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In vitro antioxidant and photoprotective activities of dried extracts from *Neoglaziovia variegata* (Bromeliaceae)

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ABSTRACT

The phytochemical screening, antioxidant and photoprotective activities of dried extracts from the leaves of *Neoglaziovia variegata* were investigated. It was also evaluated the total phenolic and flavonoid contents by the Folin-Ciocalteu and aluminum chloride methods, respectively. Antioxidant activities of the extracts were evaluated by using of 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging and β-carotene-linoleic acid bleaching and compared with ascorbic acid, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) used as reference compounds. The photoprotective effect was evaluated by the spectrophotometric method. Preliminary analysis demonstrated that the extracts were found to be positive for the presence of anthracene derivatives, flavonoids, tannins, mono and diterpenes, naphthoquinones, coumarins, triterpenoids and steroids. The most significant total phenolic and flavonoid contents was of 608.50 ± 14.74 mg of gallic acid equivalent/g and 272.50 ± 9.25 mg of catechin equivalent/g, respectively, for ethyl acetate fraction (Nv-AcOEt). The Nv-AcOEt also presented the best antioxidant activity (IC_{50} 3.51 ± 0.18 μg/ml) for DPPH scavenging. The chloroform fraction (Nv-$CHCl_3$) and Nv-AcOEt showed characteristic absorption bands in regions UVB and UVA in a concentration-dependent manner. Nv-AcOEt presented the highest sun protection factor SPF (27. 68 ± 4.03). It shows the possibility to use this extract as sunscreen in pharmaceutical preparations.

INTRODUCTION

Oxidative stress and inflammatory responses induced by ultraviolet radiation (UV) can cause a variety of harmful effects in skin, including premature photoaging and the induction of immunosupression and skin carcinogenesis (Vilela *et al.,* 2011). The harmful effects of solar radiation are caused predominantly by the UV region of the electromagnetic spectrum, which can be divided in three regions: ultraviolet A (UVA – from 320 to 400 nm); ultraviolet B (UVB – from 290 to 320 nm) and ultraviolet C (UVC – from 200 to 290 nm). UVC radiation is filtered by the atmosphere before reaching earth. UVB radiation is not completely filtered out by the ozone layer and is responsible for the damage due to sunburn. UVA radiation reaches the deeper layers of the epidermis and dermis and provokes the premature aging of the skin. Ultraviolet radiations have been implicated as a causative factor of skin cancer (Dutra *et al.,* 2004). The necessity to provide high sun protection factor (SPF) and screening efficiency against both ultraviolet A (UVA) and ultraviolet B (UVB) wavelengths has led to the development of sunscreen formulations with multiple added sunscreen chemicals (Vilela *et al.,* 2011). Plants produce a variety of antioxidants against molecular damage from reactive oxygen species (ROS), and phenolic compose the major class of plant-derived antioxidants. Among the various phenolic compounds, the flavonoids are perhaps the most important group. Besides scavenging UV-induced radicals, flavonoids might provide their protective effect against UV radiation by acting as strong UV-absorbing screens (Mambro and Fonseca, 2005). The Bromeliaceae family is distributed predominantly in Neotropical region and comprises 58 genera and approximately 3172 species (Luther, 2008). The phytochemistry of this family is characterized by the presence of flavonoids, triterpenoids, steroids, diterpenes,

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cinnamic acid derivates, lignans, nitrogen compounds among others (Manetti *et al.*, 2009). Considering the large number of species of the Bromeliaceae family few have been studied chemically so far. *Neoglaziovia variegata* belongs to the family Bromeliaceae, subfamily Bromelioideae, and is popularly known in Brazil as "caroá". This species can be commonly found in the Brazilian Caatinga vegetation (dry woodland characteristic of semi-arid regions of Northeastern Brazil) (Pereira *et al.*, 2008). Some studies realized by our research group have demonstrated that *N. variegata* have biological properties such as antinociceptive (Lima-Saraiva *et al.*, 2012a), antioxidant (Lima-Saraiva *et al.*, 2012b) and antibacterial (Oliveira-Junior *et al.*, 2012). Nowadays, one of the trends of the cosmetic market is the development of products with the use of natural resources. Several plant extracts and oils have been used in cosmetic products such as sunscreen because of the photoprotective action (Violante *et al.*, 2009). In this context, the aim of this study was to evaluate the antioxidant and photoprotective potential of dried extracts of *N. variegata* aiming the developing a new cosmetic product.

MATERIAL AND METHODS

Plant material

The leaves of *Neoglaziovia variegata* (Arruda) Mez were collected in the city of Petrolina (Coordinates: S 08°59'16"; W 40°35'20"), State of Pernambuco, Brazil, in January of 2011. The samples were identified by a botanist from Centro de Recuperação de Áreas Degradadas da Caatinga (CRAD). A voucher specimen (6441) was deposited at the Herbarium of San Francisco Valley (HVASF) of the Federal University of San Francisco Valley (UNIVASF).

