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Antioxidant, cytotoxic and UVB-absorbing activity of *Maytenus guyanensis* Klotzch. (Celastraceae) bark extracts

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ABSTRACT

Maytenus guyanensis Klotzch. is an Amazonian medicinal tree species known in Brazil by the common name *chichuá* and in Peru and Colombia by the name *chuchuhuasi*. It is used in traditional medicine as stimulant, tonic, and muscle relaxant, for the relief of arthritis, rheumatism, hemorrhoids, swollen kidney, skin eruptions, and skin cancer prevention, among others. Initially, different extraction solvents and methods were applied to dried, ground bark which made possible the preparation of extracts having both significant lethality to brine shrimp larvae (*Artemia franciscana* Leach) as well as antioxidant activity *in vitro* based on tests involving reactions with 2,2'-diphenyl-1-picrylhydrazyl (DPPH). Analysis of fractions from serial extractions with solvents of increasing polarity supports the notion that antioxidant activity is associated with compounds of intermediate polarity and cytotoxicity is associated with compounds of low to intermediate polarity. Variation of extraction time and conditions revealed that hot, continuous ethanol extraction provided good yields of bark extract in several hours. Hot extraction also provided ethanol extracts having greater lethality to brine shrimp and antioxidant activity (compared to the flavonoid rutin in semi-quantitative methods based on DPPH) than extracts obtained from maceration at room temperature. Freeze-dried ethanol extracts were prepared by: 1) maceration at room temperature and 2) hot extraction (eight hours) on several hundred gram scales and the latter extract was shown to have partial screening effects on UVB light. In this work, cytotoxic, antioxidant and potential sun-screening activity are shown for the first time in *M. guyanensis*.

KEYWORDS

brine shrimp assay, *Artemia franciscana* Leach, 2,2'-diphenyl-1-picrylhydrazyl, DPPH, beta-carotene.

Atividade antioxidante, citotóxica e absorção no UVB de extratos da casca de Maytenus guyanensis *Klotzch. (Celastraceae)*

RESUMO

Maytenus guyanensis *Klotzch. é uma árvore medicinal proveniente da Amazônia conhecida como* chichuá *(*xixuá*) e no Peru e Colombia por* chuchuhuasi. *É utilizada medicinalmente como estimulante, tônico e relaxante muscular, para o alívio de artrite, reumatismo, hemorróidas, rim inchado, erupções de pele, prevenção do câncer de pele, entre outros. Vários solventes e métodos de extração foram aplicados a cascas secas pulverizadas, possibilitando a preparação de extratos que apresentam letalidade às larvas de* Artemia franciscana *Leach, bem como atividade antioxidante em testes* in vitro *baseados em 2,2'-difenil-1-picrilhidrazil (DPPH). Análise das frações provenientes de extrações em série por solventes de polaridade crescente levou à conclusão que atividades antioxidante e citotóxica estão associadas a compostos de polaridade baixa e média. A variação do tempo e outras condições de extração revelou que extração continua a quente forneceu bons rendimentos de extrato de casca em poucas horas. Extração a quente também forneceu extratos etanólicos apresentando maior citotoxicidade para* A. franciscana *e atividade antioxidante (comparado ao flavonóide rutina em métodos semi-quantitativos baseados em DPPH) quando comparado com extrato etanólico obtido da maceração a temperatura ambiente. Extratos etanólicos liofilizados foram preparados através de maceração e extração a quente (oito horas) em escalas de centenas de gramas sendo que o extrato obtido a quente apresentou efeito protetor solar parcial na região da luz UVB. É o primeiro trabalho que demonstra a citotoxicidade, efeito antioxidante e potencial atividade de proteção solar de* M. guyanensis.

PALAVRAS-CHAVE

Artemia franciscana *Leach, 2,2'-difenil-1-picrilhidrazila, DPPH, beta-caroteno.*

