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Ecological and sustainable approach to develop natural red dyes with high stability and biological properties from five plant species of Madagascar

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Highlights

- Coloring properties of dyestuffs from typical plant species from Madagascar are highlighted.
- Plant barks are promising sustainable sources of red dyestuff materials.
- *W. fruticosa* roots and *A. excelsus* barks revealed interesting coloring and antioxidant properties.
- Pressurized liquid solvent extraction of pigment is more efficient than traditional decoction.
- Their properties generate new areas of industrial applications beyond their traditional use

Key words: Natural dyes; natural colorants; red colorants; mangrove plants; thermal stability; Pressurized liquid extraction.

Abstract

Artificial dyes from petrochemicals are extensively used in the numerous sectors. They have adverse effects on human health and environment. Economical and bio-friendly approaches are needed to extract and produce dyes in an ecological manner to replace the hazardous and toxic artificial ones. In this regard, exploiting the potential of natural dyes sources from unconventional plant species presumes a sustainable management of natural resources. In Madagascar, five plant species including *Acridocarpus excelsus*, *Ceriops tagal*, *Rhizophora mucronata*, *Woodfordia fruticosa* and *Xylocarpus granatum* are traditionally used as sources of natural red dyes by Malagasy craftspersons for textile dyeing at a very small scale. This original work is based on this assessment. Red dyestuff materials have been extracted from these five plant species by conventional and pressurized liquid extraction (PLE) method. It demonstrated that biopigments extracted *via* a low-toxicity and highly-efficient PLE method from barks or adventive roots collected from these plant species are more efficient while being

environmentally-friendly for dyes and pigments extraction. Stability tests have been performed and demonstrated both excellent pH stabilities within a pH range of 3 to 9 and thermal stability up to 200 °C was demonstrated. Further quantification of the main chemical families present showed polyphenols and tannins as the major components responsible for the coloration. These latter possess antioxidant and antimicrobial properties, well-proven in this study, which combined with their great pH and thermal stabilities, open up additional range of applications that could in a near future meet industrial application requirement. Aiming for an ecological and sustainable exploitation perspective, we are then proposing here various types of applications as well as lines of research that would improve the industrial and economic viability of the production of pigments extracted from Malagasy plant raw materials. These are mostly based on PLE technique paired with eco-friendly solvents as an alternative method for the extraction of natural dyes. The valorisation of these plant species using *green* technologies could enable the development of local sectors in Madagascar. However, issues related to development ethics, preservation of biological heritage and sustainable resource management must be carefully and thoroughly considered.

Abbreviations

ΔE : Delta E, or total color variation in the CIELAB color system

Ace: *A. excelsus*: *Acridocarpus excelsus*

ASE: Accelerated solvent extraction

ATCC: American Type Culture Collection

Cet: *C. tagal*: *Cerriops tagal*

CIELAB: Color space defined by the International Commission on Illumination (CIE) in 1976

Cyd-3-gluE: Cyanidin-3-glucoside equivalents

DPPH: 2,2-diphenyl-1-picrylhydrazyl

ECE: Epigallocatechin equivalent

FRAP: ferric reducing antioxidant power

GAE: Gallic acid equivalent

g/L: gram per liter

IUCN: International Union for Conservation of Nature

IC₅₀: 50% inhibitory concentrations

mMol: Millimoles

pH: potential of hydrogen

PLE: Pressurized liquid solvent extraction

QE: Quercetin equivalents

Rhm: *R. mucronata*: *Rhizophora mucronata*

TPTZ: 2,4,6-tripyridyl-5-triazine

UV-Vis: ultraviolet-visible spectrophotometry

v/v : volume per volume

w/w : weight per weight

Wof : *W. fruticosa*: *Woodfordia fruticosa*

Xyg : *X. granatum* : *Xylocarpus granatum*

1. Introduction

Issues of great concern nowadays include improving sustainability and reducing the amount of effluents produced and discarded by the chemical sector (Alias et al., 2019). Studies that address these issues are increasing and aim at minimizing these impacts through using natural alternatives that are beneficial to the environment (Liew et al., 2019; Yek et al., 2020). The dyestuff industry is one of the chemistry-based sectors responsible for a set of major environmental impacts due to the amounts of artificial dyes and precursors used, and the large volumes of chemicals released as effluents during their use (Venil et al., 2014). Artificial dyes, e.g. azo dyes, have been intensively used in textile, printing and dyeing, food processing, and pharmaceutical product manufacturing, because of their greater coloring properties, lower cost, and greater stability (Sigurdson et al., 2017). Despite these advantages, the findings of recent toxicological studies, coupled with consumer protection regulations and the associated search for sustainable products, have led to renewed interest in natural dyes that can replace hazardous and toxic artificial dyes (Yusuf et al. 2017). Indeed, many azo dyes which are often used in the chemical sector, like Ponceau 4R, Amaranth and Allura Red AC, show carcinogenic and mutagenic activities (Chequer et al., 2012). Unfortunately, rather large portions of these artificial dyes escape the conventional processes of wastewater treatment and exist as a potentially toxic environmental pollutant with adversities on public health and environment (Ogugbue and Sawidis, 2011; Mathiyazhagan et al., 2011).

Consequently, the dyestuff industry is continuously looking for cheaper, more environmentally friendly routes to existing artificial dyes. The non-toxicity, biodegradability and non-carcinogenicity of many natural pigments, dyes and colorants make them an attractive alternative source (Yusuf et al., 2017). As red coloring alternatives, high quality red natural colorants have a large potential market. The up-market red pigments in the world is presently carminic acid extracted from the dried bodies of female cochineals. In the plant kingdom, a wide variety of species produces red dyes, including henna and madder (from the roots of *Rubia tinctorum*) (Dufossé, 2014). These red dyes extracted from plants are traditionally used in textile and hair dyeing, and other colorants of plant origin (e.g., tomato lycopene, betalains from red beet, anthocyanins from red cabbage) are also used as color additives in food, cosmetic and pharmaceutical applications (Dweck, 2002; Andriamanantena et al., 2019). Some natural colorants have been shown to have anticancer, cytotoxic, antimicrobial (Kasiri and Safapour, 2014; Singh et al., 2005) and antioxidant (Lee et al., 2003; Grunert, 2005; Rather et al., 2017) properties. These particular biological properties open a wide potential of specific applications (such as for functional finishing of textiles, in functional foods, etc.) for natural colorants and respect to consumer's preference.

Although, dyes from renewable and sustainable plant sources should offer a sustainable alternative for the industries, they also have some limitations (Sigurdson et al., 2017). Their industrial sustainability can be limited in some cases by interactions with food ingredients, coloring powers, difficulty in reproducing colors, the need to standardize coloring, a somewhat restricted color range, and low levels

of solidity compared with artificial dyes (Gebhardt et al., 2020). Other features that limit the application of plant dyes are associated with their chemical characteristics such as sensitivity towards various environmental parameters (e.g., light, heat, or oxygen and instability in different conditions of pH) (Yusuf et al., 2017). A further obstacle to their exploitation comes from the legislation, requesting costly toxicological research, and the manufacturing costs which are often expensive. Thus, the challenges facing the color industry include industrial sustainability of their production, processing and applications. To overcome these obstacles, research for more sustainable products should involve the exploitation of novel natural sources, the valorization of subsequent by-products (biomass and waste), and the development of environmentally friendly processes including the extraction processes (Nambela et al., 2020). Different techniques can be used for the extraction of natural products like colorants from plant materials either by conventional extraction processes (decoction, solid-liquid extraction by Soxhlet...) or by alternative *green* technologies (e.g. extraction with supercritical carbon dioxide, assisted by ultrasound, enzymatic, microwave, or high-pressure systems with eco-friendly solvents) (Shahid et al., 2013). The potentiality of dye production in Madagascar is promising due to its exceptional biodiversity (Andriamanantena et al., 2019). Madagascar is one of the eight 'hottest' biodiversity hotspots based on the exceptional richness and concentrations of its indigenous flora. Some specific Malagasy dye plant species, including native plants of mangroves, are already being traditionally used by local population to dye natural fabrics into red shades (Cardon, 2014).

However, the chemical properties of a vast majority of the dyes obtained from plants – some of which are threatened with extinction – have not been systematically studied yet. The lack of scientific knowledge on these unconventional plant species limits the potential development of their uses for further applications (Andriamanantena et al., 2019). Gaining this knowledge requires learning detailed, step-by-step recipes from local practitioners, and these have often gone unrecorded. Finally, beyond their historic and academic value, natural dyes today are of great interest to many local weavers who are increasingly seeking to tap the European and North American markets which prefer natural colours and ecologically sound products. Exploiting the potential of little-known natural dyes sources such as the plant species currently used by Malagasy craftspersons presumes importance. The roots, barks, or leaves are a rich source of natural red pigments such as flavonoids, tannins, etc. No plans are made for valuing these plant species as sources of natural colorants through their conversion into value-added products in Madagascar (Andriamanantena et al., 2019).

In this context, the aim of the present study is focused on the recovery of natural red dyes - that could be potentially used to substitute their synthetic counterparts – from raw materials (e.g., barks and adventive roots) obtained from one endemic species (*Acridocarpus excelsus*) and four native species (*Woodfordia fruticosa*, *Xylocarpus granatum*, *Rhizophora mucronata* and *Ceriops tagal*) growing on the west-northwest region of Madagascar adopting pressurized liquid extraction (PLE) using eco-friendly solvents. These three last species are growing in Madagascar's mangrove ecosystem. These raw materials have been chosen because they are traditionally used by local population from Boeny

Region to dye natural fabrics in reddish hues but their up-scale exploitation potential as natural dyes sources is limited by the lack of scientific data about the chemical characteristics of the pigments extracted from these plant species. Furthermore, they present several socio-economic advantages: e.g, the two species *A. excelsus* and *W. fruticosa* are found in arid areas where only a few types of vegetation can grow. *A. excelsus* species (Malphiaceae) is an endemic tree of Madagascar and it widely spread in the west region of Madagascar. This tree is currently used as firewood by local population, and also used as a source of dyestuff materials for raffia fibers dyeing (Andriamanantena et al., 2019). However, according to the literature, the chemical composition and the characterization of the coloring properties of *A. excelsus* bark extract have never been studied so far. *W. fruticosa* (Lythraceae) is a native plant specie collected in dry areas next to the estuary of mangroves in Madagascar. The flowers, leaves and stem bark of *W. fruticosa* were demonstrated as rich in tannins and flavonoids, and leave extracts are currently used in other countries like India as natural colorants. According to Das et al. (2007), they also have some interesting medicinal properties and biological activities. So far, no information about the root extracts of *W. fruticosa*, that is the part currently used by local population in Madagascar to dye natural fabrics, were available in the literature. *C. tagal*, *R. mucronata* (Rhizophoraceae), and *X. granatum* (Meliaceae) are growing on the estuary of mangrove and are used by local population for their wood and to dye natural fabric. They are also used for their antimicrobial and medicinal properties in other parts of the world (Sur et al., 2015). These three mangrove plants, even if they should be considered as a sensitive ecosystem, are subject to a massive program of reforestation in this region Boeny and can help to the sustainability of the resources in Madagascar. In these regions, sources of income are very limited and a new sector of activity should contribute to the development of the local population.

