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Article

In vitro UV absorption properties and radical scavenging capacity of *Morella parvifolia* (Benth.) Parra-Os. extracts

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The goal of this research was to identify major compounds of the aerial parts of M. parvifolia (Benth.) Parra-Os., that could enhance its possible application as additive in dermocosmetic products, as well as evaluate the antioxidant properties. The extracts agreed with the broad-spectrum UVB/UVA absorption detected and could act as broad-spectrum sunscreens, covering the UVA and UVB range. Methanolic extracts showed an important antiradical capacity (0.46 and 0.47 g/µmol DPPH), TPC (37.58 and 51.41mg GAE/g DS) and TAC (1.12 and 3.31 mg C3GE/g DS) in fruits and leaves, respectively. M. parvifolia could be considered as a prospective source of natural UV-radiation absorbers with antioxidant capacity. Although the results have clearly demonstrated the potential photoprotection capacity, more studies are needed to enhance its application as an additive in pharmaceutical and medicinal formulations.

Keywords: *Morella parvifolia*. Potential skin barrier. UV absorption capacity. Pharmaceutical additive.

INTRODUCTION

Natural bioactive compounds isolated from plants may act as potential antioxidant, antimutagenic, anti-inflammatory and anticarcinogenic agents to reduce the UV generated ROS-mediated photodamage, immune-suppression and skin cancer in patients (Katiyar *et al.,* 1999; 2000; Stahl *et al.,* 2000; Zhou *et al.,* 2009b; Morales *et al.,* 2011). In that line, bioactive compounds such as vitamin C, vitamin E and carotenoids, phenolic acids, catechins, flavonols and anthocyanins have shown strong antioxidant activity, and all of them can be used as bio-additives in medicinal, food and dermocosmetic products (F'guyer, Afaq, Mukhtar, 2003; Duraisamy *et al.,* 2011; Jarzycka *et al.,* 2013) on account of their beneficial health effects. For a long time, plants have been used for their ethno-botanical and ethno-pharmacological relevance. However, at present day, many of them are not supported by adequate scientific reports about their potential industrial applications. For instance, information about chemical and biological properties of vegetation from ecosystems over 2000 meters above sea level is scarce. These plants are exposed to hard environmental conditions, which induce them to synthetize metabolites as natural defences against oxidative stress due to high levels of ultraviolet radiation, low temperature etc. (Crockett *et al.,* 2010). The most common compounds found in these plants are polyphenols that have demonstrated antioxidant activity. Also, it has been observed that some of them are capable of absorbing UV radiation (Agati, Tattini, 2010; Agati *et al.,* 2013).

Considering the increased exposition to solar UV radiation and the potential risk for human beings, research on new natural sources with high content of chemical compounds able to absorb ultraviolet radiation have become a hot topic in recent years (Jarzycka *et al.,* 2013; Pérez-Sánchez *et al.,* 2014). *Morella parvifolia* (Benth.) Parra-Os., a shrub that belongs to the Myricaceae family and can be found in Colombia, Venezuela, Ecuador and Perú between 1300 to 3800 meters above sea level, is exposed to adverse environmental conditions such as fluctuations of rainfall and temperature, high humidity, low temperature and high UV radiation (Parra, 2003; Gonzales de la Cruz *et al.,* 2014). These exceptional conditions induce the plants to produce

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bioactive compounds such as phenolic acids, flavonoids and high molecular weight polyphenols; all of them are natural defences against those ecological factors that are involved in UV generated ROS-mediated photodamage, immune-suppression and skin cancer (Bieza, Lois, 2001; Svobodová, Psotová, Walterová, 2003; Mellegård *et al.,* 2009). On the other hand, erythema caused by solar radiation is the best-known acute effect of prolonged human exposure to UV radiation. This electromagnetic radiation induces degenerative changes in skin cells, resulting in premature skin aging (Damiani *et al.,* 2006; Ponzo, Silvia, 2013). Furthermore, some species of Myricaceae have been used in the treatment of chest-related diseases, painful menstruation, and to enhance men's sexual performance (Ashafa, 2015). In addition, the anticancer capacity against human lung cell carcinoma was reported in extracts of *Myrica gale* L. (Sylvestre *et al.,* 2005). According to our information, studies on photoprotection and UV absorption potential of *M. parvifolia* have not yet been reported. Therefore, the aim of this study was to evaluate specifically the phytochemistry and biological activities of *M. parvifolia* grown in the Andean Mountains of Antioquia-Colombia, with regard to their *in vitro* UV-radiation absorption capacity, main phenolic compounds and antioxidant capacity, in order to propose it as non-conventional source of skin-care additives.