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INTRODUCTION

The genus *Maytenus* Molina has been the subject of a number of recent papers due mainly to the popular use of *M. ilicifolia* (Schrad.) Planch. and *M. aquifolium* Mart. (*espinheira-santa*) in the treatment of ulcers (Vilegas, *et al.*, 1999; Souza-Formigoni *et al.*, 1991; Gonzalez *et al.*, 2001; Queirroga *et al.*, 2000). In the Amazon region, two species, *M. ebenifolia* Reissek and *M. guyanensis* Klotzch., are known traditionally by the names *chichuá* in Brazil and *chuchuhuasi* in Peru. These plants are used as muscle relaxants and analgesics, for relief from arthritis, rheumatism, hemorrhoids, kidney swelling, skin eruptions, as well as skin cancer prevention, and the treatment of colds, post labor, dysentery, bronchitis and against worms (Revilla, 2000; Revilla, 2002) and finally as aphrodisiacs (Silva *et al.*, 1977); stimulants and tonics (Duke *et al.*, 1994). In phytochemical studies on *Maytenus* spp., triterpenes, flavonoids, sesquiterpene β-agarofurans and sesquiterpene evoninoate alkaloids have been isolated and studies on biological activity have revealed antitumor (Gonzalez *et al.*, 1996; Chavez *et al.*, 1998), antimicrobial (Orrabi *et al.*, 2001) and inseticidal activities for the latter compounds (Nunez *et al.*, 2004). More generally, sesquiterpene alkaloids have been isolated from *Maytenus* spp. and been shown to have cytotoxic activity (Kuo *et al.*, 1990 & 1994), and insecticide or antifeedant properties (Nunez *et al.*, 2004). In the only published phytochemical report on *M. guyanensis* to date, Sousa *et al.* (1986) isolated 4'-O-methyl-(-)epigallocatechin, proanthocyanidin A, dulcitol, sitosterol, β-sitosterone and fiedolan-3,7-dione from the trunkwood and root, as well as N,N-dimethylserine from the trunkwood. The sesquiterpene alkaloid "maytene" was also isolated from the root.

Recently, the brine shrimp assay (BSA) has been applied to the screening of a large number of Amazonian plant extracts in our laboratory (Quignard *et al.*, 2003; Quignard *et al.*, 2004). The method used is based on the original procedure developed by Meyer *et al.* (1982). BSA has grown in importance due to publications which have associated lethality to brine shrimp with *in vitro* antitumor activity (Anderson *et al.*, 1991) as well as oral lethal dose in rodents (Parra *et al.*, 2001). To our knowledge, no studies have been published utilizing BSA as a tool to identifying cytotoxicity in *Maytenus* spp..

Several methods are commonly used for the screening of plant extracts and natural products for antioxidant properties. The reduction of the stable DPPH radical is widely used to probe free radical scavenging potential and inhibition of β-carotene bleaching is used to evaluate general antioxidant effects in extracts and isolated substances. Corsino *et al.* (2003) tested the ethyl acetate extracts of leaves and root bark of *M. aquifolium* and found inhibition to β-carotene bleaching through thin-layer chromatography (TLC) autographic assay, and traced this activity to flavan-3-ols and flavanol glycosides present in these extracts.

Meanwhile, sun-screening effects are evaluated by measuring diffuse transmission rates of harmful radiation in two separate ranges of ultraviolet (UV) light. The UVA range involves lower-energy light having wavelengths in the range 315–400 nm and UVB, in the range 280–315 nm (Springsteen *et al.*, 1999). The use of the richly dark violet colored *chichuá* (*Maytenus* spp.) extracts as a purported skin cancer prevention agent might be an indication that these extracts strongly absorb cancer-inducing UV radiation.

The objective of the present work was initially to evaluate a number of *M. guyanensis* extracts for: 1) cytotoxicity in BSA, 2) radical scavenging activity via reduction of DPPH, 3) antioxidant activity by inhibition of β-carotene bleaching and 4) UV-screening / protection and then guide the development of extraction procedures which furnished highly bioactive extracts in suitable yields for technological study and future commercial use.

METHODS

COLLECTION, IDENTIFICATION AND PROCESSING OF PLANT MATERIALS

Plant materials were collected in Amazonas State, in the region near Benjamin Constant, by Dr. Juan Revilla and employees of the Botany Department at INPA, who later identified the species as *Maytenus guyanensis* Klotzch., based on voucher specimens. The bark was dried under laboratory conditions (room temperature, air-conditioning) and then pulverized.

SOLVENTS

Certified ethanol (Credie, Manaus) and technical grade dimethylsulfoxide (DMSO) were used without prior treatment. Other solvents were (fractionally) distilled before use.

SMALL-SCALE SEQUENTIAL SOLVENT EXTRACTION

Dried, ground bark (201.93 g) was extracted (2 x 10 h, with change of solvent between extractions) sequentially with: 1) hexane, 2) chloroform, 3) ethyl acetate and 4) ethanol, yielding, after rotary evaporation under vacuum using a heat bath and freeze-drying, fractions HXF1, CLF2, EAF3 and ETF4, respectively, and yields were calculated based on extracted plant material (Table 1).