Therefore, in this study, the properties of the dyes extracted from these five Malagasy species, that have not been previously reported in the literature, will be demonstrated. We compared the performance of a traditional method based on decoction and the eco-friendly PLE technique which will be applied for the extraction of natural red dyes using water and ethanol (*i.e.*, eco-friendly solvents which are allowed in the US and in the EU for the extraction of natural products) and to determine their possibilities to be used in industrial dyeing processes. The objective of these extractions is the comparison between the green and conventional methods based on similar parameters but no optimisation of each process will be conducted yet. The chemical composition of the crude pigments extracted from these raw materials, and their chemical stability (towards pH and temperature) were characterized. Finally, the antimicrobial and antioxidant properties of the pigment extracts have been determined to set up new future industrial uses based on an ecological and sustainable approach. Moreover, socio-economic development of this dyestuff sector is of interest for stakeholders from local communities to big industries. Mandatory preconditions must be acquired before any exploitation of these resources with the aim of protecting this biodiversity and avoiding overuses and species loss. Thus, we discussed the ethical and sustainable approach for the exploitation

of dye plants as an alternative source of dyestuff materials with socio-economic impacts in Madagascar.

2. Materials and methods

2.1 Plant materials

Table 1 presents the botanical details of the raw materials obtained from the five plant species investigated in this study. Barks have been collected for *A. excelsus*, *C. tagal*, *R. mucronata* and *X. granatum*. Only adventitious roots have been used for *W. fruticosa* (research authorization N° 027/18-MEEF/SG/DGF/DSAP/SCB/2018) (Fig. A.1). The identification of each species has been made by the department of flora of the zoological and botanical garden of Tsimbazaza, (PBZT/Antananarivo/, Madagascar) and a voucher specimen with the corresponding identification number was kept for each harvested species. The harvesting was done according to good practice in order to reduce adverse effect on the plants (Etheve, 2005): 150 grams of barks or roots were collected from three different trees from the same geographical and geological area (Fig. A.2), and they were then mixed to constitute one representative sample. The collection was repeated three times.

Table 1. Botanical details and known properties of the studied plant species

Species (Botanical family)	Identification number* ¹ (herbarium)	Harvested parts	IUCN Status* ² (Date assessed)	Harvest date and location	Availability and current uses in Madagascar	Other health properties	References
<i>Rhizophora mucronata</i> (Rhizophoraceae)	2017-129Rfd	Barks	Least concern (2008)	January 2018 <i>Boanamary mangroves</i>	Silk and cotton dyeing, construction	Antidiabetic	(Sur et al., 2015)
<i>Ceriops tagal</i> (Rhizophoraceae)	2017-128Rfd	Barks	Least concern (2008)	January 2018 <i>Boanamary mangroves</i>	Silk and cotton dyeing, construction	Antinociceptive, antihyperglycemic, antibacterial	(Kasmudjiastuti and Murti, 2019; Wang et al., 2012)
<i>Xylocarpus granatum</i> (Meliaceae)	2017-127Rfd	Barks	Least concern (2008)	January 2018 <i>Boanamary mangroves</i>	Silk and cotton dyeing	Antidepressant effect	(Gao et al., 2016; Pisitsak et al., 2016)
<i>Acridocarpus excelsus</i> (Malphiaceae)	2017-131Rfd	Barks	Least concern (2019)	February 2018 <i>Marosakoa highlands</i>	Raffia dyeing, craft rum production, fire woods	Astringent, rich in tannins	(Jansen et al., 2004)
<i>Woodfordia fruticosa</i> (Lythraceae)	2017-134Rfd	Adventitious roots	Least concern (1997)	January 2018 <i>Boanamary highlands</i>	Silk dyeing, traditional medicine	Antiulcer activity,	(Das et al., 2007; Kumar et al., 2016)

*¹ deposit number of the herbarium; *² www.iucnredlist.org, Least concern means that the species are not in focus of species conservation.

2.2 Chemicals

Solvents were purchased from Carlo Erba. Chemical standards including gallic acid, quercetin, epigallocatechin and cyanidine-3-glucoside were supplied by Extrasynthese. Hydrosoluble carmine (kindly provided by Pronex, Lima, Peru) and madder roots extract (Couleur Garance, Lauris, France) were used as standards. Bacteriological agar and dextrose broth were supplied by VWR.

2.3 Extractions of natural red dyes

Different techniques can be used for the extraction of natural products like pigments from plant materials either by conventional extractions (decoction, hot water, solid-liquid extraction by Soxhlet, etc.) or by alternative technologies (e.g. extraction with supercritical carbon dioxide, assisted by ultrasound, enzymatic, microwave, or high-pressure systems) (Shahid et al., 2013). Ethanol and water were chosen as extraction solvents to meet the requirements of the industries in regards to the development of ecological products and processes. Study made by Altemimi et al. (2017) also showed that ethanol was most appropriate for the extraction of phenolics or any bioactive compounds. Two different extraction processes have been compared. Hydro alcoholic solution (70% (v/v) ethanol-water) which is classified as green solvent has been used for all extractions. The first extraction process was made by decoction using either fresh materials or lyophilized materials. The color and the yield of crude pigment extract obtained from fresh and lyophilized materials were compared to verify if the drying can affect the quantity of pigment extract. The extractions were made according to Mabika et al. (2013) and Toussirot et al. (2014) with some modifications suggested by the local Malagasy dyers. Briefly, the method chosen in this study is a 3 hours hot decoction at 65 °C with 70% (v/v) hydro alcoholic solution and a ratio of 1:5 (w/w) of raw materials and solvents. A second extraction procedure was based on pressurized liquid extraction (PLE) using an accelerated solvent extraction (ASE™) Dionex 300 apparatus. Pure water was used as solvent A and absolute ethanol as solvent B. The following experimental conditions were applied for the pressurized extraction: 30% of A and 70% of B, which is the same ratio used for the above conventional extraction, a temperature of 85 °C, 6 min of preheat, and five cycles of 5 min (static time) and pressure of 100 bar. The temperature was chosen based on the work published by Pereira and collaborators (2019) and adapted according to the artisanal conditions of dyeing bath generally used by the Malagasy craftsmen. The extraction time was set according to the method used by Ju and Howard (2003). In previous published works from Pereira *et al.* (2019) and Ju and Howard (2003), PLE was successfully applied for the extraction of polyphenols, which represent the main chemical group of compounds found in this group of dye plant species (Jansen et al., 2004).

Then, all the filtrates were evaporated in a C 3900 rotavapor and the liquid extracts were lyophilized in order to generate crude dry pigment extract in powder (Fig. A.3). The crude dry pigment extracts were stored at 4 °C prior to use. The extraction yields of the crude pigment extracts (dry weight) have been compared for the uses of fresh or lyophilized plants as raw materials and the two different extraction methods. It is calculated from the following equation (Equation 1).

$$\text{Pigment extraction yield (\%)} = \frac{\text{Weight of crude pigment extract in powder (g)}}{\text{Weight of plant material (in dry weight) (g)}} \times 100 \quad (\text{Equation 1})$$

2.4 Determination of main compounds in the novel natural dyes

The analysis of the bioactive compounds present in the crude pigment extracts was done using phytochemical studies. The phenolic compounds content including flavonoids, anthocyanins, proanthocyanidins (or condensed tannins) were determined by using the standards and according to the following methods from stock solutions of 8 mg/mL of dry pigment extract dissolved in 70% aqueous ethanolic solution.

2.4.1. Total phenolic compounds were determined according to Boo et al. (2012) and Singleton et al (1999), and expressed in equivalent of gallic acid/ g of extract (GAE/g extract powder). The optical density was measured at 765 nm with a spectrophotometer model Evolution 60S (Thermo Scientific), with a methanolic solution used as blank. The same manipulation was made with gallic acid (standard) at different concentration to establish the standard curve.

2.4.2. Total flavonoid contents were determined according to Sun et al. (2011) and Ksibi et al. (2015), and expressed in quercetin equivalents/ g of extract (QE/g extract powder). The optical density was measured at 510 nm and quercetin was used as standard.

2.4.3. Total monomeric anthocyanin contents were calculated using the method based on the reduction of anthocyanin by acidic environment according to the method developed by Lee et al. (2005). The optical density was measured at 520 and 700 nm. The total monomeric anthocyanin (TMA) is expressed as cyanidin-3-glucoside equivalents. TMA was calculated using the following equations:

$$TMA = A * \frac{MW * DF * 1000}{\epsilon * l} \quad (\text{Equation 2})$$

$$A = (A_{520 \text{ nm}} - A_{700 \text{ nm}}) \text{ at pH } 1.0 - (A_{700 \text{ nm}} - A_{520 \text{ nm}}) \text{ at pH } 4.5 \quad (\text{Equation 3})$$

where: TMA: total monomeric anthocyanin content expressed in cyanidin-3-glucoside equivalents/ liter of solution (cyd-3-gluE/l solution extract); A: Absorbance of the sample; MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor; l = pathlength in cm (1 cm); ϵ = 26 900 molar extinction coefficient, in L/mol/cm, for cyd-3-glu; and 1000 = factor for conversion from g to mg.

2.4.4. Total condensed tannins contents were measured according to Sun et al. (2011) and expressed in epigallocatechin equivalent/ g of extract (ECE/g extract powder). 100 μ l of the stock solution was mixed with 6 mL of 4% vanillin solution in methanol and 3 mL of concentrated H₂SO₄ (0.5 N) and left for 5 min at 30 °C. Epigallocatechin was used to produce the standard curve at 500 nm (Sun et al., 2011).

