



Evaluation of Mangrove (*Rhizophora mangle* L.) products as coloring, antimicrobial and antioxidant agents

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Abstract

Mangrove populations were selected in Multiple Use Natural Reserve of Monterrico, Guatemala, for the chemical characterization and biological evaluation of leaves, roots and bark. Ethanol extract were prepared and secondary metabolites identified by macro and semi-micro tests. The antioxidant activity was determined by ABTS and DPPH tests and antibacterial activity by a microdilution test. The best extraction yields of leaves was obtained with ethanol (43%), most common secondary metabolites were identified as flavonoids and tannins. Leaf samples demonstrated the greatest number of flavonoids expressed as chlorogenic acid; in the bark and roots, the most common secondary metabolites were tannins. Ethanol extracts of root and leaf showed the highest antioxidant activity by DPPH and ABTS tests (IC₅₀ 0.21 and 0.15 mg/mL, respectively). The extracts showed moderate antibacterial activity against *Escherichia coli* and *Salmonella typhi* at 1 mg/ml. The tests showed a possible cosmetics use as coloring extracts; it can be used at concentrations of 1, 3 and 5%, which had coloration from yellow to orange as possible substitutes for artificial yellow dyes such as No. 5 and 6. Tinctures showed good stability a different pH (3, 4, 5 and 7), especially leaves tinctures at pH 5 such as Red No. 40. Phytochemical analysis showed the presence of various metabolites of therapeutic and cosmetic significance. This paper demonstrates that Mangrove products could be used as coloring and antioxidant agents in the cosmetic, pharmaceutical and food industry. Utilization of these products might help in preserving Mangrove from depredation and sustainable exploitation in ecological reserve areas.

Keywords: Antioxidant activity, Antibacterial activity, Colorant properties, Mangle



Introduction

The term mangrove is used to designate halophytic (salt loving) and salt resistant marine tidal forests comprising of trees, shrubs, palms, epiphytes, ground ferns and grasses, which are associated in stands or groves. Mangroves are usually only in tropical climates, as they need consistently warm conditions for development and survival.¹

Covering over 4,000 km², mangrove forest is probably the most important coastal ecosystem along the Pacific Coast of Central America. This region contains highly diverse and structurally developed mangrove forest.²

Red mangroves are probably of greatest value for their environmental benefits, since they (and mangroves in general) play a vital role in supporting marine food chains, protecting coastal areas, and improving water quality. Traditionally, mangroves have been exploited for firewood and charcoal and were used in construction of dwellings, furniture, boats and fishing gear, and production of tannins for dyeing and leather production. Mangroves provide food and a wide variety of traditional products and arti-

facts for mangrove dwellers. Numerous mangrove plants have been used in folklore medicine. Use of extracts from mangroves and mangrove-dependent species to prohibit growth of human, animal and plant pathogens are reported in limited investigations without identify the metabolites responsible for their bioactivities.^{3,4}

Metabolites from mangrove have been characterized, some with novel chemical structures belonging to variety of chemical classes. Aliphatic alcohols and acids, amino acids and alkaloids, carbohydrates, carotenoids, hydrocarbons, free fatty acids including polyunsaturated fatty acids (PUFAs), lipids, pheromones, phorbol esters, phenolics, and related compounds, steroids, triterpenes, and their glycosides, tannins, other terpenes are among these classes.⁵

In the New World, *Rhizophora mangle* L. is the dominant pioneer mangrove species colonizing emergent substrates.⁶⁻⁹ The bark and leaves are used in folk medicine for the treatment of diabetes, angina, boils, minor bruises, and fungal infections, diarrhea, dysentery, elephantiasis,



fever, malaria, leprosy, plaster for fractured bones, tuberculosis, and antiseptic. The bark is used for the treatment of sore throat and against hemoptysis in pulmonary tuberculosis for its hemostatic properties. It has been reported to have antifungal and antibacterial activity, gastric antiulcer properties, and show efficacy in wound healing.