SMALL-SCALE PREPARATION OF OTHER EXTRACTS

Dried, ground bark (40.89 g) was infused in water (800 ml; 15 min), followed by hot filtration, rotary evaporation, and freeze-drying to yield crude water extract (WTE). Another portion of dried, ground bark (162.72 g) was continuously extracted in a soxhlet apparatus with methanol (2 x 6 h, changing solvent between extractions) followed by the same evaporation procedure as above, to yield methanol extract (MTE). A third portion of ground bark (38,42 g) was macerated in ethanol (500 ml; 3 days) and after filtration and total evaporation as

previously described, yielded crude ethanol extract (ETM1).

STUDY ON CONTINUOUS ETHANOLIC EXTRACTION TIMES

Separate portions of powdered bark (132.56, 101.75, 103.98 and 104.96 g) were extracted with ethanol in soxhlet apparatus for 4, 6, 8 and 12 h, respectively, yielding, after total evaporation, crude extracts ETS4, ETS6, ETS8 and ETS12, respectively.

PILOT-SCALE EXTRACT PREPARATION: MACERATION IN ETHANOL

Powdered bark (3.30 kg) and ethanol (6 l) were placed in a stoppered, clear glass jar. After 3 days, the jar was drained and the volatile materials totally removed by the methods described previously, to yield powdered, crude extract (ETM).

PILOT-SCALE EXTRACT PREPARATION: CONTINUOUS EXTRACTION IN ETHANOL

Powdered bark (2.00 kg) was placed in six soxhlet apparatus. Each extractor was filled twice with ethanol (0.5 l) which was allowed to siphon to the flask (ETS).

SCREENING FOR ANTIOXIDANT ACTIVITY

Radical scavenging activity: DPPH method.

Qualitative analysis. 2 μL of methanol solutions of each extract at different, known dilutions were spotted onto a normal-phase, commercial thin-layer chromatography (TLC) plate along with 2 μL of rutin (antioxidant positive control) solution in methanol (25 $g \cdot l^{-1}$) as positive control. The uneluted plate was immersed in 0.2 % DPPH methanol solution and sample spots were evaluated for radical scavenging activity (Choi *et al*., 2000).

Semi-quantitative analysis. A commercial TLC plate was spotted with different amounts of sample (50, 25 and 12.5 μg) as well as rutin standard (50 μg) by applying 2 μL of methanol solutions of each sample. The uneluted plates were immersed in 0.2 % DPPH methanol solution (Brand-Williams *et al*., 1995). Preliminary analyses showed that 40 μg of ETM and 12.5 μg of ETS were as active to DPPH as 50 μg of rutin. Refined comparison of ETM (50, 40, 30, 20 μg), ETS (20, 10, 5 e 2.5 μg) showed that 35 ± 5 μg of ETM, and 13 ± 5 μg of ETS had DPPH reducing activity similar to 50 μg of rutin. For the other extracts and fractions the tests began with a mass of 50 μg. The activity of CLF2, MTE, ETS4, ETS6, ETS8 e ETS12 was greater than 50 μg of rutin. Next, 25, 12.5, 6.3 and 3.1 μg of each sample was spotted on TLC plates and tested with DPPH solution against 50 μg of rutin.

QUALITATIVE COMPARISON OF TLC / ANTIOXIDANT PROPERTIES OF ETS4 - ETS12

mg samples of ethanol extracts ETS4, ETS6, ETS8 and ETS12 were charged on TLC plates and eluents were systematically varied to establish suitable conditions for comparison. Next, solutions of ETS4, ETS6, ETS8 and ETS12 in methanol (5 $mg \cdot l^{-1}$) were prepared. Aliquots (2 μL) of each extract solution were spotted on TLC plates (individual extract charge: 10 μg) using a pneumatic micropipette and elution was performed using hexane, hexane: chloroform (2:8 and 1:1), DCM: MeOH (8:2 and 7:3) and AcOEt. The resulting chromatograms were illuminated with a UV lamp (254 and 366 nm), iodine vapor, p-anisaldehyde and β-carotene.

