [21]or mainly as an ingredient of soups in Hong Kong, China [22]. Traditionally, the dried kernel of Coco de Mer was used by ayurvedic practitioners as antidiabetic medicine. The water of the green fruit and its soft kernel exhibited antiacid and antibilious effects [23]. In 2017 Seychelles' kernel export was

banned, but after two years, in 2019, this restriction was loosened with a new regulation where the exportation of the unprocessed kernel of a Mature Nut is permitted for scientific, diplomatic and any other justified purpose [24].

Literature offered only one contribution on the morpho-anatomy of the fruit at different stages of development [25]. Previous phytochemical works consisted in the characterization of the chemical composition of fruit kernel by gas chromatographic techniques to determine volatile aroma, sterol and fatty acid profiles [20][26].

In the light of the gaps of literature and in the framework of the enhancement of a threatened endemic species, the present work was aimed at studying 'Coco de Mer' fruit coupling a morphological and a phytochemical research approach. To this purpose, we examined: (i) the micromorphological features of the mature fruit, with special reference to the exocarp and the mesocarp, parts of the fruit that are normally discarded. The mesocarp can be subdivided in three layers: the most internal one about 0.5 cm thick in average; the intermediate one 0.3 cm and the most external layer only about 0.1 cm thick (see fig. 2C in Romanov et al.[20])We used histochemical techniques to localize in situ the different compounds of interest by means of light microscopy (LM) and (ii) Gas Chromatography-Mass Spectrometry (GC-MS) to analyze the composition of the exocarp and mesocarp fruit essential oil, extracted by hydro-distillation.

Results and Discussion

Fruit morphology and anatomy

The anatomy of the outermost layers of mature fruit is shown in Figure 2A. The exocarp was composed of single-layered palisade cells, defined as cells with the major axis orthogonal to the fruit surface. The mesocarp was composed of several cell layers. The outermost one, forming the hypodermis, exhibited irregularly distributed brachysclereids; below, tangentially elongated sclereids were organized in vertical rows of 35-40 cell layers (Fig. 2A). The innermost layers were composed of parenchymal cells and of numerous brachysclereids (solitary or organized in nests) and in particular, regularly developed vascular bundles with stout sheaths (Fig. 2D). Romanov et al. (2011) [25] described the multi-layered mesocarp of the mature fruit of *L. maldivica*, distinguishing six topographic zones. Figure 2A shows the first two zones of the mesocarp underlying the exocarp: the first is represented by the hypodermis, and the second by the innermost layers composed by parenchymal cells with brachysclereids and vascular bundles.

Concerning the histochemical investigation, the Fluoral Yellow-088 stain evidenced the lipidic components of the cuticle and the waxes covering the palisade cells of the exocarp (Figures 2B and 2C). Lipids were not found in the hypodermis or the immediately underlying region. In the second zone of the mesocarp, lipidic aggregates were consistently distributed in the lumen of the vessels grouped in bundles and in the parenchymal cells surrounding them (Figure 2D).