2.5 Determination of the coloring properties of the novel natural dyes

The crude dry pigment extracts were dissolved in 70% (v/v) ethanol-water solution at different concentrations, *i.e.*, from 1.0 to 32 mg/mL of dry powder extract in solution, to show the evolution of the shade of the extract by their color value measured in the international CIELab color system in terms of L* (lightness), a* (greenness versus redness), b* (blueness versus yellowness), C* (chroma expresses saturation or intensity and vividness degree of the color), and h* (hue angle indicates the observed color) values. L*a*b coordinates values were measured by a spectrophotometer and C* and h* values are calculated from a* and b* values. The value of chroma C* ($C^* = \sqrt{a^{*2} + b^{*2}}$) is 0 at the center and increases according to the distance from the center. The hue angle h* ($h^* = \arctan(b/a)$) is defined as starting at the +a* axis and is expressed in degrees: 0° would be +a* (red), 90° would be +b* (yellow), 180° would be -a* (green), and 270° would be -b* (blue). Hue values correspond to the angle of the a*/b* coordinates of the points. The optical density measured at different visible wavelengths, e.g., at 400-450 nm, 450-490 nm, 490-520 and 520-580 nm will confirm if the extracts have major yellow, orange, red and purple-red pigments, respectively. A spectrophotometer model CM-3500d (Minolta) has been used to identify the color coordinates in the CIELab color system, and the spectrophotometer model Evolution 60S (Thermo Scientific) for absorbance analysis.

2.6 Physico-chemical stability tests of the novel natural red dyes

The stability towards pH and temperature of each crude pigment extracts has been compared with those of two commercial red dyes, carmine and madder extract which are natural red colorants obtained from the cochineal (*Dactylopius coccus*) and madder roots (*Rubia tinctorium*), respectively.

2.6.1. pH stability: To demonstrate the stability of the crude pigment extracts towards pH, pigment solutions (extract powder in water-ethanol solution) were prepared in different pH values (pH 3, 5, 7, 9 and 11) following the method used by Torskangerpoll and Andersen (2005). The color value measured in terms of L*, a*, b*, C* and h* values, and delta E (ΔE) *i.e.* total color variation between the initial (neutral) and the final (acidic or basic) state were recorded for all the samples using a spectrophotometer model CM-3500d (Minolta). ΔE was calculated using the following equation (Marzec et al., 2019; Toussirot et al., 2014) :

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (\text{Equation 4})$$

where ΔL^* is the difference between the final and the initial value of L*, Δa^* is the difference between the final and the initial value of a* and Δb^* is the difference between the final and the initial value of b*.

2.6.2. Thermal stability: Three step assays were performed to study the thermal stability of these pigment extracts. A first test was consisted in heating the pigment solutions (extract powder in water-ethanol solution) for 3 hours in a water bath at 90 °C). Second test was conducted by combining pH and temperature parameters: pigment solutions with different pH (from 3 to 11) were heated at 90 °C

in order to have acid hydrolytic and alkaline hydrolytic conditions, and variations of the color were observed after 3 hours. The last test was a thermal stability assay and was performed directly on dry powder extract: 120 mg of dry powder extract were heated in an oven at 105, 205, 250 and 305 °C for 30 min according to the method developed by Marzec et al. (2019). Then, 15 mL of 70% (v/v) ethanol-water were added to these thermally-treated pigment extracts to obtain a final concentration of 8 mg/mL of dry powder extract in solution. The physicochemical modification and/or the thermal degradation of the pigments was monitored by measuring the total color variation ΔE between the non-thermally treated solutions and the thermally treated ones.

2.7 Antimicrobial and antioxidant activities

Antimicrobial activities were evaluated on Petri dishes following the method described by Siva et al. (2011) by using the diffusion method from a filter paper disc impregnated with different concentrations of crude pigment extracts on several common pathogens including *Staphylococcus aureus* (ATCC 6538), *Salmonella typhi* (ATCC 14028), *Bacillus cereus* (ATCC 80876), *Escherichia coli* (ATCC 8739), and *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Propionibacterium acnes* isolated at the laboratory of biotechnology of Tshwane University of Technology (Pretoria). Nutritive agar was sterilised at 120 °C for 15 min. The nutritive agar is composed of 1.0 g/L of meat extract, 5.0 g/L of peptone, 2.0 g/L of yeast, 8.0 g/L of sodium chloride and 15.0 g/L of agar. Then, lyophilized microorganisms were put in a nutritive broth composed of 10.0 g/L of casein peptone, 5.0 g/L of yeast extract, and 10.0 g/L of sodium chloride and spread on the agar nutrient medium. Pigment extracts were dissolved in distilled water containing 2.5% DMSO. 10 μ L drop of extracts was then placed on 5 mm discs and dried in an oven at 45 °C. Two different concentrations of 5 mg/mL and 10 mg/mL were respectively deposited on the discs for each Petri dish. A third disc impregnated with 2.5% DMSO was used as a negative control while ampicillin was used as positive control. Finally, the petri dishes containing the nutrient medium, the microorganisms and the disks were incubated at 35 °C overnight and the zone of inhibition was evaluated.

Then, the antioxidant activity was determined using two classical methods. The first method is based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability as described by Yeo et al. (2019). The second method based on ferric reducing-antioxidant power (FRAP) was adapted from Thatoi et al. (2014). Briefly, for both methods, 2000 μ g of the crude dry PLE extract (obtained after lyophilization, i.e. contained >99% dry matter) have been weighted and diluted at different concentrations from 2000 μ g/mL to 15.62 μ g/mL per solution in 96-well plates. A diluted solution of DPPH or FRAP has been added to the wells and the reduction has been observed after incubation by a spectrophotometer at respectively 515 nm and 593 nm. The same manipulation has been performed on pure standard compounds, i.e., by using 2000 μ g of acid ascorbic or butylated hydroxyanisol diluted in the same dilution range.

2.8 Statistical analysis

The statistical analysis for each test was performed using analysis of variance (ANOVA) and expressed as mean \pm standard deviation of three replicates. The procedure was followed by Duncan test to determine the statistical significance ($p < 0.05$) (Boo et al., 2012; Sun et al., 2011).

3. Results and discussions

3.1 Extraction yields and evaluation of colorimetric characteristics

Two different extraction techniques have been used here to obtain crude pigment extracts in order to use environmentally friendly processes and materials based on the experience of the dyers in the west region of Madagascar. Firstly, hot decoction which is the most common method used by craftsmen was used as conventional process. Secondly, a pressurized liquid extraction (PLE) using eco-friendly solvents was developed as an innovative green technology for the recovery of red dyes from the raw materials. PLE is an alternative greener extraction process which is underused for the extraction of plant pigments (Otero et al., 2018).

As shown in Figure 1, which describes the extraction yields of the pigment from 5 different plant species, the best pigment extraction yields were observed for barks of the three mangrove plants, and more particularly from ones belonging to Rhizophoraceae family: 30.7% (w/w) for *C. tagal* and 28.8% for *R. mucronata* after material decoction. These pigment extraction yields were higher than the ones reported by previous studies performed on stem barks. Baliarsingh et al. (2012) obtained an average extraction yield of 15% (w/w) for several dyes extracted from barks, and a value ranging from 15 to 25% (w/w) was reported in the study performed by Kechi et al. (2013). Then, our results showed that the alternative high-pressure PLE extraction (with five cycles of 5 min) is less efficient for pigment extraction from dry barks of *C. tagal* and *R. mucronata*, compared to the traditional decoction (after 3 hours) performed on this study. The pigment extraction yields obtained from barks of *C. tagal* were 30.7% and 21.1% (w/w) after dry material decoction and PLE extraction, respectively. Concerning the pigment extraction yields from barks of *R. mucronata*, 23.2% (w/w) was reported after PLE extraction compared to 28.8% (w/w) after fresh material decoction. Nevertheless, the pomace obtained after the PLE extraction is still colored for these two last plant species, and obviously still contains non-extracted compounds.

Otherwise, results obtained by this alternative high-pressure PLE extraction revealed some differences for the three others dye plant species investigated here. Interestingly, this alternative extraction method appeared as significantly more efficient than the conventional decoction for pigment extraction from barks of *X. granatum* and *A. excelsus*, and from roots of *W. fruticosa*, by respectively +100%, +88% and +62% higher in term of pigment extraction yield (Fig. 1). The PLE, compared to the decoction traditionally used, is more sustainable, green and economically advantageous due to smaller volumes of solvents required for a given quantity of raw material (improved extraction capacities due to the combination of increased pressure and temperature). The extraction times are also reduced by PLE.

For instance, PLE was completed after five cycles of 5 min, while decoction required several hours at ambient pressure (3 hours here). Comparatively, less energy is required for the extraction by PLE. Additionally, the use of increased (nitrogen) pressure during the extraction protects the compounds of interest from oxygen oxidation. Thus, the simultaneous use of higher pressure, higher temperature and environmentally benign solvents (mix of ethanol and water) allows to significantly reduce the extraction time, the amount of solvent needed, as well as the potential degradation of target biochemicals due to pH, O₂, temperature oxidation and/or harsh solvents exposure, among others. According to our knowledge, this study is the first investigation on pigment extraction by PLE from these kinds of plant species (e.g. roots and barks of tropical plants like mangrove ones). And it displays interesting results for this alternative green and sustainable method. Nevertheless, even if the two Rhizophoracea species (e.g., *C. tagal* and *R. mucronata*) demonstrated lower extraction yield with this alternative extraction technique, the PLE process reported here offers a standardized way of extracting water-soluble pigments from raw plant materials. Furthermore, this process can be still optimized and improved separately for each plant materials. Finally, the results demonstrated that the PLE is an adequate alternative to conventional methods for the extraction and recovery of red dyes from natural bioresources and more particularly from roots and barks.

Concerning the decoction of fresh materials, the results showed an extraction yields from 7.27% (w/w) for *W. fruticosa* and up to 28.80% (w/w) for *R. mucronata*. Decoction of dry raw materials (*i.e.*, lyophilized barks or roots) demonstrated an extraction yield from 8.03 up to 30.73% (w/w) for *W. fruticosa* and *C. tagal*, respectively. Thus no significant difference between the extraction yields obtained from dry materials and those obtained from fresh materials could be concluded (see statistical significances in Fig. 1). Then, the raw materials conditions (fresh or dry) did not seem to impact the overall extraction yield, neither the color of the extracts (no difference was observed). The findings suggested that the fresh raw materials (roots and barks) collected from these dye plant species listed above can be pre-dried and stored at room temperature by the craftsmen at least for few days before the dyeing process, without changing their coloring properties for dyeing application.
