SCREENING FOR GENERAL CYTOTOXICITY USING BSA

Screening at a single concentration. Brine shrimp (*Artemia franciscana* Leach) eggs (BrineShrimpDirect, USA) were hatched in silicate, phosphate and nitrate-free artificial brine (17.5 g $seasalt \cdot l^{-1}$ distilled water) under the light of a lamp for 48 h, yielding second instar larvae. Extracts were dissolved in DMSO. The wells of a test-plate each received artificial brine and ten larvae were transferred to each well using a Pasteur pipette. The final volume in each well was 1 mL. The concentration of extract in each well during the test was 500 $\mu g \cdot ml^{-1}$ and the máximum DMSO concentration in the wells was 1 %. Each test was performed in triplicate. After 24 h, live larvae were counted in each well and mortality was evaluated as a percentage of the initial number of live larvae.

EVALUATION OF MEDIAN LETHAL CONCENTRATIONS (CL_{50}) WITH BSA

Extracts presenting lethalities over 50 % in the above assay were further evaluated to establish CL_{50} (Table 1). Extract test concentrations of 1000, 500, 250, 125 and 62.5 $\mu g \cdot ml^{-1}$) were used in triplicate in what is otherwise essentially the same method used above. The CL_{50} value was obtained through application of the probit method as described by Meyer et al. (1982).

ABSORPTION AND TRANSMISSION OF UV BY ETHANOL BARK EXTRACT

The method used was that described by Springsteen *et al.* (1999) with several modifications. Briefly, different concentrations of extract, as well as a commercial sunscreen (Sun Protection Factor, SPF 15), in spectrophotmetric grade methanol (Omnisolv, EMD Chemicals, Canada) were analyzed at a resolution of 1 nm in absorption and transmittance modes using a UV-visible spectrophotometer (Femto, model 8000xi) in the ranges 280 a 315 nm (UVB) e 315 a 400 nm (UVA), after baseline establishment with a methanol blank in quartz cuvettes. Bark ethanol extract ETS (c = 0.25 $g \cdot l^{-1}$) presented λ_{max} = 275 nm and absorbance (A) of 1.8 absorbance units (A.U.). From the relation $A = \varepsilon \cdot c \cdot d$, where d is the path length, it was possible to calculate an approximate extinction coefficient of $\varepsilon_{275nm} = 7.2$ $A.U. \times cm^2 \times mg^{-1}$.

RESULTS

In general, the extracts prepared from *M. guyanensis* bark are of a dark, violet color and after freeze-drying were powdery and non-hygroscopic. The percentage yields for each extraction, as

Table 1 - Extraction methods, yield data, BSA lethality and free-radical scavenging activity for *M. guyanensis* extracts.

Extract	Extraction method[a]		Yield		*A. franciscana*	Antioxidant Effect
	Solvent	Time, h	g	%[d]	CL_{50}, μg · mL^{-1}	DPPH, μg[e]
HXF1	Hexanes	2 x 10	2.1	1.0	363 ± 123	>50
CLF2	$CHCl_3$	2 x 10	4.4	2.2	17 ± 23	9
EAF3	AcOEt	2 x 10	14.9	7.4	> 1500	>50
ETF4	EtOH	2 x 10	34.7	17.1	> 1500	>50
MTE	MeOH	3 x 6	63.5	39.0	> 1500	18
WTE	H_2O[b]	0.25	4.5	11.0	> 1500	>50
ETM1	EtOH[c]	72	8.8	22.9	1150 ± 150	40
ETM	EtOH[c]	72	756.1	20.6	1230 ± 195	35
ETS4	EtOH	4	132.6	22.0	281 ± 48	6
ETS6	EtOH	6	101.8	20.1	301 ± 55	6
ETS8	EtOH	8	104.0	26.5	588 ± 132	13
ETS12	EtOH	12	105.0	25.0	660 ± 553	13
ETS	EtOH	8	494.7	24.6	512 ± 135	13

[a]soxhlet extraction, unless indicated otherwise. [b]infusion. [c]maceration. [d]based on mass of powdered plant material extracted, [e]mass of extract (± 5 μg) with effect comparable to 50 μg of rutin by comparison on TLC plate.

well as lethality towards *A. franciscana* larvae, are presented in Table 1.