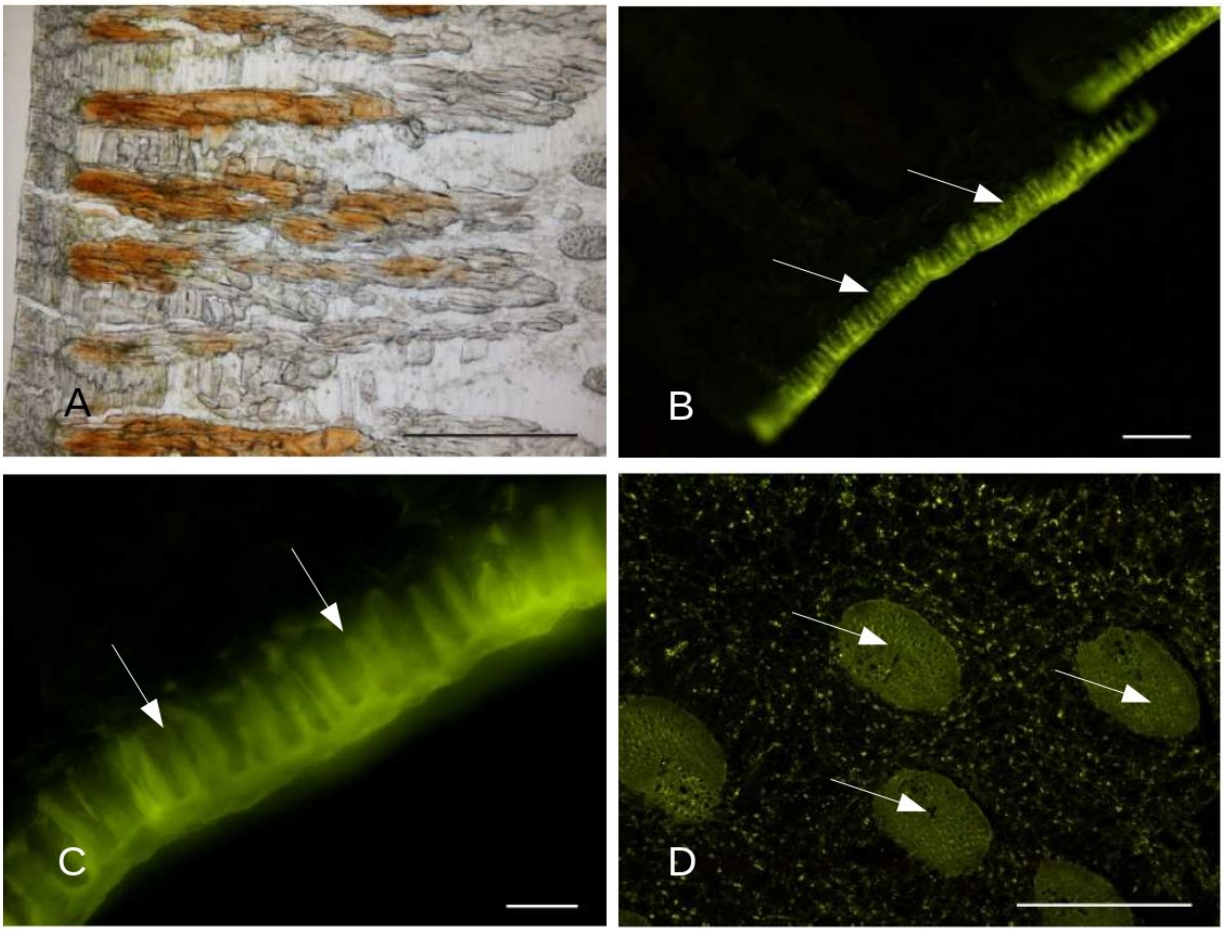

Figure 2. Cross-sections of the mature fruit of *L. malvidica*. (A) LM image showing the pericarp external layer and the two outermost zones of the mesocarp, without staining. (B) Fluorescence images with Fluoral Yellow-088, showing the positivity to lipids covering the exocarp. (C) a detail of Fig. 1b. (D) Second zone of the mesocarp stained with Fluoral Yellow-088. Scale bars: (A, D) = 500 µm; (B) = 100 µm; (C) = 25 µm.

Figure 3A shows the exocarp and the peripheral region of the mesocarp which was characterized by sclereids with thickened lignified walls as evidenced through the phloroglucinol staining. The distribution pattern of the sclereids below the exocarp is evident: some of them were grouped close to the palisade cells forming a compact layer, more inside the brachysclereids were arranged in bundles forming multiple layers which can reach the overall thickness of a few mm. The innermost zone of the mesocarp was characterized by numerous vascular bundles with lignin sheath and by sclereids located in the parenchymatous tissue (Figures 3B and 3C). Furthermore, the vascular bundles were characterized by the presence of terpenoidic content evidenced by the NADI reagent (Figure 3D). Scattered droplets of terpenoidic nature were also observed in the parenchymal tissue.