< Place Figure 1 near here >

The spectrophotometric characteristics (expressed in terms of CIE L*, a*, b*, C* and h* values) of the pigment extracts dissolved at different concentrations in water-ethanol solution was assessed, and the results are given in Table 2. A strong variation of the hues of the extracts, from yellowish to reddish hue, was observed when increasing the concentrations of the dry pigment powder in solution. For instance, the crude pigment extracts obtained from the barks of *R. mucronata* and *C. tagal* appeared as golden yellow to orange color at low concentration (*e.g.*, from 1 to 8 mg/mL) and turned to red at high concentration of more than 16 mg/mL, as shown in Figure 2. For the crude pigment extracts obtained from roots of *W. fruticosa*, and from barks of *X. granatum* and *A. excelsus*, the hues appeared reddish

with concentration from 8 mg/mL. Thus, the pigment extracts from these three above mentioned plant materials, *W. fruticosa*, *X. granatum* and *A. excelsus*, showed the higher red coloring properties (*i.e.*, best red tinctorial power) to be used as natural sources for industrial applications. As demonstrated by Willemen et al. (2019), the use of total plant extract is considered to be advantageous over the use of individual dye compounds in terms of solubility, color and stability. The other non-coloring compound may play an important role in coloring properties. Extracts obtained either by decoction or by the new PLE extraction process with ethanol-water are not purified pigments, and they can contain other compounds extracted by the hydroalcoholic solution from the plant material.

< Place Figure 2 near here >

Table 2: Color expressed in terms of CIE L*, a*, C* and h* values in the CIELab colour system of the red dyes extracted by PLE depending on pigment concentration range in hydroalcoholic solution.

Dissolution of the dry color extracts in hydro alcoholic solution at different concentrations						
	Species	L*	a*	C*	h*	Visual color
2 mg/mL	<i>W. fruticosa</i>	68.32±0.86	25.96±0.86	66.92±0.57	67.16±1.00	
	<i>X. granatum</i>	70.19±3.20	25.06±2.84	62.82±2.72	66.52±1.74	
	<i>A. excelsus</i>	68.88±2.97	28.12±3.46	73.07±3.66	67.19±3.79	
	<i>R. mucronata</i>	66.65±1.37	36.40±4.76	112.27±5.99	71.12±1.62	
	<i>C. tagal</i>	77.93±2.61	20.70±3.62	85.30±2.48	75.90±2.93	
		L*	a*	C*	h*	
8 mg/mL	<i>W. fruticosa</i>	32.41±2.69	54.82±0.49	78.04±2.92	45.29±2.63	
	<i>X. granatum</i>	38.83±3.06	57.35±2.05	84.12±2.49	47.02±0.32	
	<i>A. excelsus</i>	38.54±2.51	58.62±2.77	88.26±0.82	48.36±1.99	
	<i>R. mucronata</i>	52.65±6.38	50.17±6.32	103.04±3.78	60.90±2.88	
	<i>C. tagal</i>	52.15±5.70	50.32±4.59	102.31±5.94	60.34±4.85	
		L*	a*	C*	h*	
16 mg/mL	<i>W. fruticosa</i>	18.22±2.55	49.23±2.34	58.34±4.38	32.31 ±2.50	
	<i>X. granatum</i>	27.58±1.97	58.78±0.14	73.86±0.26	37.28±0.08	
	<i>A. excelsus</i>	27.12±3.14	59.13±3.12	75.22±2.64	38.19±1.45	
	<i>R. mucronata</i>	37.24±1.75	57.58±1.60	86.02±2.32	47.90±2.58	
	<i>C. tagal</i>	36.97±4.36	57.79±1.78	85.86±4.33	47.52±4.25	

Captions: Mean values of three replicates are indicated in each box of concentration for the L*, a*, C* and h° values; L describes the lightness, a* is the red value, C* and h° describe the chroma and the hue angle (or tone) of the color, respectively.

Interestingly, the three crude pigment extracts exhibiting high red coloring properties, *i.e.* extracts from *W. fruticosa*, *X. granatum* and *A. excelsus*, showed similar UV-visible spectral characteristics as depicted in Figure 3. Indeed, the three extracts showed two absorption maxima (λ_{max}) at the following

visible wavelengths, 450 and 580 nm, confirming the presence of both orange and purple-red pigments, respectively, in these three closely related red dyes from plant origin (Fig. 3a). Not surprisingly, the two other pigment extracts from the mangrove plants of the Rhizophoraceae family, *C. tagal* and *R. mucronata*, exhibited λ_{\max} in the visible wavelengths 400, 450 and 480 nm (in addition with a lower absorbance at 580 nm), which especially confirmed the presence of yellow, orange and red pigments (in addition with minor purple-red pigments), respectively, in these extracts (Fig. 3b). All the extracts also exhibited absorption maxima in the ultra-violet region, at about 300, 310 or 330 nm. This finding clearly revealed the presence of colored aromatic compounds like polyketide-based pigments (e.g. flavonoids, condensed tannins or anthocyanins ...). Interestingly, the shift of the absorption spectrum towards the red “region” of the spectrum (*i.e.*, towards longer wavelengths in the range of 500 to 550 nm) by increasing the concentration of the pigment powder in the solution was confirmed by the experimental results (Fig. A.4).

< Place Figure 3 near here >

3.2 Chemical composition of the red dyes extracted by PLE

The dyeing properties of these dye plant species should be linked to their richness in total polyphenols (tannins, etc.) as reported by Jansen et al. (2004). The polyphenol contents of the raw materials obtained from the five dye plant species investigated in this study are presented in Figure 4. The total polyphenolic contents, flavonoids contents, total condensed tannins and total monomeric anthocyanin contents of the five samples showed important variations from one another. The polyphenol content of the barks of *A. excelsus* was found to be notably high (up 3 to 10 time higher) as compared to the other plant extracts and reached 977 mg equivalent of gallic acid/g of extract. For comparison, the pigment extracts from *R. mucronata* roots contain about 193 mg of total polyphenols per gram which is a value slightly greater than the ones (150 mg/g) obtained by Banerjee et al (2008) for *R. mucronata* roots collected in the Sundarbans mangroves (Bay of Bengal). The flavonoid content of *C. tagal* and *R. mucronata* barks (*i.e.*, in the range of 17.6 to 23.2 mg quercetin equivalent/g crude pigment powder) is very similar to the one of the crude pigment extracts obtained from the three other dye species investigated in this study, *X. granatum*, *A. excelsus*. Flavonoid content of *W. fruticosa* appears to be lower than the other species. Extracts of *W. fruticosa*, *X. granatum* and *A. excelsus*, which turned to have red deep hues, also showed high content of condensed tannins, (702 - 880 mg epigallocatechin equivalent/g of crude pigment extract), as compared to the two others closely related species of the Rhizophoraceae family, *C. tagal* and *R. mucronata* yielding respectively 160 to 236 mg of total condensed tannins. These findings confirmed the ones previously observed by Pisitsak et al. (2016) which have analysed the tannin content in the bark extracts of *X. granatum*. Furthermore, the pigment extracts from roots of *W. fruticosa* which showed the deeper red shade, contained the highest amount of monomeric anthocyanin with 0.23 mg cyanidin-3-glucoside equivalent /L of solution. As might be expected, the anthocyanin contents of all the crude extracts were found to be much lower

than those generally observed in fruits and flowers (Chandra et al., 2001). These results highlighted that the coloring properties of the three species, *W. fruticosa*, *X. granatum* and *A. excelsus* are strongly related to their contents in phenolic compounds, and more particularly in proanthocyanidins (condensed tannins), and flavonoids, which is coherent with previously reported studies on pigment content in barks and roots (Willemen et al., 2019) and barks are often composed by polyphenols such as flavonoids (Andersen and Markham, 2006), condensed tannins (Tanner, 2004) and anthocyanins (Torskangerpoll and Andersen, 2005). Beyond to their coloring properties, polyphenols are well-known for their positive bioactivities highly wanted in food and health industries, presuming further applications for the polyphenol-rich raw material hereby investigated. The cytotoxic and antioxidant activities of these phenolic compounds is attributed to their ability to act as reducing agents for free radicals. They have received attention because their intake is correlated to the lower incidence of degenerative diseases, such as cancer, diabetes, Alzheimer, and cardiovascular diseases (Gutierrez-Grijalva et al., 2016; Rodriguez-Amaya 2016).

< Place Figure 4 near here >

3.3 Stabilities of the red dyes extracted from plant species

pH and temperature are the main factors that may strongly affect the coloring properties of the pigment extracts under conditions of industrial application. Ngamwonglumlert et al. (2017), described that the temperature is the second major factor, after the pH, that affect the color. For instance, in natural dyeing industries, pH variations is a method used by dyers to modify the color by acidifying with acidic fruit or vinegar or by adding ashes for the alkaline environment (Cardon, 2014; Etheve, 2005). On the other hand, temperature is important for the stability of the color for their uses in industrial process requiring high temperature (such as pasteurization). Most of the pigments extracted from fruit or leaves, such as chlorophylls, are temperature sensitive consequently limiting their use in the industries (Boo et al., 2012; Ngamwonglumlert et al., 2017). In addition, currently in use natural red pigments in industry consists essentially from the anthocyanins from red cabbage and the betalain from red beet, and both have their limits in stability (pH, oxidation, and heat-degradation) and vividness upon used in industrial processes (Ngamwonglumlert et al., 2017). This instability is probably due to the aromatic acyl groups which influence the aglycone chromophore by inter-/intramolecular association, and change the tones (Torskangerpoll and Andersen, 2005). Thus, for the five novel natural red dyes investigated in this study to be any industrial interest, it is necessary to confirm that the pigment extracts are stable under different industrial processing conditions (pasteurization, fabrication of bio-composites, etc.). If the natural colorants are too labile, their industrial applications will be limited. The pH stabilities of the five pigment extracts obtained from the endemic and native plant species to Madagascar are presented in Figure 5 (Tab. B.1 to B.5). The effect of pH conditions on the color changes was evaluated by measuring and comparing the color coordinates. The color variations under different pH conditions (at room temperature) have been

compared to those of carmine and madder roots extract submitted to similar pH conditions (Tab. B.6-B.7).

Carmine was the best stable standard towards pH changes as shown in Figure 5, *i.e.*, no color variation was observed within a pH range of 3 to 11 (Tab. B.7). Madder extract was particularly sensitive towards pH changes because the red color becomes more pronounced in an alkaline environment (the hue angle strongly decreased; Fig. 5). When compared to madder extract, all the crude pigment extracts from these five endemic and native dye plant species of Madagascar were found to be more stable with respect to pH condition, and more particularly within a pH range of 3 to 9 (Fig. 5; Tab.B.6). From pH 3 to pH 9, the color of the sample expressed in terms of lightness, as well as chroma and hue angle didn't change significantly for the five pigment extracts investigated in this study (Fig. 5). However, when the pH of the solution was set at pH 11, the L^* and chroma C^* coordinates, as well as the hue angle strongly decreased for all pigment extracts indicating a browning of the dye (Fig. 5), while the a^* (red) value increased (Tab. B.1 to B.5). This was also true for the control madder extract, but not for the colorant carmine which remained unaffected by the pH changes as mentioned above. Indeed, carmine as previously reported by Borges et al. (2012) and Dufossé (2014), and confirmed in this study, is highly stable towards pH in acidic and alkaline environments. Thus, these findings highlighted that the five new red dyes described in this study, compared to other natural pigments extracted from fruits or vegetables currently used in the industries, are interesting for different coloring applications both for acidic and alkaline environments. For instance, anthocyanin pigments obtained from red cabbage are very unstable to pH (Torskangerpoll and Andersen, 2005) and betacyanin pigments extracted from berry juice are only stable until pH 7 (Ngamwonglumlert et al., 2017), while these five new red dyes are stable within a pH range of 3 to 9.