M. guyanensis is used traditionally in the prevention of skin cancer (Revilla, 2000; Revilla, 2002). One of the possible mechanisms of this preventive action could conceivably be filtering or absorption of detrimental solar radiation in the ultraviolet range, the latter often associated with different forms of skin cancer. The absorption spectra in the UVA e UVB regions for ETS (Spectrum 1 and 3) are similar to those of a relatively strong commercial sun-screen (Spectrum 2 and 4). Extract ETS presented very low transmittance at the high energy end of the UV spectrum (< 285 nm) as well as in the UVB range and also exhibited diminished transmittance (< 35 %) at the concentration used for comparison with the commercial sun-screen, which in the UVB range showed a near 0 % UVB transmittance (Spectrum 6). Transmittance in the UVA region for the extract and commercial sun-screen are also shown (Spectrum 7 and 8). These data show a diminished transmittance of UVB light for ETS which means that this extract has some sun-screening capacity. The method utilized does not allow calculation of SPF for the extract.

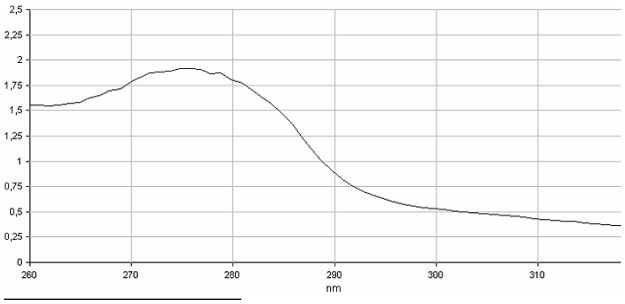
Another mechanism for cancer prevention might be radical scavenging of free radical oxygen and other species associated with cancer cell development. Due to the intense, dark colors of the bark extracts, tests for antioxidant activity using β-carotene were not conclusive. However, the results from the semi-quantitative comparative tests for radical scavenging activity with DPPH showed that of the 13 samples tested, nine are more active on a mass basis than a pure standard of the flavonoid rutin.

Sequential extraction revealed that the substances responsible for the mortality in the *A. franciscana* were of low to medium polarity, as evidenced by the lethality of HXF1 and especially of CLF2, towards brine shrimp larvae (Table 1). CLF2 also was quite active towards DPPH suggesting that the antioxidant substances in *M. guyanensis* bark are of intermediate polarity.

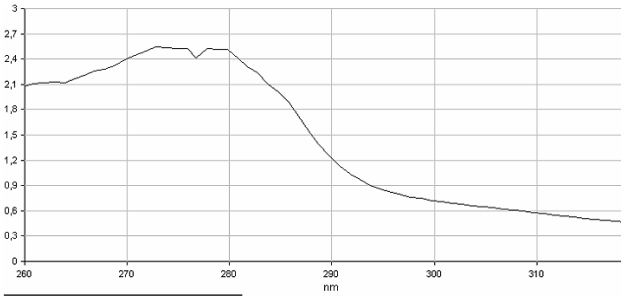
Freeze-dried methanol and ethanol extract yields were high in general in light of our own experiences with the preparation of large numbers of extracts under similar conditions based on other plant species (Quignard *et al.*, 2003; Quignard *et al.*, 2004; Pohlit *et al.*, 2004). Also, hot, continuous ethanol extractions (ETS) furnished proportionally better yields than maceration (ETM). Cytotoxic and radical scavenging activity of ethanol extracts diminished as a function of extraction time. An extraction time of 8 h provided good percentage yield of extract (ETS) on a nearly half kg scale using standard Soxhlet apparatus and without significant sacrifices in cytotoxicity (as evaluated in BSA) or radical scavenging activity. ETS also presented greater antioxidant effect towards DPPH and greater cytotoxicity in brine shrimp larvae than ETM.

Thus, we have demonstrated the preparation of dry extract (ETS) on a pilot scale which is rapid, efficient and safe and shown the extract to have UVB filtering, cytotoxic, and antioxidant (radical scavenging) activity. The antioxidant and cytotoxic activity are directly traceable to a chloroform extractable fraction (CLF2) of the bark. and future work should reveal the substance(s) responsible for the biological activity detected to date.