Healing effect of *R. mangle* bark on gastrointestinal ulcers has been proven by several mechanisms of action, like cytoprotection, anti-secretion, prostaglandin PGE2 depletion and inhibition and wound healing.^{11,12}

Chemical constituents reported in bark are sulphur containing nitrogenous compounds.¹³⁻¹⁵ Previously isolated compounds include polyphenols, carbohydrates, fatty acids and sterols.¹⁶

The bark extract showed activity against a variety of cancer cells (carcinomas, melanomas and lymphomas) and whole plant showed anti-hyperglycemic effects.⁵ The aqueous extract of the bark, its main component and high molecular weight polyphenols' fraction were studied for antioxidant activity using the deoxyribose assay. The total extract and its fraction showed scavenging activity of hydroxyl radicals and the ability to chelate iron ions.¹⁰

Materials and methods

Collection of plant materials and extracts preparation

Materials were collected in the Multiple Use Natural Reserve of Monterrico, which lies on the Pacific coast of southwest Guatemala in the Chiapas Sector of the Chiapaneca-Hondureña Province. It belongs to the Guatemalan System of Protected Areas and has a predominantly estuarine and coastal marine ecosystem, it supplies medicine to the local people as well; due to lack of economic resources, many people rely on natural sources.³⁶

One kilogram of leaf, root and bark of *R. mangle* was collected in five points, in different geographic coordinates from the Multiple Use Natural Reserve of Monterrico. These samples were processed according to the chosen techniques. The biologist Luis Alvarez established the identity of the samples, and voucher samples of the specimens deposited at CEMAT-Farmaya Ethno botanical Herbarium (CFEH 1183 and 1190).

The fractionated extraction was prepared from dry plant materials using hexane, dichloromethane, ethyl acetate and ethanol in polarity sequence. The extracting solvent was added to 200-500 g of dry plant material; it was percolated and repeated for five consecutive days by continuing replacement of the solvent. The extraction was concentrated by reduced pressure at a temperature below 45°C using a rotary evaporator. The secondary metabolites present in the extracts were screened by phytochemical procedure.¹⁷

Determination of antioxidant activity

Total Phenolic Compounds (TPC). Total phenolic compounds were determined by a standard macrometric method using the Folin-Ciocalteu reagent according to Phipps et al.,¹⁸ read in a Thermo Genesys 10 Spectrophotometer at 765 nm, and concentration estimated by a regression curve expressed in µg of gallic acid equivalent/

mg of dry extract.

1,1-diphenyl-2-picrylhydrazyl (DPPH). Qualitative evaluation was done by a standard thin layer chromatography (TLC) method in 60F₂₅₄ silica gel plates and sprayed with DPPH.¹⁹ Macrometric method was performed in tubes using acetate buffer, methanol, DPPH (0.0219%), and extract; after agitation and incubation for 30 min at room temperature, the results were read in a Thermo Genesys 10 Spectrophotometer at 517 nm against blank, and the IC₅₀ was calculated.²⁰ Micrometric determination was performed in a similar setting, but taking into consideration the scaling down needed to maintain the system in a 96-well plate, which was evaluated in an Elisa reader (Bio-Tek ELx-800) at 490 nm, followed by IC₅₀ calculation in mg of dry extract from the regression line or TDAC.²¹

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Discoloration of ABTS was evaluated according to Re et al.²² The ABTS radical cation was produced by mixing ABTS solution (7 mM) with potassium persulfate (2.45 mM), kept in the dark at room temperature for 16-18 h. For analysis, the reagent was diluted in ethanol until the absorbance at 734 nm was 0.70 ± 0.02 at 30°C. Extract dilutions were added to the diluted reagent and read at 1, 4 and 6 min. For each dilution, a curve was prepared in 60-70% inhibition, and the IC₅₀ was calculated.

Determination of Biocidal Activity

Antibacterial. Activity against bacteria and yeast was determined by an agar plate dilution method according to Mitscher et al.²³ Antibacterial screening of extracts was performed in agar, by preparing MHA with 1.0 mg/ml of the extract (MHA-E). Bacteria were inoculated in tubes with broth for 24 h at 36°C, and a 1:100 dilution in sterile distilled water was prepared. Each strain was streaked in quadruplicate (error <0.05) on the MHA-E surface and incubated at 36°C for 24 h. Bacterial growth was evaluated, and the minimal inhibitory concentration (MIC) was performed by microdilution method using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) according to the National Committee for Clinical Laboratory Standards guidelines.²⁴

Antifungal. Antifungal activity was assessed by evaluating the growth in agar containing extracts according to Brancato & Golding²⁵ adapted to natural products. It consists in purifying fungi in Mycosel agar, inoculating in sporulation medium (Takashio), and incubating at 25°C for 21 days; the spores were collected, counted, and the suspension of 1x10⁵ spores/ml was standardized and stored at 4°C. Sabouraud agar was prepared with 1.0 mg/ml of the extract or fraction; four holes of 8 mm diameter were inoculated with 30 µl of spore suspension and incubated for 21 days. To determine the MIC the same procedure was used, but with decreasing concentrations. The diameter (D), measured in mm of growth halo, was compared with a negative control using the formula: % = Dm / Dc X 100. For the percent inhibition of greater than >75%, the extract was set active (+); if it was less than 25%,

the extract was identified as an inactive (-).