< Place Figure 5 near here >

Additionally, the thermal stability of the different samples has been evaluated following three step tests. Thermal stability is critical because thermal treatments are applied in the dye industries: for example, the dyeing textiles process usually involves high temperatures at 90 °C with duration time between 30 to 180 min (Etheve, 2005; Cardon, 2014). First, experimentation was conducted to evaluate the stability of the pigment powder extracts dissolved in hydroalcoholic solution at a temperature range of 40-90 °C for 3 h at a neutral pH was evaluated. It was observed that all the samples were stable, *i.e.*, no change in color was observed when measuring the CIELab color coordinates. Then, a second assay was performed by combining both pH and temperature conditions. The colored solutions at different pH values (from 3 to 11) were put in a heated bath (90 °C) for 3 h. This generated acid hydrolytic conditions (pH 3 or 5 at 90 °C) and alkaline hydrolytic conditions (pH 9 or 11 at 90 °C). The changes in color between the initial time ($t=0h$) and the final state (after 3 h of exposition at acidic or alkaline hydrolytic conditions) was observed. The results indicated that a browning due to the high alkaline condition (pH 11) as described previously was observed for all the

extracts, but the thermal hydrolytic conditions did not significantly affect the color of the solution in the pH range of 3 to 9 (Fig. A.5). Thus, a third thermal stability assay was carried out on the pigment powder extracts in order to define more precisely the thermal stability and the degradation of the pigmented molecules with respect to the exposition temperature. The pigment powder extracts were therefore exposed for 30 min in an oven to different high temperatures, *i.e.* 105, 155, 205, and 305 °C. The total color variation (ΔE) in the CIELab color system between the non-thermally treated and the thermally treated pigment extracts was monitored as shown in Figure 6. In comparison with the carmine control (thermally stable until 105 °C, $\Delta E < 10$), the results confirmed that four of the new red dyes investigated in this study were also stable until 105 °C for 30 min of exposure (which is the maximum temperature used by craftsmen for natural fabric dyeing), *i.e.*, $\Delta E < 10$ at 105 °C for the pigment extracts of *W. fruticosa*, *X. granatum*, *A. excelsus*, and *C. tagal* (Fig. 6; Tab. B.8 to B.11). However, the pigment extract from *R. mucronata* showed a different profile as shown in Fig. 6 and Tab. B.12, indicating that it was less stable ($\Delta E: +23.7$) than the previous ones when it was subjected to 105 °C for 30 min of exposure. Its heat stability was comparable to that of madder extract ($\Delta E: +28.6$ at 105 °C for 30 min), which was less heat stable than carmine. In higher exposition temperatures, the four pigment extracts from *W. fruticosa*, *X. granatum*, *A. excelsus*, and *C. tagal* have excellent heat stability up to 155 °C for 30 min of exposure ($\Delta E < 10$ for all these samples; Tab. B.8 to B.11). Interestingly, only the pigments from *W. fruticosa*, *X. granatum* and *A. excelsus* remained relatively stable even when samples were exposed up to 205 °C for 30 min ($\Delta E: +28.8$, $+36.9$, and $+50.9$, respectively; *i.e.*, the pigments retained more than 50% of initial intensity after the thermal exposure condition) (Tab. B.8 to B.10). In this thermal exposure condition at 205 °C, the pigment extracts from *C. tagal* and *R. mucronata* have lost more than 90% of their color intensity ($\Delta E: +89.6$ and $+110.6$, respectively; Tab. B.11-B.12). Lastly, when temperature reached 305 °C, results concluded that color of all the pigment extracts became darker, they completely lost their original color ($\Delta E: +97.5$ to $+113.2$) (Tab. B.8 to B.12). These pigment degradations were also confirmed by measuring the losses in optical density at the pigment specific absorbance wavelengths, *i.e.* 400–580 nm; the optical density of the solution tended to zero at 305 °C (Fig. A.6). These findings confirmed the previous study performed by Mazec et al. (2019) on thermal denaturation of azo dyes, a class of synthetic colorant commonly used in the dye industries that are also denatured from 300 °C. Compared to madder root extract, which is one of the most commonly used natural red dyes in textile industries and which is recognized as a good indicator for heat stability (Deveoglu et al., 2012), the five pigment extracts characterized here, and more particularly those from roots of *W. fruticosa* and from barks of *X. granatum* and *A. excelsus*, presented excellent thermal stability up to 205 °C (under the thermal exposure condition performed in this study). Such results imply that these red dyes presented higher retention of the color intensity during thermal treatments (pasteurizing, fabrication of bio-composites, etc.) than other natural pigments.

< Place Figure 6 near here >

3.4 Antimicrobial and antioxidant activities of the red dyes extracted by PLE

A screening of the antimicrobial activities of the crude pigment extracts was carried out on the main pathogenic microorganisms commonly tested in the food industry, cosmetics and pharmacology. The method investigated here made it possible to assess the zones of inhibition in three categories: good antimicrobial activity, moderate antimicrobial activity and no antimicrobial activity observed. The extracts obtained with PLE have demonstrated interesting antimicrobial activities as described in Table 3. All the crude extracts have revealed good antimicrobial activities against *Staphylococcus aureus* and displayed promising antimicrobial activities against *Bacillus cereus*, more particularly for the bark extract of *A. excelsus*. Interestingly, the crude extracts obtained from barks of *A. excelsus* and roots of *W. fruticosa* have revealed good antimicrobial activities for the pathogenic *Pseudomonas aeruginosa* at least. All bark extracts demonstrated moderate antimicrobial activities against *Salmonella typhi*. It is worth noting that the bark extract of *A. excelsus* demonstrated good antimicrobial activity on *Propionibacterium acnes*, a pathogenic bacterium encountered in cosmetics. On the other hand, unfortunately, no extract demonstrated significant antimicrobial activities against *Escherichia coli* and *Enterobacter aerogenes* (Tab. 3). These coloring properties and antimicrobial properties can be used together to be applied in several products such as for the functional finishing of textiles.

Table 3. Screening of antimicrobial activities of the red dyes extracted by PLE

Plants/Bacteria	<i>Propionibacterium acnes</i>	<i>Salmonella typhi</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Bacillus cereus</i>	<i>Enterobacter aerogenes</i>	<i>Pseudomonas aeruginosa</i>
<i>C. tagal</i>	-	+	++	-	+	-	+
<i>R. mucronata</i>	-	+	++	-	+	-	-
<i>X. granatum</i>	-	+	++	-	+	-	-
<i>A. excelsus</i>	++	+	++	+	++	-	++
<i>W. fruticosa</i>	-	-	+	-	+	-	++

++: good antimicrobial activity, +: moderate activity, -: no antimicrobial activity observed

Moreover, all the extracts obtained by using PLE have demonstrated high antioxidant activities as described in Table 4. Antioxidants are important for the protection of the cells against free radicals and are also used as preservatives in several industries.

Table 4. Antioxidant activities of the red dyes extracted by PLE

	IC 50 means ($\mu\text{g/mL}$)*	mMol Trolox equivalent/g*
<i>C. tagal</i>	396 \pm 47	9.37 \pm 0.99
<i>R. mucronata</i>	485 \pm 23	8.31 \pm 0.81
<i>X. granatum</i>	441 \pm 80	7.49 \pm 0.37
<i>A. excelsus</i>	521 \pm 80	7.40 \pm 0.44
<i>W. fruticosa</i>	493 \pm 88	6.61 \pm 0.11

* Each value is a mean of three independent replicates; Lower IC₅₀ or higher mMol Trolox equivalent mean higher antioxidant activity.

The inhibitory concentrations (IC₅₀) of the five pigment extracts, ranging from 396 to 521 µg/mL, were globally lower than ascorbic acid (753 µg/mL) and butylated hydroxyanisol (605 µg/mL), two famous antioxidants used as controls. This result indicated that all the pigment extracts from Malagasy plants characterized on this study had higher antioxidant activity as compared to both standards. The best antioxidant properties were exhibited by the pigment extracts of *C. tagal* and *X. granatum*: 396 and 441 µg/mL, respectively (Tab. 4). At least two different methods should be used to demonstrate the antioxidant activity of plant extracts, then, these first results obtained by DPPH reducing method were confirmed by a second technique based on FRAP method. The latest method showed that the extracts of *C. tagal* revealed also the higher antioxidant values of 9.37 mmol Trolox equivalent per gram among these five extracts (Tab. 4). Also the study made by Yea and Shahidi (2019) have demonstrated that the color of the dye plant extract may affect the measurements of the absorbance after the reaction. However, it does not affect the measurements made at 515 nm for DPPH method and 593 nm for FRAP method because all of these plant extracts have insignificant absorbance at both wavelengths. Lastly, the results confirmed the ones from previous studies performed by Thatoi et al. (2014) and Das et al. (2019). Indeed, they have also demonstrated in the same way that mangrove plants such as species from *Xylocarpus*, *Avicennia*, *Bruguiera* and *Ceriops* genera exhibited high antioxidant activities, as effective as ascorbate and tocopherols. The antioxidant properties of these mangrove plants should be attributed to the presence of high amounts of phenolic compounds (proanthocyanidins, flavonoids and anthocyanins).

3.5 Range of potential applications of these new sources of natural dyestuffs

In this study, it has been demonstrated that these new sources of red natural colorants are rich in phenolic compounds such as proanthocyanidins, flavonoids and anthocyanins. The red colorants can be easily extracted from their stem bark or roots *via* green extraction technology and eco-friendly solvents. Moreover, the results demonstrated that the colorant extracts from these dyestuffs displayed greater color stability towards acidic and alkaline environments and high temperature (up to 205 °C for some of these red dyes), as compared to madder roots extract. So, these crude extracts can be classified as stable ecological dyes, like carmine (E-120) which is the most sustainable and stable red dye currently used in several industries for red coloring. In the range of experimental conditions evaluated in this study, these five new sources of natural red dyestuffs showed a good potential for applications in heat pasteurized food matrices, and more particularly under the acidic conditions generally encountered in foods. The pigment extraction yield, the polyphenols content, and the high antimicrobial and antioxidant activities give to these red dyestuffs a promising added value and open up potential applications for further exploitation in the industries.