MATERIAL AND METHODS

Plant materials, chemicals, extraction and phytochemical analysis

Fresh vegetal material, leaves and fruits (mature stage) of *M. parvifolia* (Benth.) Parra-Os. were collected in March 2013 during rainy season and flowering stage, at Llanos de Cuivá, Santa Rosa de Osos, Antioquia, Colombia at 2700 meter above sea level (Geographical coordinates: 6°49'50.6"N; 75°29'29.9"W). A voucher specimen was deposited in the Herbarium of the University of Antioquia (HUA) under registry code HUA192390.

2,2-Diphenyl-2-picrylhydrazyl (DPPH) stable radical, gallic acid, rutin, benzophenone 3 (B3), butyl methoxydibenzoylmethane, (BMDM) and ethylhexyl methoxycinnamate (EHMC) were obtained from Sigma Chemical Co. (St. Louis, MO). Myricetin-3-glucoside, myricetin-3-rhamnoside and cyanidin-3-rutinoside were purchased from Extrasynthese (Lyon, France). Folin-Ciocalteu phenol reagent, methanol, ethanol, hydrochloric acid 37%, potassium chloride, sodium acetate and sodium carbonate were obtained from Merck Chemical Supplies (Damstadt, Germany). Formic acid was obtained from Fermont (Monterrey, NL, México). 2-thiobarbituric acid (TBA), butylated hydroxyl toluene (BHT) and methyl linoleate (MeLo) were purchased from Alfa Aesar (Ward Hill, MA).

All fresh vegetal material was processed in March 2013. Leaves and fruits were dried at room temperature, protected from sun and artificial light for one week. Then, the dry sample (DS) was crushed using an electric grinder (IKA, A11 basic S1). Next, dry and powdered leaves or fruits of *M. parvifolia* (c.a. 1.0 g) were mixed with 50 mL methanol and 0.5 mL HCl (37%, v/v) for 24 h at room temperature and magnetically stirred at 900 rpm. The resultant solution, previously filtered, was evaporated to dryness in a rotary-evaporator under reduced pressure at 40 °C. Then, extracts were redissolved in methanol and keep at 4 °C before analysis. The content of phytochemicals such as phenols, flavonoids, anthocyanins, quinones, and tannins was analysed using dry material dissolved in ethanol:water (1:1) according to methods described by Dohou (Dohou *et al.,* 2003).

Total polyphenol content (TPC)

The total polyphenol content in leaves and fruits extracts was determined using the Folin-Ciocalteu colorimetric technique with some modifications (Mejía-Giraldo *et al.,* 2016). Briefly, leaf and fruit extract solutions (c.a. 0.1 mL) were added to 0.125 mL of the Folin-Ciocalteu reagent and 1.25 mL of 20 % (w/v) Na_2CO_3. After incubation for 90 min at room temperature, the absorbance was read at 760 nm using an Evolution 60S spectrophotometer (Thermo Fisher Scientific, Inc., Shanghai, China). The results were expressed as gallic acid equivalents per gram of dry extract (GAE/g DS).

Total anthocyanin content (TAC)

Total anthocyanins were estimated by a pH differential method (Lee, Durst, Wrolstad, 2005) with some modifications. An aliquot (c.a. 1.0 mL) of solution of extract was mixed with 2 mL of buffer at pH 1.0 (hydrochloric acid/potassium chloride; 0.025 M) and another at pH 4.5 (acetic acid/sodium acetate; 0.4 M). After that, absorbance (A) was measured in a Thermo Scientific Evolution 60S UV-Visible spectrophotometer at 510 and 700 nm. The anthocyanin pigment concentration was expressed as cyanidin-3-*O*-glucoside equivalents using the following equation:

$$\text{Total Anthocyanin Content (TAC, mg/L)} = (A \times MW \times DF \times 1000)/(\varepsilon \times l)$$

where A= absorbance = $(A_{520nm} - A_{700nm})_{pH1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}$; MW is the molecular weight for cyanidin-3-*O*-glucoside (cyd-3-glu) = 449.2 g/mol; DF is the dilution factor established; l is the path length of the cell in cm; ε is the molar extinction coefficient of cyd-3-glu = 26 900 L/mol cm; and 10^{+3} is the conversion factor.