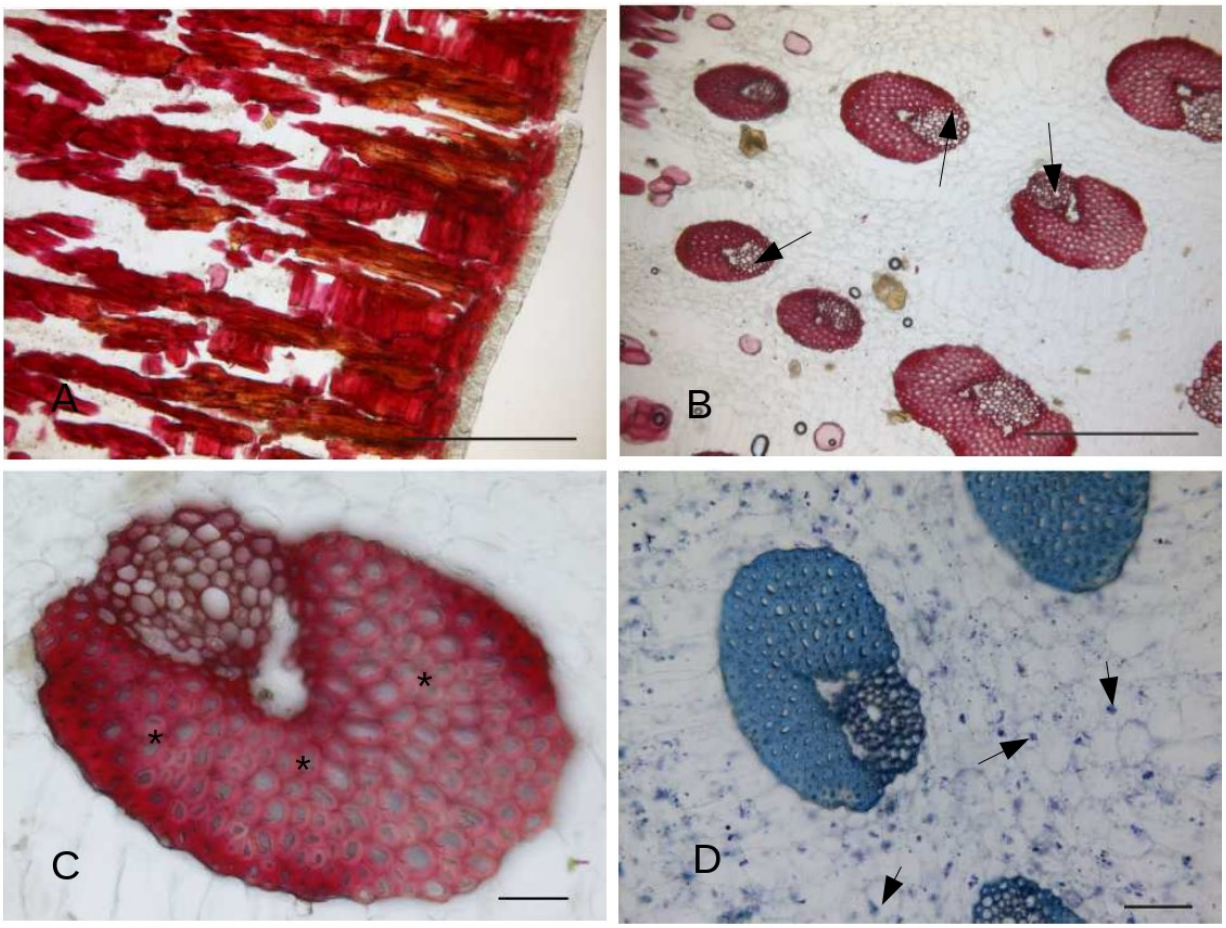

Figure 3. LM images showing cross-sections of the mature fruit of *L. malvidica.* (A) exocarp and hypodermis, stained with phloroglucinol. (B) mesocarp stained with phloroglucinol. Vascular bundles surrounded by sclerenchyma are indicated by arrows. (C) detail of a vascular bundle surrounded by sclerenchyma "helm" (asterisks). (D) mesocarp stained with NADI reagent showing vascular bundles surrounded by the parenchymal tissue. NADI positive bodies (arrows) can be observed inside the parenchymal cells. Scale bars: (A, B) = 500 µm; (C) = 50 µm; (D) = 100 µm.

The ferric trichloride staining revealed the presence of polyphenols filling the lumen of sporadic cells in the second zone of the mesocarp (Figures 4A and 4B). In the aerenchyma, the third zone of the mesocarp (Figures 4C, 4D, 4E, and 4F), cells with phenolic contents were grouped forming aggregates. Comparing the second and the third zones of the mesocarp, the cell morphology and distribution appeared different, along with the degree of intensity in the brown colouration of the stained cells, directly related to the amount of phenols. We found that the cells were stained brownish (Figures 4A and 4B) when they were scattered or gathered in small groups of two/three cells (Figures 4C), while when the number of the cells was higher, their coloration turned to an intensive dark brown (Figures 4D and 4E). Sporadically, they appeared reddish (Figure 4F).