Concerning the underutilized dye species, *A. excelsus* and *W. fruticosa*, which until now have mainly been used to dye natural fibers like raffia and silk in Madagascar, their uses could be extended to other industrial fields such as functional finishing of textiles, pharmaceutical, cosmetics and plastics coloring. For instance, the processing temperature for the fabrication of bio-composites is around 160 °C. All the colorant extracts investigated here are suitable for such industrial applications because their colors are stable at this temperature. In the production processes of bioplastics using higher temperatures up to 200 °C, pigment extracts from *W. fruticosa* or *A. excelsus* with high temperature stability could be particularly suitable. Moreover, pharmaceutical industries need red dyestuffs for capsules and tablets. The colorant extracts obtained from *A. excelsus* and *W. fruticosa* in view of their great color stability and antioxidant properties could be particularly useful. Cosmetic industries do not require such high temperatures and the products are used in neutral and acidic pH conditions. Therefore, *A. excelsus* and *W. fruticosa* colorant extracts, stable in acidic environment, could be good natural alternatives as coloring agents in this line of industrial applications. Preliminary experiments were performed in the lab to produce colored soap using colorant extracts of these plant species with promising results (Fig. A.7). The next focus should be the full characterization of the toxicological properties of these colorant extracts, in order to investigate their potential effect on animal and human health. Toxicological studies are in progress on zebrafish and albinos' mice models. Some biological properties of these colorant extracts could enlarge their range of applications. For instance, other studies demonstrated that *R. mucronata* extracts have medicinal properties such as antidiabetic activity by reducing the blood glucose level in type 2 diabetic rats, as well as neuroprotective effects (Tekupalli et al., 2020) and antimicrobial activities (Sur et al., 2015) that are particularly interesting properties to be associated with the coloring power. Particularly, antioxidant properties can also be used for specific applications such as in functional finishing of textiles, cosmetics, pharmaceutical, etc.

3.6 Extrapolation of PLE techniques in a large scale within the local communities

PLE has been widely used and recognized as a more adequate tool for natural products extraction essentially due to reduced time of extraction and amount of solvent required, as well as the less denaturing profile compared to conventional solvent extraction (Gil-Chávez et al., 2013; Zhang et al., 2018). Moreover, PLE enables highly efficient biocompounds extraction using only water and/or other GRAS (Generally Recognized As Safe) type solvents. Another net advantage of such technique is that PLE can be more easily automated compared to solvent extraction methods, consequently appearing as a more suitable candidate for industrialization and scaling-up (Osorio-Tobón and Meireles., 2013). Although PLE seems as the best option on the market, the initial investment as well as the operation and maintenance costs have limited its successful scale-up at industrial level or small businesses so far. However, some perspectives need to be taken before deciphering on the overall economical and environmental pertinence of the use of PLE. Indeed, very little information is available with actual numbered cost estimations between different extraction processes and for what

use. Indeed, if the endpoint added-value of a biomolecule is a key driver in the design and cost-effectiveness of one process, it can be easily counteracted by operational, supply and maintenance costs (Vega et al., 2020). Interestingly, a recent study (Vega et al., 2020) has carried out a case study of two polyphenols from red wine pomaces extraction methods, namely a PLE and conventional acetone or ethanol solvent extraction method. By combining both Techno-Economic Analysis (TEA) and Life Cycle Analysis (LCA), the study was able to draw central parameters that impact the most on the final economical and environmental pertinence of each extraction method. Solvent to dry weight biomass ratio and extraction yields were concluded in this study by Vega et al. (2020) as key parameters. The conclusions reported that either method can outperform the other one when sufficient optimization is done. Moreover, the same study clearly highlighted that the petrochemical origin of ethanol and acetone along with the health and safety issues related to this latter significantly and negatively impacted the LCA. In the current study, when compared to decoction methods using the same mixture of ethanol and water to carry out the PLE, higher extraction yields (see section 3.1) were obtained with a very little amount of solvent (seven times less than decoction method) and significantly reduced time (six times less than decoction method) by PLE techniques for *X. granatum*, *A. excelsus* and *W. fruticosa*.

With respect to TEA, this study offers a new prospect towards extrapolation of PLE techniques within the Malagasy local community using bio sourced and ecological solvent such as ethanol and water. Vega et al. (2020) demonstrated as well that the petrochemical origin of extraction solvents negatively impacted both LCA and TEA up to a 2-fold higher level than increased energy consumption would do due to pressure and heat requirements. It is interesting to mention that bioethanol has recently been produced in Madagascar, consequently offering an excellent opportunity to further promote and ease the development of this process with the local supply of the main solvent. Additionally, the cost and supply of compressed nitrogen becomes more affordable, especially because the nitrogen used does not necessarily have to be food- or laboratory-grade for this process. The initial concern of the expensive cost of very specific equipment can be overcome by the design of homemade PLEs. As surprising as it may sound, few studies were already working on PLE systems with their own designed and assembled PLEs (Sanagi et al., 2005; Santos et al., 2011), and with final extraction yields equivalent to the ones obtained with commercial apparatus (Alcázar-Alay et al., 2017). Such home-made PLEs have been successfully applied to similar uses as in this paper as the examples of pigments extractions from biomasses, namely anthocyanins and carotenoids from plant materials. Therefore, there is no reason not to be able to use similar strategy and build artisanal and tuned PLE systems to suit the needs of an integrated local circular economy.

Thus, PLE, in the long term, has high potential as extraction method for local Malagasy communities against solvent extraction if well and thoroughly designed. The creation of cooperative institutions, such as Cooperative Farms, where farmers from different counties would deliver and be reattributed based on their harvest would be an efficient way to reduce the overall capital (equipment

and localisation, building and /or rent), operation and maintenance costs as well as enable better management of the consistency of the extraction processes and lower potential health and safety concerns. Last, this type of cooperative institutions completely falls into economic and social development strategies and would most likely be partly if not completely founded by governmental and/or non-governmental financial supports.

3.7 Ethical and sustainable approach for the exploitation of dye plants as an alternative source of dyestuff materials with socio-economic impacts in Madagascar

Further industrial exploitations of these plant species could be interesting as described above. Nevertheless, exploitation of the aforementioned dye plants is a sensitive subject both environmental and political point of view because some of them are part of the protected ecosystem program. Mangrove forests are subject to deforestation in many regions of the world, including Madagascar. This ecosystem has strongly declined in these few last decades in Madagascar due to human activities and to climate change (Gilman et al., 2008; Giri and Muhlhausen, 2008). On the other hand, it has been observed previously that large scale use of some plant species in the absence of a program devised to ensure the sustainability of such use results in additional threats to biodiversity. For instance, *Prunus africana* has been over-exploited for years in countries like Cameroon and Madagascar for its medicinal properties, with the result that, after a few years, it had to be classified as endangered species (Stewart, 2003). The same has been observed for plant sourced rubber in Madagascar where the exploitation of this natural resource has led to biodiversity loss during a few years (Danthu et al., 2016). However, this doesn't mean that natural resources shouldn't be exploited. The exploitations must be monitored by a strict sustainable management program initiated by the relevant stakeholders including the local population, government, industries and primarily conditioned by the cultivation of these species. Provided that an adequate management program is set up with the participation of all the stakeholders, the valorization of these alternative sources of dyestuff from these species will represent a new income source for the local population and will contribute to its economic development. The sustainable production of natural colorants would be particularly useful in the concerned areas where sources of income are reduced mostly to fishing and silkworm breeding.

Some of the species considered in this article grow in environments which make their exploitation interesting from a social and economic point of view. *A. excelsus* and *W. fruticosa*, are widely spread in dry wild areas where very few species of plants can grow. Their exploitations as new sources of colorants may be viewed as promising new activities by for local population. These two plants are not endangered species and don't belong to protected ecosystem. Moreover, their cultivation at large scale for their coloring properties could contribute to the environment preservation by covering dry, bare areas. Concerning the mangroves species, e.g. *C. tagal*, *R. mucronata* and *X. granatum*, even though they are growing in a protected area and a sensitive ecosystem, a positive factor is that a reforestation program is already ongoing in the mangroves of Boanamary estuaries

conducted by local associations and some non-governmental organizations. Although the choice of the species used for this reforestation program does not yet take into the economic potential of these trees here studied as sources of natural colorants, the realization of this potential will constitute a further incentive to their plantation. It is our belief that reforestation can allow a sustainable exploitation of these mangrove plants as crops with potential industrial uses as natural colorants for textile dyeing and functional finishing, and/or coloring the materials in the food and non-food industrial sectors (pharmaceutical, cosmetics, plastics, paints and coating). It is further to be remarked that the use of these mangroves trees as lumber for the construction of house roofs and boat floors by local population is permitted after previous authorization from government authorities. Concerning, *A. excelsus*, this plant specie is widely used as firewood and for the production of charcoal. The current population of this species would not yet allow any semi-industrial or industrial exploitation. In a first stage, colorant extraction could represent a new valorization of the waste products (i.e. barks) from lumber or firewood exploitation. At a later stage, provided that sustainable programs of cultivation of the concerned species are implemented, they would provide new regular sources of natural colorants at industrial scale.

Exploitation of these plants can contribute to the preservation of the biodiversity and the local expertise. According to the experiences of local population, the current methods used to collect the barks of *A. excelsus*, *C. tagal*, *R. mucronata* and *X. granatum* allow bark regeneration after cutting and debarking wood. It could be still improved with further scientific experimentations. Concerning *W. fruticosa*, their adventitious roots are very long, which facilitates their harvest while preserving the plant. It means that this kind of uses, if well managed, are not going to be a threat to biodiversity. If these raw materials can be sustainably used to produce reliable sources of income, the local population will definitely plant trees and participate to the reforestation. Not only the biodiversity should be preserved but the local expertise as well. Consequently, any use of the products of biodiversity and of traditional knowledge must consider the recommendations of the Nagoya Protocol on Access and Benefit Sharing (ABS) (Rambinintsotra, 2014) and the convention on biological diversity (Pechard, 2005).

As a contribution to the knowledge on these dye plants and on natural dyeing fields, this study offers some solutions for the research of natural ecological colorants to substitute chemical sources and for the creation of new sources of income for the local population. Sustainable exploitation of raw materials can contribute to the conservation of the biodiversity and creates both environmental and socio-economic benefits. Industries are researching new sustainable and environmentally friendly products and these red dyes extracted from raw materials obtained from endemic and native dye plants species of Madagascar, which are available and easy to grow, are one of the solutions for this research of new sources of natural dyestuffs.