In vitro DPPH radical assay

This technique is based on the reduction of the DPPH radical (purple-coloured) into the corresponding yellow hydrazine (DPPH-H) by the antioxidant (H-transfer reaction) (Pereira, Tavano, 2014). The radical scavenging capacity of each extract in different concentrations was estimated according to the method described previously (Brand-Williams, Cuvelier, Berset, 1995) with some modifications; the effective relative concentration (EC_{50}) at which 50% of DPPH has been removed was expressed as mg of extract/µmol of DPPH radical, based on the following equation:

Efficient Concentration (EC50) = concentration of test at steady state/concentration of DPPH t=0.

The initial DPPH exact concentration (90.84 µmol/L) in the reaction system was calculated according to a calibration curve (Y = 1.145E-2X – 4.192E-3; r = 0.9999; where Y= absorbance and X= concentration of DPPH) at 514 nm). All experiments were performed in triplicate. All spectrophotometric data were obtained using a Thermo Scientific Evolution 60S UV-Visible spectrophotometer. Disposable cuvettes (1 cm x 1 cm x 4.5 cm) were used for visible absorbance measurements for analyses. BHT standard was used as positive control.

In vitro lipid peroxidation inhibition of methyl linoleate (MeLo)

A solution of MeLo 10 mM containing 0.02% (w/v) of BHT and 0.02% (w/v) of each dry extract was exposed to thermal oxidation at 40±5 °C for 5 days in test tubes. After accelerated oxidation, each sample was dissolved in 1 mL of ethanol. The conjugated diene hydroperoxide (CDH) was measured spectrophotometrically at 234 nm. The samples were diluted to 1:25 with ethanol. An extinction coefficient of 29 000/M cm, was utilized to quantify the concentration of CDH formed during oxidation. The peroxidation level was expressed as mmol CDH/kg MeLo (Farhoosh *et al.,* 2012). On the other hand, the level of lipid peroxidation was also expressed as malondialdehyde (MDA) content and was determined as 2-thiobarbituric acid reactive species (TBARS). Briefly, 50 µL of sample were mixed with 350 µL of ethanol, 100 µL BHT 0.2% w/v in ethanol and 500 µL of TBA 0.37% w/v in HCl (0.25 mM) in a test tube. The resulting mixture was heated for 30 min in a sand bath at 90±5 °C. After that, the solution was quickly cooled in an ice bath and the flocculated precipitate was removed by centrifugation at 1100 rpm/5 min. The absorbance of the samples was measured at 535 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. MeLo standard was used as positive control. The peroxidation level was expressed as mmol MDA kg^{-1} MeLo using a molar extinction coefficient of 156 000/M cm (Puertas-Mejía, Ruiz-Díez, Fernández-Pascual, 2010).

UVA-UVB absorption capacity assay

The UVA-UVB absorption capacity was determined using the absorption coefficient (absorbance DS/mg mL) measured at 290, 310, 340 and 380 nm. Briefly, an aliquot (c.a. 100 µL) of crude extract was added to 2000 mL of methanol in a quartz cuvette and its absorption spectra (wavelengths 200 - 400 nm) was acquired using a Thermo Scientific Evolution 60S UV-Visible spectrophotometer against a blank containing methanol. Benzophenone 3 (B3), butyl methoxydibenzoylmethane (BMDM) and ethylhexyl methoxycinnamate (EHMC) were used as conventional sunscreen standards.

HPLC-DAD-IT-MS^n characterization of the main compounds

Analysis was performed using an Agilent 1200 series system (Agilent Technology, Palo Alto, CA, USA) equipped with a diode array detector (G1315B), a model G1379B degasser, a binary gradient pump (G1312A), an autosampler G1367B and a column thermostat (G1316A). The analytical column (Waters Symmetry 4.6 mm x 75 mm x 3.5 µm) was thermostated at 30 °C. The mobile phase consisted of a gradient mixture of (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol. The gradient program was as follows: 30% B (0.5 min), 30-90% B (6.5 min), 90% B (1 min), 90-30% B (1 min), 30% B (2 min). The phenolic compounds were monitored at 290, 310, 340, 380 and 520 nm, and the diode array detector was set to an acquisition range from 190 nm to 700 nm. The flow rate was 0.5 ml/min, and the injection volume was 2 µL (Mejía-Giraldo *et al.,* 2016).