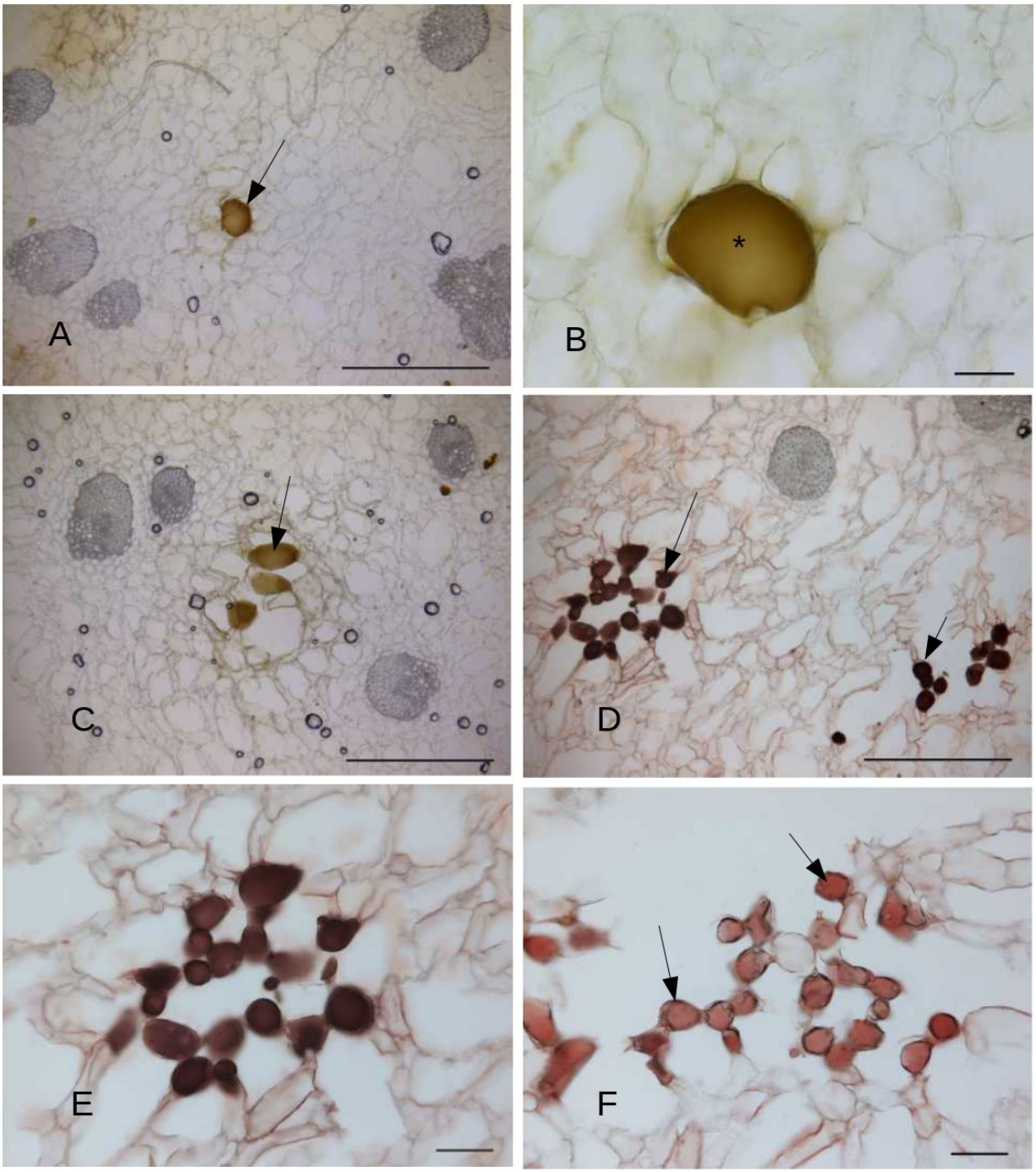

Figure 4. LM images of the cross-section of the mesocarp. (A) Ferric chloride III-positive idioblast (arrow) in the parenchyma. (B) Detail of the idioblast (asterisk). (C) Aerenchyma with presence of cells filled with tannins and phenols (ferric chloride III-positive, arrows). (D) other aerenchyma zone with darker ferric chloride III-positive (arrows). (E) Detail of Fig. 4D. (F) Large intercellular spaces of the aerenchyma are surrounded by ferric chloride III-positive cells (arrows). Scale bars: (A, C, D) =500 µm; (B) = 50 µm; (E, F) = 100 µm.

Figure 5A showed polysaccharidic material evidenced by PAS reagent in the parenchymal tissue and the vessel lumen of the vascular bundle. These components occurred in the parenchymal cells of the second zone (Figure 5C) of the mesocarp where differently-sized aggregates (20-60

μm) were irregularly distributed, but even in the aerenchyma where they constituted larger clusters (Figure 5B). It was noteworthy the presence of a proteic envelope covering some organelles within the parenchymal cells (Figure 5D).