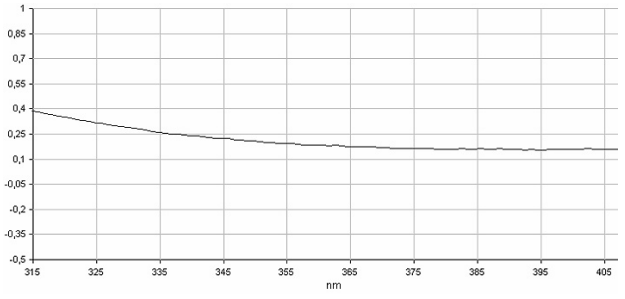
This work is part of the project entitled "Development of two phytotherapeutic and one cosmetic product from Amazonian



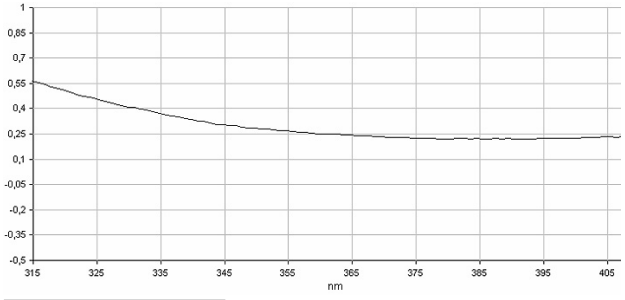
Spectrum 1 - UVB absorption of ETS (0.25 g · L^{-1} MeOH).



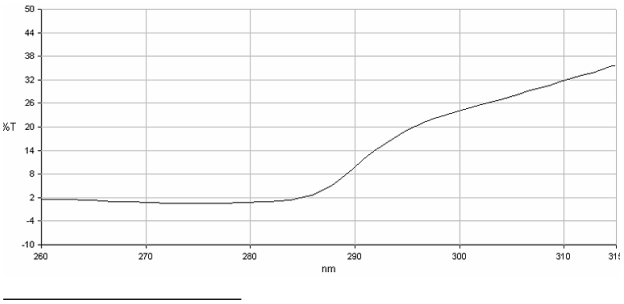
Spectrum 2 - UVB absorption SPF 15 sun-screen (0.25 g · L^{-1} MeOH).



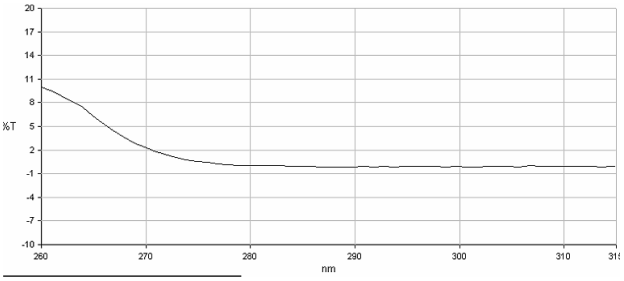
Spectrum 3 - UVA absorption of ETS (0.25 g · L^{-1} MeOH).



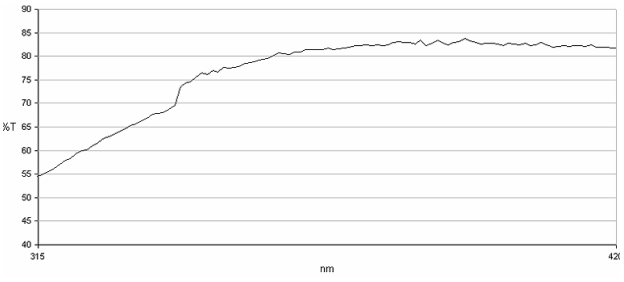
Spectrum 4 - UVA absorption SPF 15 sun-screen (0.25 g · L^{-1} MeOH).



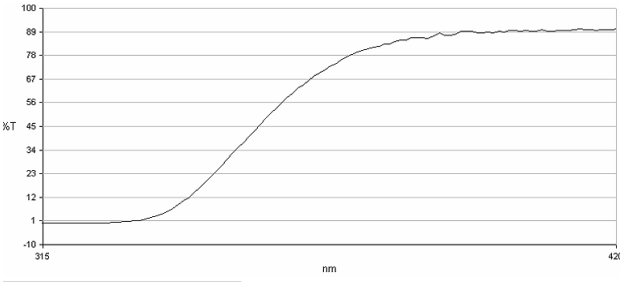
Spectrum 5 - UVB transmittance of ETS (0.25 g · L^{-1} MeOH)



Spectrum 6 - UVB transmittance SPF 15 sun-screen (0.25 g · L^{-1} MeOH).



Spectrum 7 - UVA transmittance of ETS (0.25 g · L^{-1} MeOH)



Spectrum 8 - UVA transmittance of SPF 15 sun-screen (0.25 g · L^{-1} MeOH)

plants", whose objective it is to identify and develop plant extracts of potential commercial importance. Future work should yield the bioactive substances present in the extracts in pure form (biomarkers) as well as standardized methods for *M. guyanensis* bark extracts.

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