The IT-MS analysis was performed on a Thermo Scientific system equipped with an Accela autosampler

and an Accela 600 quaternary pump (Thermo Scientific). The mobile phase was composed as described in the analysis of HPLC-DAD, and the injection volume was 10 µl. The column effluent was analysed by ESI-MS in positive ion mode with an LCQ Fleet Ion Trap (Thermo Scientific). The capillary voltage was set to 35 V, the spray voltage at 4.5 kV and the tube lens offset to 80 V. The sheath gas (nitrogen) flow rate was set to 45 (arbitrary units) and aux gas flow rate to 5 (arbitrary units). The capillary temperature was 300 °C. Data were acquired within 100-1000 m/z range. MS^n experiments were performed in the "Data dependent Ion tree" mode with normalized collision energy of 35 (arbitrary units). For data analysis, the Xcalibur 2.1 (Thermo Scientific) and MZmine 2 (MZmine Development Team) software were used (Pluskal *et al.,* 2010).

Statistical analysis

Results were expressed as mean ± standard deviation (at least three replicates). Analysis of variance and significant differences among means and correlation analysis were performed with one-way ANOVA. The experimental data were analysed using SPSS V.21.0 software.

RESULTS AND DISCUSSION

Certain promising topical treatments for skin aging, including herbal extracts, vitamin and antioxidant supplements, have been widely accepted to scavenge free radicals from skin cells and restore skin elasticity. In this line of work, the search for new natural sources of photoprotective and antioxidant functional compounds has been a challenge. The TPC (expressed in mg GAE/g DS) from selected aerial parts are shown in Table I. The highest concentration of TPC was found in leaf extracts. These results are convenient because leaves are more accessible and easier to collect throughout the year than fruits. On the other hand, the DPPH radical scavenging method is a common practice used for screening the ability of a substance or extract to scavenge free radicals. The EC_{50} values found were 0.46 and 0.47 g/µmol DPPH (fruits and leaves, respectively). According to these findings it is suggested that both leaves and fruit extracts showed strong antiradical capacity and *M. parvifolia* could be used, *e.g.* as raw material with application in topical formulations.

Concerning the anthocyanin contents, the results were similar to those obtained with TPC and fluctuated from 1.12 to 1.31 mg C3GE/g DS, with the highest content in the leaf extract (Table I). Anthocyanins are particularly abundant in some berries such as blackberry and bilberry (Faria *et al.,* 2010; Garzón *et al.,* 2010). Although, the anthocyanin content in *M. parvifolia* was too close to that reported for bayberry pomace (Zhou *et al.,* 2009a), in this situation, TAC was higher on leaves than fruits. These results could be linked to some molecular mechanism to reduce the oxidative stress initiated by UV radiation on its organelles and cellular tissue. Therefore, the extracts showed an important scavenging capacity that could be associated with the protection of plants from high altitude against oxidative stress due to UV radiation exposure. Furthermore, the assessment of accelerated oxidation of MeLo on each *M. parvifolia* extract confirmed the antioxidant outcome on the tested substances. Moreover, leaves and fruit extract did not show significant differences (P-value > 0.05) with respect to BHT and formation of MDA. In addition, our results were similar to that reported on methanolic extracts of fractions of *A. reticulata* and *Ziziphusjujuba* bark species, which showed effective antioxidant

TABLE I - Extraction yield and *in vitro* antioxidant capacity of *M. parvifolia* (Benth.) Parra-Os

Extract	Yield %	TPC mg GAE/g DS	EC_{50} g DS/µmol DPPH	TAC mg C3GE/g DS	MDA mmol/kg MeLo	CDH mmol/ kg MeLo
Leaves	36.79±0.69[a]	51.41±0.44[a]	0.47±0.01[a]	1.31±0.40[a]	0.18±0.11[a]	N.D.
Fruits	26.42±0.41[b]	37.58±0.67[b]	0.46±0.02[b]	1.12±0.41[a]	0.10±0.07[b]	N.D.
MeLo	-	-	-	-	12.1±1.9[a]	9.2±2.1[a]
BHT	-	-	0.112±0.01	-	-	-
MeLo+BHT	-	-	-	-	N.D.	N.D.