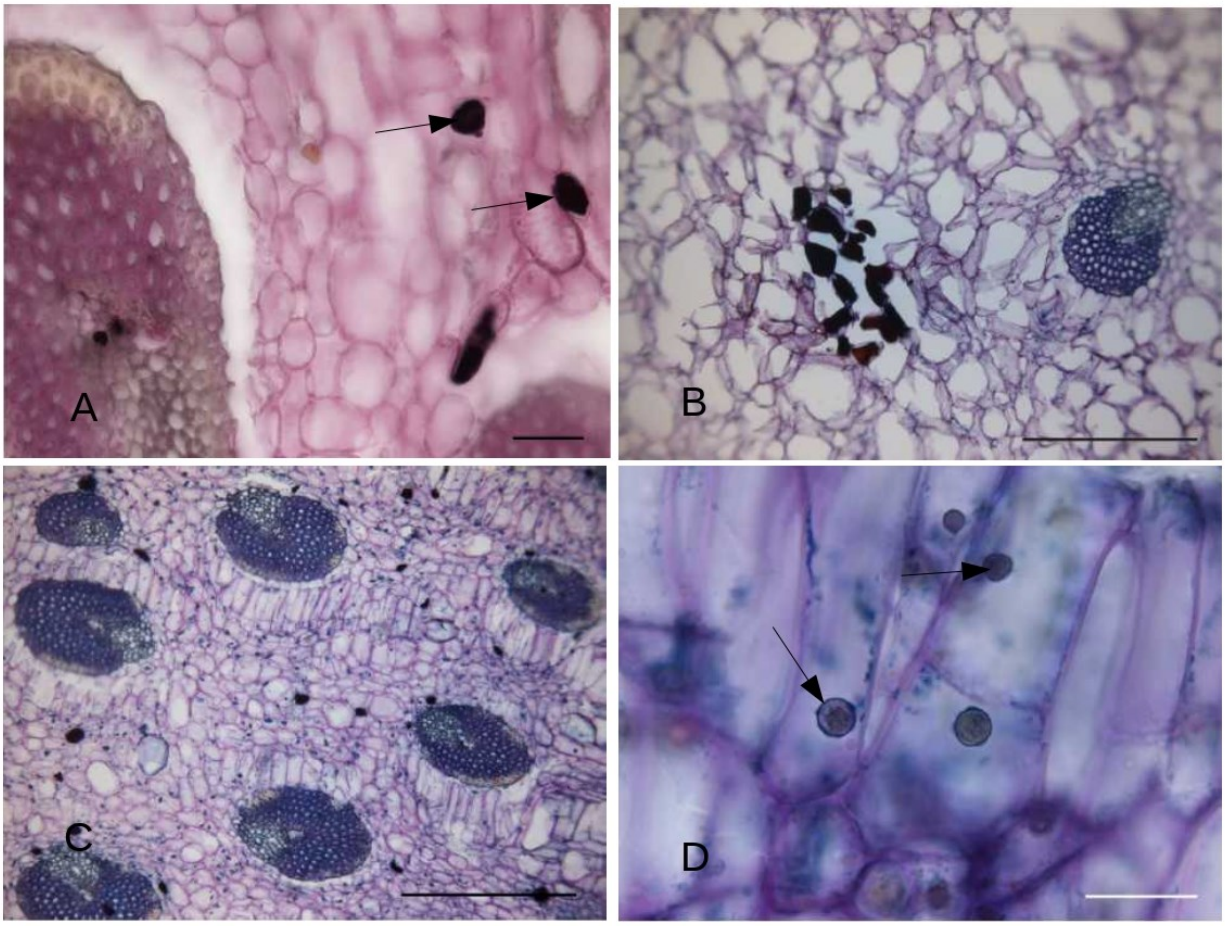

Figure 5. LM images of the cross section of the mesocarp. (A) PAS positivity of some cell contents (arrows). (B) cross-section of the aerenchyma zone. (C) mesocarp, stained with PAS reagent and Aniline Blue-Black. (D) outermost layers of the mesocarp; a proteic envelop surrounds some bodies (arrows) in the parenchyma cells, stained with PAS reagent and Aniline Blue-Black. Scale bars: (A) = 50 μm; (B, C) = 500 μm; (D) = 25 μm.

The second zone of the mesocarp was rich in protein content as shown in Figure 6A. Protein aggregates approximately were of the same range of dimensions (about 10-20 μm) and uniformly distributed inside the parenchyma cells and even in the lumen of vascular bundles. Figures 6D and 6C revealed the distribution of cellulose matrix in the aerenchyma, which was found mainly in the primary wall of the cells and not in the vascular bundles (Figure 6A) whose cells were characterized by lignified walls. Figures 6D and 6C showed the intracellular spaces of varying shape inside the aerenchyma network formed by elongated thin-walled cells.