4. Conclusions and prospects

The dyestuff materials extracted from the endemic *A. excelsus*, and the native *C. tagal*, *R. mucronata*, *X. granatum*, and *W. fruticosa* can offer an important reservoir for significant novel and biologically active biopigments for several sectors. Most of these extracts which can give a wide range of colors, from yellowish to red hues, have been found to be insensitive to variations of pH and temperature. Comparing to less stable natural colorants encountered in the markets, the greater stability for these extracts are promising for several range of applications that required specific pH and thermal conditions. Furthermore, they contain active substances such as polyphenols which probably give them their antimicrobial and antioxidant properties. The future will tell us if there may be any industrial large-scale application of natural colorants coming from Malagasy dye-yielding plants in the industries. PLE extractions performed of these plant species have been demonstrated as more efficient than conventional decoctions in terms of extraction yields, time and quantity of solvent used and the methods described on this study can be still optimised. Hence, PLE extraction could be the ecological and sustainable way for the exploitation of alternative sources of red dyes from plant materials. The scaling-up of PLE extraction could be achieved locally if the exploitation is accompanied by sustainable management of the plant resource as it was already been shown in other part of the world. As an example, in Guadeloupe – France, dye plant species from its biodiversity have been recently being put forward by a private local company as sources of natural colorants (Green-ingredients.com). The production of natural colorants from dye plants of Madagascar by local dyers should be an adequate biodiversity-related activity for the future. This potential activity could contribute to maintaining the complex and fragile relationship between the preservation of plant biodiversity, economic and social development, and the material and spiritual well-being of the local populations. It could also prevent biodiversity loss in Madagascar by developing natural capital knowledge through scientific research and sustainable socio-economical program with the collaboration of all stakeholder. The interest for dye plant species is not only limited to these species and the north-west part of this country, there is still so much to be done in other regions of Madagascar and in the Indian Ocean region. It is also possible that this biodiversity contains other species which may provide natural blue or green dyes and biological properties of interest to the dye industries.

Author contributions

YC and TP conceived the idea to conduct the study. YC, TP, and PD designed the study and planned the implementation. MA conducted the laboratory work and conducted the sample analyses. MA and YC interpreted the resulting data, with inputs of TP and PD. MA and YC wrote the manuscript with inputs from all authors. All authors have made critical revisions and have approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure captions

Figure 1. Extraction yields of the pigment from 5 different plant species and using different extraction methods.

(Rhm: *R. mucronata*, Cet: *C. tagal*, Xyg: *X. granatum*, Ace: *A. excelsus*, Wof: *W. fruticosa*. PLE: pressurized liquid solvent extraction of dry materials using an accelerated solvent extraction apparatus. Mean values are calculated from three independent experiments and the bars represent the standard error. Means with the same letter in column are not significantly different at $p < 0.05$ level by Duncan's multiple range test and variance analysis have been conducted only between the extraction methods and not between plant species).

Figure 2: Color of the crude pigment extracts from the five red dyestuffs investigated in this study (*W. fruticosa*, *X. granatum*, *A. excelsus*, *C. tagal* and *R. mucronata*) measured in terms of hue angle (h^*) value in the CIELab color system, according to the concentration of the pigment (crude powder) dissolved in aqueous ethanolic solution.

(Variation of h^* value from 90° to 0° represents variation of yellowness to redness hue, respectively).

Figure 3: Overlay of UV-visible spectra from 280-800 nm of the crude pigment extracts from the five red dyestuffs. Figure at left shows UV-visible spectra of the three closely related red dyes from *W. fruticosa*, *X. granatum* and *A. excelsus* exhibited λ_{\max} at 310, 450 and 580 nm; Right figure indicates UV-visible spectra of the two others closely related red dyes from *C. tagal* and *R. mucronata* exhibited λ_{\max} at 300/330 nm, 400, 450, 480 and 580 nm.

Figure 4. Polyphenol contents of the crude pigment extracts. Mean values are calculated from three independent experiments and the bars represent the standard error. Means with the same letter in column are not significantly different at $p < 0.05$ level by Duncan's multiple range test. The analysis of variance has been conducted separately for each chemical family). **A:** Total polyphenol content expressed in gallic acid equivalent (mg GAE/g of extract powder); **B:** Flavonoid content expressed in quercetin equivalent (mg QE/g extract powder); **C:** Total condensed tannins expressed in epigallocatechin equivalent (mg ECE/g extract powder); **D:** Total monomeric anthocyanin expressed in cyanidin-3-glucoside equivalent (mg C3GE/l solution extract).

(Wof: *W. fruticosa*, Xyg: *X. granatum*, Ace: *A. excelsus*, Rhm: *R. mucronata*, Cet: *C. tagal*).

Figure 5. Effect of pH on color characteristics and stability of the crude pigment extracts expressed in terms of lightness (L^*), hue angle (h^*), and chroma (C^*) values in the CIELab color system.

(Ace: *A. excelsus*, Cet: *C. tagal*, Rhm: *R. mucronata*, Xyg: *X. granatum*, Wof: *W. fruticosa*. Carmine (Car) and Madder (Mad) were used as standards).

Figure 6. Variation of the intensity of the color (in terms of chroma C^* value) of the crude pigment extracts from the five red dyestuffs (A, B, C, D & E) exposed at different temperatures (from 30 to 305 °C) during 30 min., and the total color variation (ΔE) in the CIELab system compared with the control (non-thermally treated dye) for the entire temperature range.

(A: *W. fruticosa*, B: *X. granatum*, C: *A. excelsus*, D: *C. tagal*, and E: *R. mucronata*. Carmine (F) and madder extract (G) are used as standard for comparison purpose. The region of the box below the broken red line describes the thermal condition which exhibit the ΔE_{50} value, i.e., a colour variation above 50 colour units compared with the control).

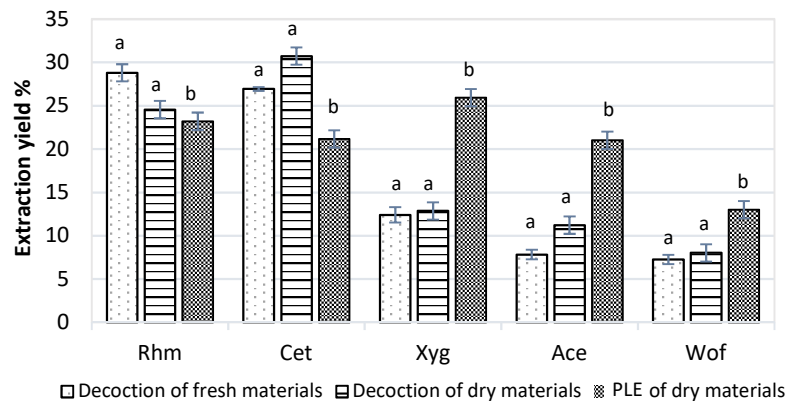


Figure 1.

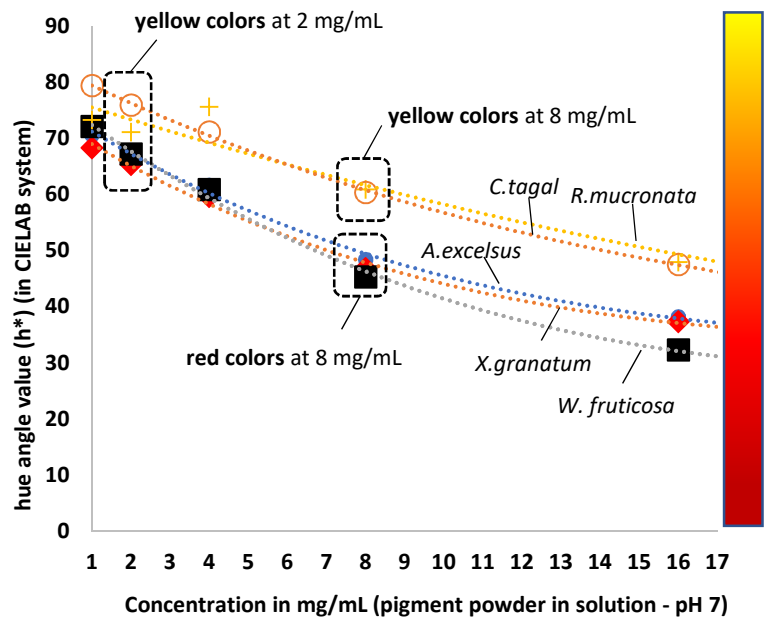


Figure 2:

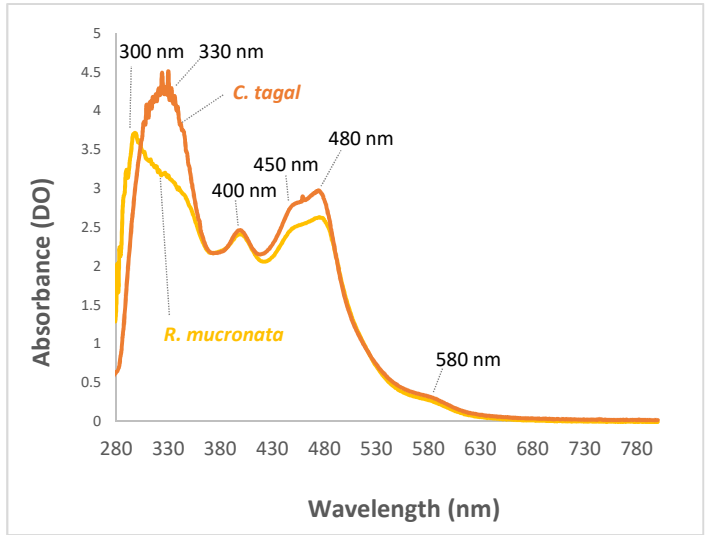
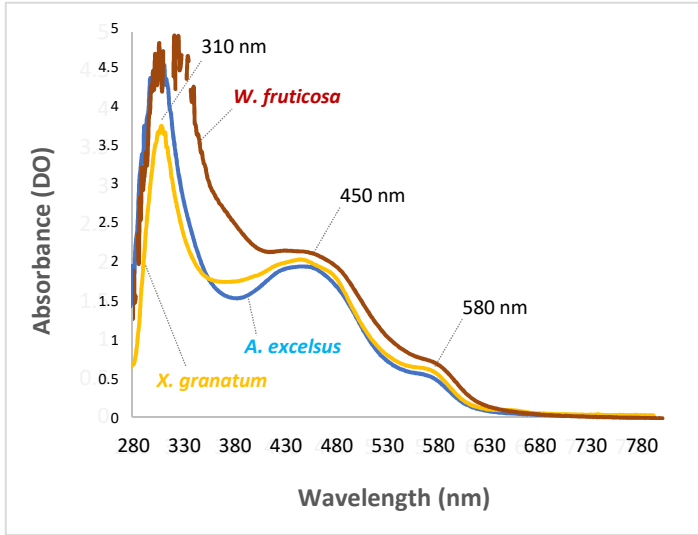