Results are expressed as the mean value ± standard deviation (n=3). For each parameter, different letters (a,b) denote significant differences by the LSD test (P < 0.05). TPC (total phenolic content), GAE (gallic acid equivalent), EC_{50} (efficient concentration at 50%), TAC (total anthocyanin content), C3GE (cyanidin-3-O-glucoside equivalents), DS (dry sample), MDA (Malondialdehyde), CDH (Conjugated diene hydroperoxide), BHT (Butylated hydroxy toluene), MeLo (Methyl linoleate), N.D. (No detected).

and antiinflammatory properties (Kandimalla *et al.,* 2016a; 2016b). Along these lines, our result showed a satisfactory antioxidant effect of the extracts in the last phase of lipid peroxidation of MeLo.

Plants' phenolic compounds play an important role on human diet for their beneficial health effects, mainly associated with their antioxidant capacity and ability to scavenge free radicals, chelate metal catalysts, activate antioxidant enzymes, etc. The qualitative study in this work, showed the presence of phenols, flavonoids and anthocyanins in both extracts. These outcomes are in good agreement with data reported by some authors (Wollenweber *et al.,* 1985; Santos, Waterman, 2000; Geyid *et al.,* 2005) who have detected the presence of flavonoids in Myricaceae species. According to these results, we found correlation between the yield obtained in both extracts as well as in TPC, TAC content and DPPH analysis. In addition, the EC_{50} values agree with those reported on methanolic extracts of *M. rubra* (Myricaceae) (Akazawa *et al.,* 2010). Furthermore, the TPC value obtained was higher than that described for common cultivars, including strawberries (0.844 mg GAE/g) (Kajdžanoska, Petreska, Stefova*,* 2011) and common legumes (0.57 to 9.60 mg GAE/g) (Xu *et al.,* 2007).

On the other hand, Ion Trap MS analysis delivers better qualitative structural analysis because of its capability to achieve consecutive fragmentation (MS^n) with high sensitivity, which is ideal to elucidate the fragmentation pattern. In our case, the HPLC-DAD-IT-MS analysis showed major presence of four components, which were identified as myricetin-3-O-glucoside, myricetin-3-O-rhamnoside, rutin and cyanidin-3-O-rutinoside (Table II), and all of them are considered flavonoids that are present in many plants, grapes and cranberries.

Therefore, extracts of *M. parvifolia* showed an interesting UVA-UVB absorption capacity, but both showed a broad-spectrum range similar to the standards (Figure 1). In addition, the absorption coefficients of the extracts were determined and compared at 290, 310, 340 and 380 nm, the selected regions for the UVA and UVB solar spectrum (Figure 2).

The compounds identified showed absorption bands in the UVA and UVB region and the enriched *M. parvifolia* extract could be of great interest as additives in broad-spectrum sunscreen formulations. In addition, an appropriate analysis of phenolic compounds depends on multiple factors such as their chemical nature, sample particle size, storage time, extraction and quantification methods and presence of interferences.

Otherwise, the use of synthetic UV absorbers as sunscreens in commercial cosmetic formulations must offer a broad-spectrum (UVB/UVA) that can be used for different skin pigmentations and environmental conditions, however, these chemical compounds can cause skin irritations. For that reason, screening of natural extracts is a suitable photoprotection strategy to reduce the UV generated ROS-mediated skin damage. Although fruits as well as leaves displayed broad-spectrum coverage, it was much higher in the UVB range for the fruit extract. This result has been associated with the presence of phenolic compounds in the extracts that have evidenced absorption of UV radiation (Stevanato, Bertelle, Fabris*,* 2014). The presence of cyanidin-3-rutinoside and myricetin derivatives possibly increased the ability of both extracts to act as absorbers of UV radiation (Velasco *et al.,* 2008). These phytochemicals have been widely studied due to their anticarcinogenic, antimutagenic, anti-inflammatory and antioxidant actions (Agati, Tattini 2010; Anunciato, da Rocha Filho 2012).

TABLE II - HPLC-DAD-ESI-MS/MS characterization of extracts from *M. parvifolia* (Benth.) Parra-Os. leaves

Peak	T_R min	UV spectra λmax (nm)	$[M+H]^+$	Major MS^2 and MS^3 fragments	Compound*
1	4.6	257sh, 262, 298, 356	481	MS^2: 481 → 319, 181 MS^3: 319 → 181	Myricetin-3-glucoside
2	4.9	262, 308sh, 350	465	MS^2: 465 → 319 MS^3: 319 → 273, 245, 165	Myricetin-3-rhamnoside
3	5.8	227, 256, 264, 300, 354	611	MS^2: 611 → 465, 449, 303; MS^3: 465 → 303; MS^3: 303 → 285, 257, 229, 165, 153, 137, 111	Rutin
4	7.4	520	595	MS^2: 595 → 449, 287; MS^3: 449 → 287	Cyanidin-3-rutinoside

*Peak compared with standards.