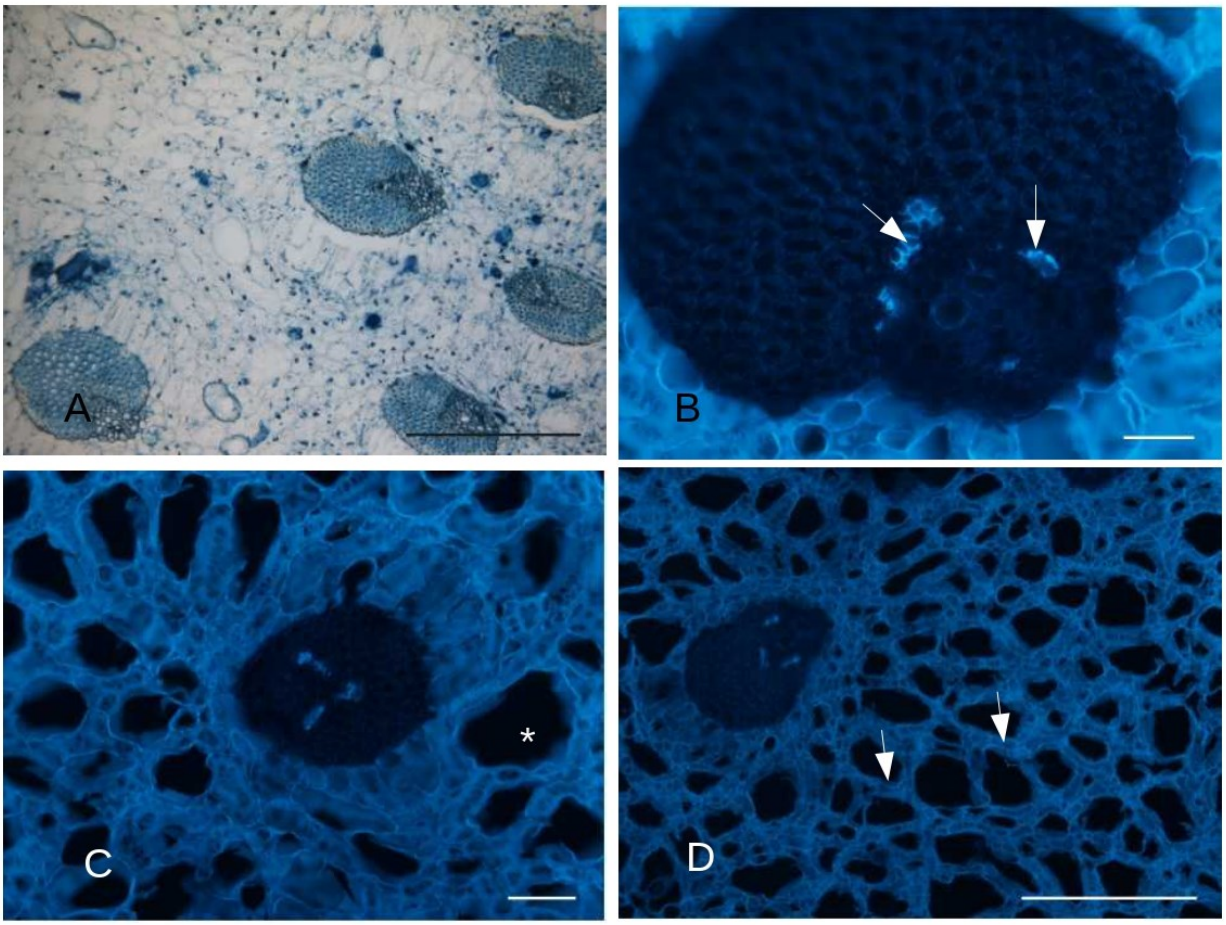

Figure 6. LM images of the cross-section of the inner zone of the mesocarp. (A) stained with Aniline Blue-Black staining showing the large amount of aniline blue-positive cells in the aerenchyma. (B) Calcocluor positive walls (arrows) between the xylem and the sclerenchyma. (C) Aerenchyma and vascular bundle within, stained with calcofluor. The intercellular spaces are up to 150x100 µm (asterisk) of dimension. (D) Aerenchyma and vascular bundle within, stained with calcofluor. Calcofluor positivity appears to be particularly intense along a line (arrows) inside the walls. Scale bars: (A, D) = 500 µm; (B) = 50 µm; (C)= 100 µm.

Essential oil analysis by GC-MS

The essential oil profile is shown in Figure 7 and the identified compounds are listed in Table 1, following their gas-chromatographic elution and reported with their respective CAS numbers. We performed a semi-quantitative analysis on the basis of peak area calculation of each compound with respect to the sum of all the peak areas. The dominant class was represented by acyclic sesquiterpenoids (53.95%), followed by bicyclic sesquiterpenoids (31.69%). monoterpenes (11.89%) and monocyclic sesquiterpenoids (2.44%).

In particular, 19 compounds were detected. The major compounds were β-caryophyllene (31.69%), aromadendrene (18.23%) and bicyclogermacrene (16.35%), followed by bornyl acetate (9.27%), *β*-maaliene (3.98%) and ledene (3.95%). *α*-Gurjunene, *α*-humulene and neo-allocimene exhibited relataive amounts in the range 3.0%-2.0%, whereas the remaining compounds accounted for percentage lower than 2.0%. The comparison with literature is not possible since the only previous contribution [20] referred to the fruit volatile profile obtained by a different technique. However, only one common compound was present, *i.e.* bornyl acetate.

Concerning the ecological role ascribed to the major compounds, *β*-caryophyllene was known to be involved in attracting pollinators [27] [28]as well as protection against pests [29] [30]. Generally, these actions were increased in synergy with α-humulene.

Aromadendrene was documented to be active against GRAM+ and GRAM- bacteria and yeast [31]. In addition, this compound was known to possess insecticidal properties [32], as well as byciclogermacrene [33]and ledene [34], acting in particular as larvicidal. Bornyl acetate, detected even

in the essential oils of *Rosmarinus officinalis* [35] and *Juniperus horizontalis* [36], was recognized as acaricidal [37]. *β*-Maaliene was found as one of the main components of the essential oil obtained from the roots of *Artemisia annua* and is toxic against the coleopteran beetles *T. castaneum* [38]. The presence of allo-aromadendrene is of interest since it was considered efficient in reducing juglone-induced oxidative stress resistance in *Caenorhabditis elegans*, even improving its life span[39]. The presence of this sesquiterpenoid in *L. maldivica* may suggest potential antioxidative and antiaging effects in humans.

Also germacrene, as other sesquiterpene lactones, could be considered involved in interorganism relationship, again possibly with insecticidal properties [40]. In addition, larvicidal properties were recognized to minor compounds such as sinularene as a component of *Ziziphora clinopodioides* essential oil [41], *α*-cubebene and *β*-cubebene, as components of several profiles (for instance in *Dendropanax morbifera*: [42], *Verbena officinalis* and *Lantana camara*: [43]), calarene and δ-cadinene [44]. In addition, the emission of δ-cadinene was documented to be activated in cotton by the presence of fungi, leading to the synthesis of phytoalexins [45], enhancing the defending role of this terpene.

Beside deterrent roles, other of the identified terpenes appear to be involved in insect attraction, such as neo-alloocimene attracting wasp in the fruit of *Ficus sycomorus* [46] and α-copaene, recognized as a potent attractant for male Mediterranean fruit fly, *Ceratitis capitata* [47][48].

Concerning the biological activity, terpenic compounds are characterized by a wide range of properties i.e. anti-inflammatory, cancer prevention effect, anti-hyperglycemic, and antiparasitic [49]. Terpenoids are used as main components in essential oils to treat bronchial disturbs, beyond to rheumatic and neuralgic disease [50]. Inhibiting the Low-Density Lipoprotein (LDL) oxidation, this class of compounds prevents the correlated pathologies i.e. atherosclerosis and heart disease [20][51]. With regards to *L. maldivica* essential oil, β -caryophyllene and α-humulene were both involved in anticancer activities [52]. A non-cytotoxic concentration of β-caryophyllene was documented to increase the anticancer activity of α-humulene and isocaryophyllene on MCF-7 human tumor cell line. α-Humulene was considered partly responsible for the cytotoxicity of *Abies balsamica* essential oil by induction of intracellular glutathione depletion and an increase in the production of reactive oxygen species [52], while β-caryophyllene would not inhibit tumor cell growth itself. However, β-caryophyllene was known to increase the intracellular accumulation of anticancer agents, enhancing cytotoxicity [53]. *α*-Humulene was also shown to be capable of reducing the edema formation induced by carrageenan in rats [54], showing hence an anti-inflammatory activity.