Cytotoxicity and larvicidal activity, *Artemia salina* assay was done according to Solis et al.,²⁶ using extract dilutions of 1,000, 500 and 250 µg/mL; LC₅₀ was calculated by non parametric regression analysis using a Finney program for Basic. One, two and three instars larvae (*Aedes aegypti* and *Anopheles albimanus*) were used against the same dilutions, after 24 h, death larvae were evaluated visually.²⁷

Extraction and quantification of colorants

Colorants extraction. Dry material (100-200 g) was percolated with 95% ethanol:0.1N hydrochloric acid (85:15) in a 1:5-1:10 proportion for 24 h, eluate was concentrated to a honey grade, then dried in a desiccator and stored in refrigeration.

Colorant stability, Stability at different pH (3, 4, 5 and 7) was evaluated in a 0.5% solution in buffer. Samples were read at maximum absorbance wavelength in the visible region of the standard spectrum, at 40°C and 70% humidity. Reads were recorded daily until absorbance was less than 80% of initial value.²⁸

Quantification of flavonoids. Plant material (1 g) was added to 50 mL of hot water in a 100 ml volumetric flask in water bath for 60 min. The solution was cooled to room temperature and volume adjusted with distilled water, filtered and diluted 1:10. Dilutions were prepared (10, 20, 30, 40, 50, 60 and 70 ppm) of the standard chlorogenic acid and a curve was prepared at wavelength 324 nm. The chlorogenic acid curve was used to determined the metabolite concentration in the sample.²⁹

Tannins determination. Total tannins content was determined by phosphomolybdenum tungstic acid. Solution was prepared with 10 g of sample and 500 mL of 50% ethanol, shaken for 6 h, stand for 8 h and shaken again for 30 min and filtered. The filtrate was transferred to a volumetric flask of 50 mL and diluted with distilled water to the total volume, using tannic acid as the standard, read in wavelength of 700 nm.³⁰

Results

Total yield of ethanolic extracts ranged from 35.16% to

43.38% (Table 1). This implied that most of the soluble components have a high polarity.

Antioxidant capacity of ethanolic extracts were evaluated by three methods (DPPH, ABTS and TEAC) comparing with five standards. Leaf extracts showed the best antioxidant activity by the three methods (IC₅₀ 0.15 ± 0.02, 0.26 ± 0.04 and 1.01 ± 0.02 µg/ml, respectively). Total phenolic contents in leaves of *R. mangle* were 637.3 ± 31.3 mg/g. Flavonoids expressed as chlorogenic acid on leaves showed the highest concentrations (110.1 ± 0.6 ppm). Quantification of tannins showed that highest concentration in the root samples (5.4 ± 0.1%).

The antibacterial activity in samples of leaves, bark and roots extracts of *R. mangle* were screened by agar dilution method; positive extracts were further tested to determine the MIC in microplate. All extracts showed antibacterial activity against *Salmonella typhi* and *Bacillus subtilis* sub sp. *spizizenii* at a concentration of 1 mg/mL. However, ethanol extract showed a moderate activity only against two bacteria. The MIC values of extracts were better for Gram negative bacteria.

Absorption spectra of *R. mangle* tinctures were recorded using a UV-visible spectrophotometer. The change in the maximum absorbance (Amax) at varying wavelengths (λmax) presented the change in the color intensity. Results showing higher absorbance at pH 3, 4 and 5 for tinctures leave samples while at pH 7 a higher absorbance was observed for bark (Table 3). Tinctures showing maximum absorbance were at different wavelengths 322-323 nm, bark 210-306 nm and for roots at 208 and 585 nm. The leaves showed highest color equivalent compared as Red 40 in ethanol HCl (382.4 ± 2.8 ppm/g), while bark (300.7 ± 2.3 ppm/g) and roots (310.1 ± 3.6 ppm/g) showing to buffer pH 7 (Table 2).