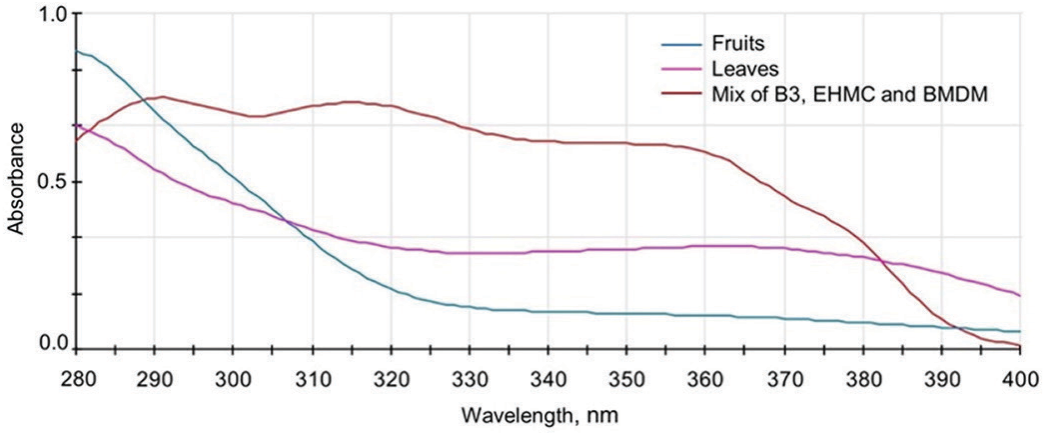

FIGURE 1 - UV spectrum of *M. parvifolia* extracts (at 0.019 mg/mL, all of them) and a mixed sample of benzophenone 3 (B3), butyl methoxydibenzoylmethane (BMDM), ethylhexyl methoxycinnamate (EHMC) (at 0.017 mg/mL, all of them).

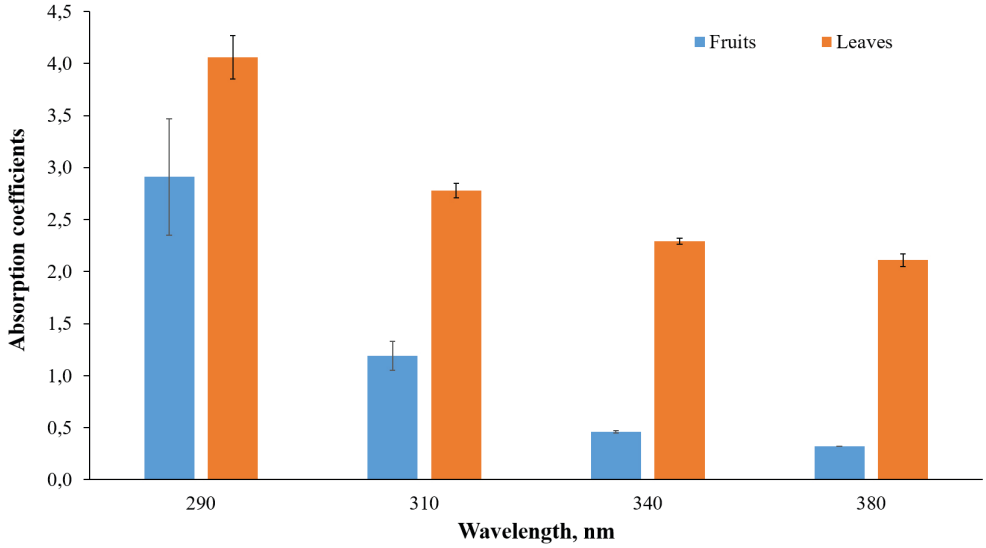

FIGURE 2 - UVA-UVB absorption coefficient (absorbance/ mg DS x mL) at 290, 310, 340 and 380 nm.

CONCLUSIONS

The photoprotective, phytochemical and antioxidant assays demonstrated that *M. parvifolia* extracts are a prospective source of natural photoprotection and antioxidant substances, then *M. parvifolia* could be evaluated in combination with commercial sunscreens to potentially produce a synergistic effect with promising photoprotection capacity. Furthermore, a deep chemical study of this material must be completed to detect additional compounds that could contribute and enhance these biological properties against the adverse effects of ultraviolet radiation.

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