Asakura et al. [55] showed that α-eudesmol was able to block Ca^{2+} channel in the rat brain, with a protective effect against brain injury after focal ischemia.

Bicyclogermacrene and β-caryophyllene were proposed as a possible herbal candidate with anti-viral activity against SARS-CoV-2 [56].

Furthermore, neo-alloocimene was detected in the essential oil of Chinese medical herb *Evodia rutaecarpa* [57]and it was one of the main volatile compounds found in *Syzygium cumini* [58] and *Zanthoxylum schinifolium* [59]fruits.

α-Humulene was found in the sub-fraction from the essential oil of *Salvia officinalis* [60] and *Syzygium aromaticum* [61].

α-Gurjunene is an important constituent of the patchouli oil and the gurjun balsam oil, used as fixative in perfumes [62]and it may be one of the compounds making *L. malvidica* as a potential precursor of perfumes.

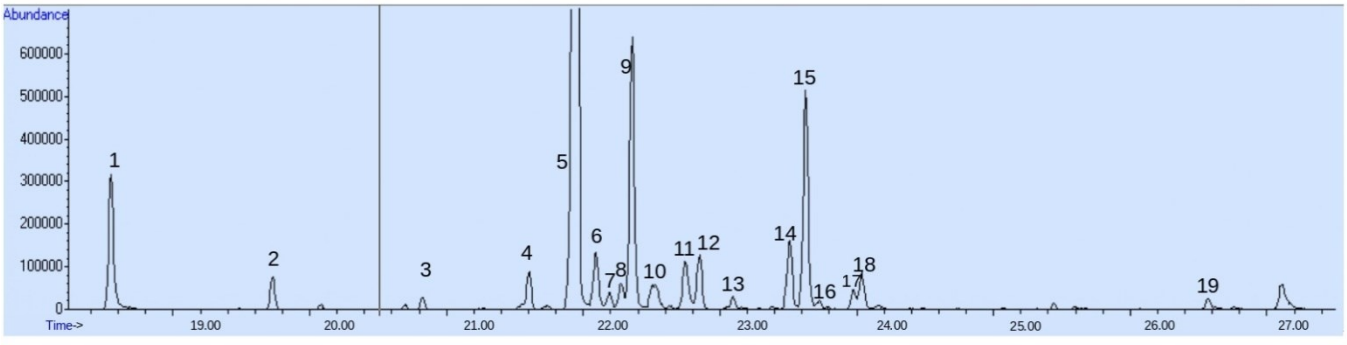

Figure 7. GC-MS profile of the essential oil extracted by "Coco de mer" fruit showing 19 different volatile compounds.

Table 1. Volatile components identified by GC-MS with their corresponding CAS (*Chemical Abstract Service*) number. The percentage is relative at the integration of the peak area and is calculated with respect to the sum of all the peaks. The relative percentage are hence to be considered approximate and the analysis only as semi-quantitative.

Peak number	Compound	CAS	Relative Percentages
1	Bornyl Acetate	5655-61-8	9.27%
2	α- Cubebene	17699-14-8	1.56%
3	α- Copaene	3856-25-5	0.35%
4	α- Gurjunene	489-40-7	2.05%
5	β-Caryophyllene	87-44-5	31.69%
6	β-Maaliene	489-29-2	3.98%
7	Calarene	17334-55-3	0.88%
8	Selina 3,7(11)diene	6813-21-4	1.05%
9	Aromadendrene	489-39-4	18.23%
10	Allo-Aromadendrene	25246-27-9	1.69%
11	α-Humulene	6753-98-6	2.44%
12	Neo-alloocimene		2.62%
13	β-Cubebene	13744-15-5	0.52%
14	Ledene	21747-46-6	3.95%
15	Bicyclogermacrene	24703-35-3	16.35%
16	Sinularene		0.35%
17	Germacrene	23986-74-5	0.94%
18	δ-Cadinene	483-76-1	1.65%
19	α-Eudesmol	473-16-5	0.40%
	Total		**99.97%**
	Monoterpenes		**11.89%**
	Acyclic sesquiterpenoids		**53.95%**
	Monocyclic sesquiterpenoids		**2.44%**
	Bicyclic sesquiterpenoids		**31.69%**

Conclusions

A morphological and phytochemical analysis was performed on 'Coco de mer' fruit. In addition, the in-depth histochemical survey allowed to describe the sites responsible for the synthesis and storage of the investigated volatiles.

The anatomical investigation showed the presence of idioblasts and droplets positive to NADI reagent for terpenes in the mesocarp. In the same anatomical zone, other groups of cells accumulated polyphenols (positivity to ferric trichloride), as well as in the aerenchyma.