Figure 3:

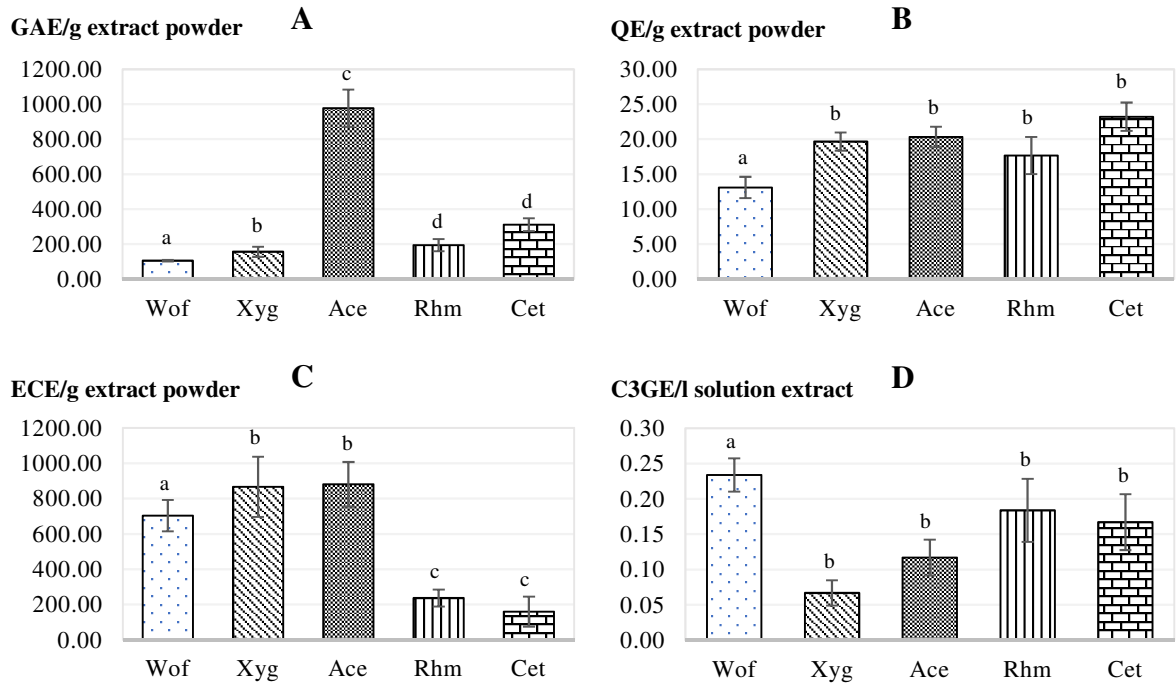


Figure 4.

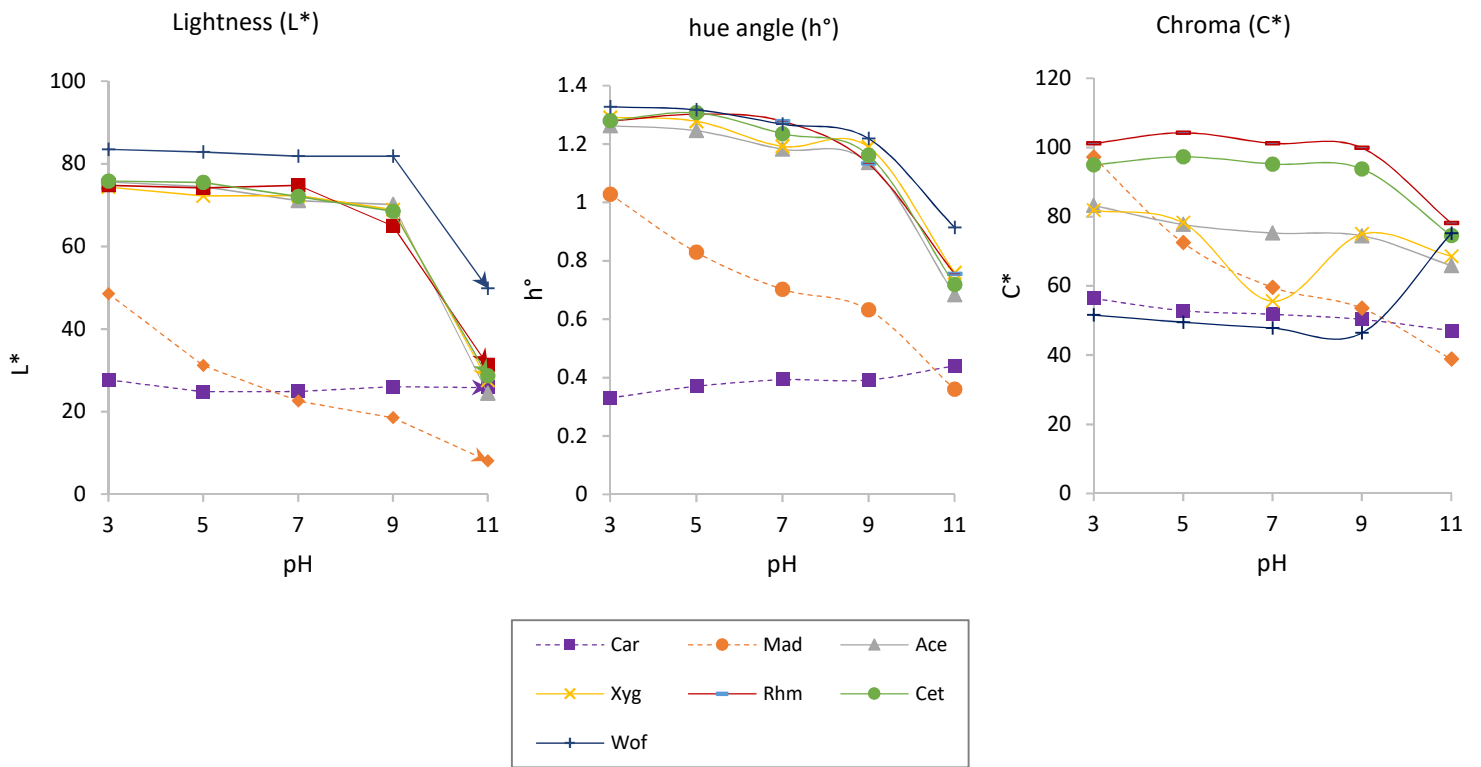


Figure 5.

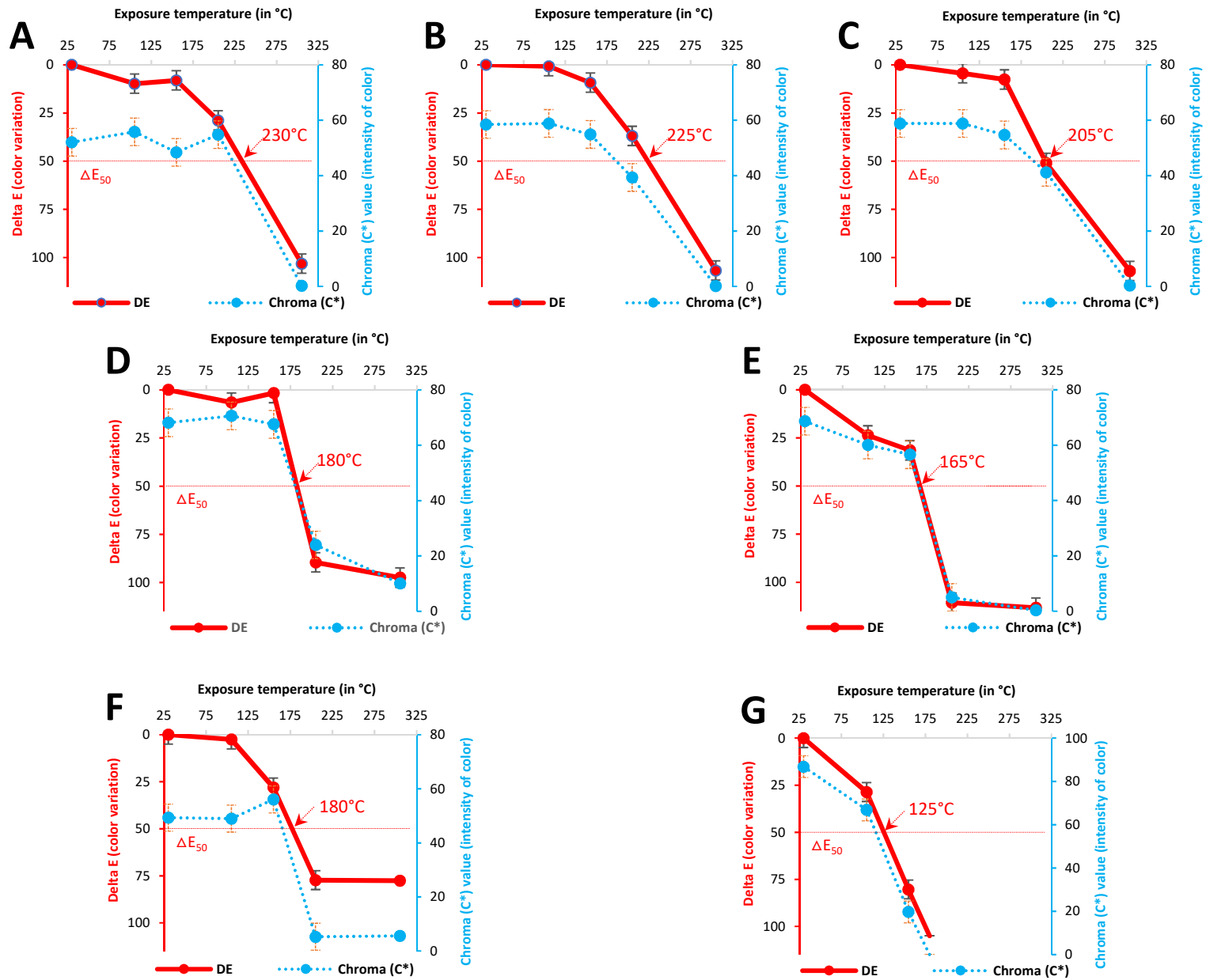


Figure 6.