Discussion

Mangrove was selected because they are species generally undervalued, overexploited, and poorly managed.³¹ Yet, their importance to human, wildlife, and global carbon balance is paramount.^{32,33} Human activities have destroyed 35% of the worlds' mangrove forests over the last two de-

Table 1. Extraction Yield and Antibacterial Activity of *Rhizophora mangle* (Red Mangrove) Extracts

		Sample		
		Leaves	Barks	Roots
Total Yield in ethanol extraction by maceration (%)		43.38	35.16	36.56
Metabolite Quantification	Flavonoids as chlorogenic acid (ppm)	110.1 ± 0.6	42.9 ± 0.9	90.2 ± 0.6
	Tannins	4.5 ± 0.2	4.2 ± 0.1	5.4 ± 0.1
Antibacterial Activity (mg/mL)	<i>Staphylococcus aureus</i> ATCC 25923	> 1	> 1	> 1
	<i>Salmonella typhi</i> ATCC 14028	1	1	1
	<i>Mycobacterium smegmatis</i> ATCC 607	> 1	> 1	> 1
	<i>Bacillus subtilis</i> ATCC 6051	> 1	> 1	> 1
	<i>Pseudomonas aeruginosa</i> ATCC 27853	> 1	> 1	> 1
	<i>Bacillus subtilis</i> subsp. <i>spizizenii</i> ATCC 6633	> 1	> 1	> 1
	<i>Escherichia coli</i> ATCC 8739	1	1	1

Table 2. Antioxidant Activity of *Rhizophora mangle* (Red Mangrove) Extracts

Bioassay	Organ from the plant			Standards				
	Leaf	Bark	Root	Vit. C	Vit. E	Quercetin	Rutin	TBHQ
DPPH (TLC)	++++	++++	++++	++++	+++	+++	+++	+++
DPPH IC ₅₀ (mg/mL)	0.15 ± 0.02	0.24 ± 0.01	0.21 ± 0.01	0.14 ± 0.03	0.72 ± 0.01	0.16 ± 0.04	0.16 ± 0.01	0.29 ± 0.02
ABTS IC ₅₀ (mg/mL)	0.26 ± 0.04	0.32 ± 0.01	0.35 ± 0.01	4.04 ± 0.03	0.19 ± 0.02	0.10 ± 0.04	0.05 ± 0.02	0.09 ± 0.02
TEAC(mmol/g)	1.01 ± 0.02	0.81 ± 0.03	0.88 ± 0.04	–	–	–	–	–
Phenolics (µgGA/mg)*	637.3 ± 31.3	538.9 ± 40.3	598.6 ± 71.7	–	–	–	–	–

Table 3. Evaluations of Potential Colorants From *Rhizophora mangle* Extracts

	pH of the Buffer	Abs (λ)	Sample		
			Leaves	Barks	Roots
Stability of tinctures at different pH	pH3	λ	322	306	585
		Abs	0.63	0.29	0.01
	pH4	λ	323	–	–
		Abs	0.64	–	–
	pH5	λ	322	305	–
		Abs	0.64	0.25	–
	pH7	λ	279	210	208
		Abs	1.03	3.48	2.87
		λ	322	280	280
		Abs	0.62	0.73	0.40
λ		–	–	318	
Abs		–	–	0.14	
Color equivalent compared with synthetics colorants (ppm/g)	EthOH:HCl	Red 40	382.4 ± 2.8	–	115.9 ± 2.4
		Yellow 5	96.2 ± 0.5	68.3 ± 0.3	46.8 ± 0.5
	pH3	Red 40	166.4 ± 1.0	71.3 ± 0.8	–
		Yellow 5	18.9 ± 0.6	–	–
	pH4	Red 40	155.3 ± 3.2	–	–
		Yellow 5	18.9 ± 0.6	–	–
	pH5	Red 40	198.1 ± 0.4	53.3 ± 0.2	75.6 ± 0.1
		Yellow 5	–	300.7 ± 2.3	310.1 ± 3.6
	pH7	Red 40	–	300.7 ± 2.3	310.1 ± 3.6
		Yellow 5	87.4 ± 2.0	68.7 ± 1.7	57.3 ± 1.2

cedes,³⁴ and mangrove conservation and sustainable use as a zone of critical transition between land and sea needs to be better appreciated.³⁵

Red mangrove extract was produced by percolation using different solvents. Results have shown that ethanol extract yields were higher in all organs of the plant, mainly in the leaves (43%). The quantification of flavonoids demonstrated the maximum value expressed in chlorogenic acid in leaves (110 ppm). It is accepted that several classes of flavonoids play a significant role in many physiological processes, show antioxidant and fungicidal activity, and are natural antihistamines. Flavonoid and flavonol-lignan derivatives inhibit lipid peroxidation and are potent quenchers of triplet oxygen.³⁷ Several investigators have established that mangroves are also rich in polyphenols, among which flavonoids are a significant group.³⁸

Tannins are used in the manufacture of plastics, paints, ceramics and water softening agents. Members of the families Avicenniaceae, Rhizophoraceae, and Sonneratiaceae are rich source of tannins.³⁹ Increasing attention is attributed to the use of tannins as antimicrobial agents (e.g.