The terpenes and terpenoids occurring in the essential oil profile and particularly those detected in higher amount, were in large part known for their activity against insect larves, while others were antioxidants. The antioxidant properties were probably also enhanced by the phenolic

content. In addition, bicyclogermacrene and β-caryophyllene, abundantly present in *L. maldivica* fruit, have been proposed as a possible herbal candidate with anti-viral activity against SARS-CoV-2.

Experimental Section

Materials and Methods

Fragments of the exocarps were collected in the Seychelles islands and brought to the University of Florence upon the permission of the local Government. The material was conserved at -20°C until the micromorphological and phytochemical analyses.

Light Microscope Analysis

The exocarp and the mesocarp of *L. maldivica* were reduced with a hacksaw and then with a razor blade into coarse fragments of volume equal to 1-2 cm^3. The obtained samples were cut with a Cryostat; the so produced sections were 10-20 µm thick and were stained with different histochemical techniques [63]. The employed stains were: Fluorol Yellow 088 (FY088) for total lipids [64], phloroglucinol for lignin [65], NADI reaction for terpenes [66], $FeCl_3$ (ferric trichloride) for polyphenols [67], Periodic Acid- Schiff (PAS) for polysaccharides [68], Aniline Blue Black for proteins [68] and Calcofluor for cellulose [69]. A Leitz DM-RB Fluo Optic microscope (Wetzler, Germany) equipped with a digital camera Nikon DS-L1 camera (Tokyo, Japan) was used for observations. Series of 5 digitized images were treated with the python program ALLAMODA2.0 [70] to reduce noise.

Extraction of essential oils

Coarse fragments of exocarp and mesocarp of volume equal to 4-5 cm^3 (119 g in total) were obtained with a hacksaw. They were subjected to hydro-distillation in a Clevenger-type apparatus for 1h 30'. The essential oil was diluted in diethyl ether, stored in sealed vials under refrigeration prior to GC-MS analysis.

GC-MS Apparatus

The GC-MS analysis was carried out using an HP 5890 Series II Gas Chromatograph coupled to a HP 5971A Mass Selective Detector single quadrupole mass spectrometer, with EI source. The GC-MS was equipped with a ZB-5MS column (30 m × 0.25 µm i.d., 0.50 µm film thickness) with column guard (Phenomenex). The oven temperature program was the following: initial temperature 40°C maintained for 3 min, then to 300°C at 10°C/min, held isothermally for 1 min. The temperatures of the injector and transfer line were 250°C and 270°C, respectively. The carrier gas was He, at 1 mL/min flow rate at 40°C. The injection was performed in split mode (1:20 split ratio). Full EI MS spectra were recorded from 40 m/z to 450 m/z at 1.8 scan/sec. Solid Phase MicroExtraction (SPME), with a 100 µm PDMS fiber, was used to trap the volatiles in the headspace of the vial containing the essential oil (1 min exposure time): the analytes were thermally desorbed in the GC injection port, exposing the fiber for 3 minutes. Data acquisition and processing were performed using HP ChemStation software (version D.02.00).

The identification of the components was made by comparison of their mass spectra with those of commercial (NIST 98 and WILEY) and home-made library mass spectra database built up from pure compounds and MS literature data.

Approximate semi-quantitative relative amount calculation for each compound was done by measurement of the corresponding peak area and expressed as relative (percent) areas by normalization with respect to the total ion chromatogram as, for instance, in Barroso et al. [71]. and method 3 in Ruiz-Henandez et al. [72]. The analysis can be considered only as semi-quantitative, since, lacking the use of the standards, the quantification is subjected to an error depending on the calculated peak areas and heights measurement, the degree of peak asymmetry and relative peak size [73]. even if, in our case, the peaks were relatively well separated and not particularly narrow, with a low signal/noise ratio. Error in peak calculation would amount in less than 0.1% in optimal conditions after Dyson[73]. However, in our case, the total error was probably increased by the heterogeneity of the essential oil.

Author Contribution Statement

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References

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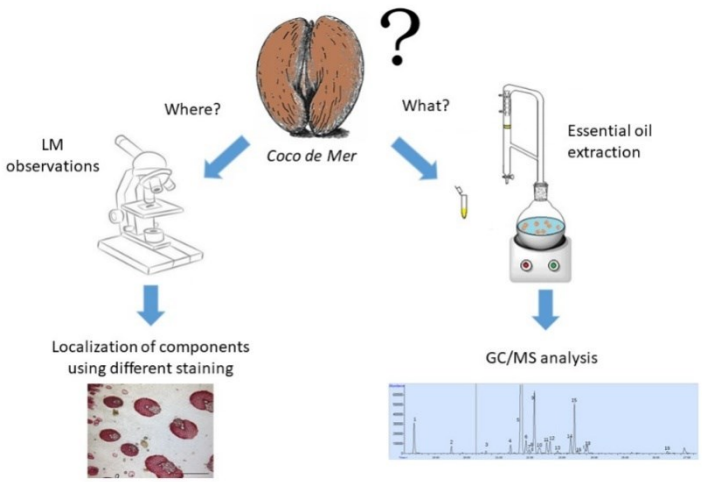

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