wood preservation) or prevention of dental caries. They are important molecules in flavor and color of wines. Recently, evidence has been obtained in support of potential values as cytotoxic or antineoplastic agent.⁵ In the chemical characterization studies of the extract, tannins are the main components, although the presence of other compounds such as epicatechin, catechin, chlorogenic, gallic and elagic acids, as well as galotannins have been also described. The extracts of some mangrove species indicated significant antioxidant activity,^{40,41} and we supposed the active compounds responsible for antioxidant activity are tannins. The mangrove tannins have substantial reducing power, DPPH as well as ABTS-free radical-scavenging abilities, that are comparable to the synthetic standards and other commercial tannins evaluated.⁴² The structures of the main monomers constituting the condensed tannins were identified as catechin, epicatechin, epigallocatechin, and epicatechin gallate.⁴³ In mangrove species, condensed tannins are abundant components (as high as 20% dry weight) which prevent damage from herbivores,⁴⁴ but they also show a diversity

of other biological activities of historic and potential importance to humans.⁴⁵

Free radical scavenging capacity was analyzed using DPPH, ABTS, TEAC method, was determined in root, bark and leaf samples. A higher activity was found in ethanolic extract in leaves (0.15 mg/ml, 0.26 mg/ml and 1.01 mmol/g, respectively) comparable with standards as vitamin C, quercetin and rutin. Previous studies demonstrated that aqueous extract of *R. mangle* bark and its fractions showed antioxidant activity, achieved by the scavenging ability observed against hydroxyl radicals and iron chelating properties.¹⁰ These results are in agreement with those for others plant extracts that contain polyphenols.⁴⁶

R. mangle aqueous extract had antioxidant properties in vivo and its major polyphenolic fraction was responsible of this effect. Antioxidant activity of the extract and its fractions can explain, at least in part, the wound healing accelerator effects shown in previous studies.¹⁶

The higher total phenols content was demonstrated in leaves (637 µg galic acid/mg extract) related to antioxidant activity. The antioxidant activities of polyphenolics have received considerable attention because of their links to human health,^{47,48} and in plants, for their association with multiple stress responses. Quercetin, the major flavonol on which the mangrove non-tannin flavonoids are based, is particularly effective in this function. The diversity of mangrove polyphenolics reflects factors ranging from the biochemical to the evolutionary scales.⁴⁹ Polyphenols are the major plant compounds with antioxidant activity. The results strongly suggest that phenolics are important components in these plants, and some of their pharmacological effects could be attributed to the presence of these valuable constituents.

Antibacterial assays for ethanol extracts in bark, leaves and roots and the controls, were also carried out. All extracts showed moderate activity against *Escherichia coli* and *Salmonella typhi* (1mg/mL). According to own and others studies,^{50,51} it has been recorded that, a number of mangrove plant extracts of methanol, ethanol and water showed antibacterial activity against pathogenic isolates as well as antibiotic resistant bacteria. Insecticidal,¹⁴ and antiviral⁵² activities are found in extracts of different parts of the plant. Compared to other species tested, *R. mangle* showed only little activity against fungal skin infections.^{53,54} However, the antibacterial activity of mangroves may be influenced by factors such as the habitat and the season of collection, different growth stages of plant and experimental methods. Although a variety of solvents have been employed in screening the mangroves for their antimicrobial activity, it is still not clear what kind of solvent is the most effective and suitable for extract preparation. The tests showed the cosmetics use as coloring extracts; it can be used in concentrations of 1, 3 and 5%, which showed colorations from yellow to orange as possible substitutes for artificial yellow dyes such as # 5 and 6.

For a therapeutic success in the development of new formulations in the pharmaceutical and cosmetic industry, the new product should be stable, which makes the stabil-

ity evaluation a key factor. This can be measured through a study of accelerated stability, which aims to verify, in specific, controlled, conditions the ability of a product to maintain the same characteristics and properties during its useful life. The red mangrove tinctures showed good stability at different pH (3, 5 and 7), under accelerated temperatures, but this is a matter to be confirmed in future shelf stability study.

Conclusion

Guatemala has very rich biodiversity including a large variety of native and introduced plants, rich in natural colorants. *R. mangle* represents an ethnobotanically relevant specie in different countries, traditionally used for different medical applications. The result obtained showed that some extracts have a potential as antioxidant and colorant, which make its exploration and utilization possible, particularly because of the high activity by the leaves. There is an unexplored and expressive potential for extraction, production and use of natural colorants in the country. This is unknown and deserves higher attention as new materials in the development of new ingredients as natural colorants; these extracts might have several applications on food, cosmetics, and leather and textile industries, becoming a new income source for small rural producers.

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