Extraction

The dried and powdered leaves (1174 g) were macerated with ethanol 95% at room temperature for 72 hours. The extractive solution was concentrated under vacuum in a rotatory evaporator oven at 45 ºC, producing 64 g of crude ethanol extract (Nv-EtOH). The Nv-EtOH was suspended in a mixture of H_2O:MeOH (7:3) and extracted successively with hexane (Hex), chloroform ($CHCl_3$) and ethyl acetate (AcOEt) in crescent order of polarity to obtain the respective fractions (Nv-Hex, Nv-$CHCl_3$ and Nv-AcOEt). Subsequently, all fractions were dried in an oven at 40 °C for 1 hour for complete elimination of solvent. The dried extracts were used to perform all tests in this study.

Qualitative analysis of phytochemicals

The extracts were evaluated on thin layer plates of silica gel 60 F_{254} aluminum supports, applied with a micropipette and eluted in different solvent systems as described by Wagner and Bladt (1996) and Sobrinho et al. (2012), seeking to highlight the main groups of secondary metabolism (Table 1).

Total phenolic content

Total phenolic contents were assayed using the Folin-Ciocalteu reagent, it is based on the method reported by Slinkard and Singleton (1997), and only the volumes have been reduced (Almeida *et al.*, 2011). An aliquot (40 µl) of a suitable diluted Nv-EtOH, Nv-Hex, Nv-$CHCl_3$ and Nv-AcOEt extracts was added to 3.16 ml of distilled water and 200 µl of the Folin–Ciocalteu reagent, and mix well. The mixture was shaken and allowed to stand for 6 min, before adding 600 µl of sodium carbonate solution, and shake to mix. The solutions were left at 20 °C for 2 hours and the absorbance of each solution was determined at 765 nm against the blank and plot absorbance vs. concentration. Total phenolic contents of the extracts (three replicates per treatment) were expressed as mg gallic acid equivalents per gram (mg GAE/g) through the calibration curve with gallic acid. The calibration curve range was 50 – 1000 mg/l (R^2 = 0.9928). All samples were performed in triplicates.

Determination of total flavonoid content

Total flavonoid content was determined by using a colorimetric method described previously (Dewanto *et al.*, 2002). Briefly, 0.30 ml of the Nv-EtOH, Nv-Hex, Nv-$CHCl_3$ and Nv-AcOEt extracts, or (+)-catechin standard solution were mixed with 1.50 ml of distilled water in a test tube followed by addition of 90 µl of a 5% $NaNO_2$ solution. After 6 min, 180 µl of a 10% $AlCl_3.6H_2O$ solution was added and allowed to stand for another 5 min before 0.6 ml of 1 M NaOH was added. The mixture was brought to 330 µl with distilled water and mixed well. The absorbance was measured immediately against the blank at 510 nm using a spectrophotometer (QUIMIS, Brazil) in comparison with the standards prepared similarly with known (+)-catechin concentrations.

The results were expressed as mg of catechin equivalents per gram of extracts (mg CE/g) through the calibration curve with catechin (R^2 = 0.9948). The calibration curve range was 50 – 1000 mg/l.

Table. 1: Elution systems and revelators used to characterize the main secondary metabolites from the extracts of leaves of *Neoglaziovia variegata* by thin layer chromatography.

Phytochemicals	Elution systems	Revelators
Alkaloids	Toluene: ethyl acetate: diethylamine (70:20:10, v/v)	Dragendorff reagent
Anthracene derivatives	Ethyl acetate: methanol: water (100:13.5:10, v/v)	10% ethanolic KOH reagent
Coumarins	Toluene: ethyl ether: (1:1 saturated with acetic acid 10%, v/v)	10% ethanolic KOH reagent
Flavonoids and tannins	Ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26, v/v)	NEU reagent
Lignans	Chloroform: methanol: water (70:30:4, v/v)	Vanillin sulfuric reagent
Mono and diterpenes	Toluene: ethyl acetate (93:7, v/v)	Vanillin sulfuric reagent
Naphthoquinones	Toluene: formic acid (99:1, v/v)	10% ethanolic KOH reagent
Triterpenes and steroids	Toluene: chloroform: ethanol (40:40:10, v/v)	Liebermann-Burchard reagent

DPPH free radical scavenging assay

The free radical scavenging activity was measured using the 2,2-diphenyl-1-picrylhydrazil (DPPH) assay (Falcão *et al.*, 2006). Sample stock solutions (1.0 mg/ml) of extracts were diluted to final concentrations of 243, 81, 27, 9, 3 and 1 µg/ml, in ethanol. One ml of a 50 µg/ml DPPH ethanol solution was added to 2.5 ml of sample solutions of different concentrations, and allowed to react at room temperature.

After 30 minutes the absorbance values were measured at 518 nm and converted into the percentage antioxidant activity (AA) using the following formula: AA% = [(absorbance of the control – absorbance of the sample)/ absorbance of the control] x 100. Ethanol (1.0 ml) plus plant extracts solutions (2.5 ml) were used as a blank. DPPH solution (1.0 ml) plus ethanol (2.5 ml) was used as a negative control. The positive controls (ascorbic acid, BHA and BHT) were those using the standard solutions. Assays were carried out in triplicate.

β-Carotene bleaching test

The β-carotene bleaching method is based on the loss of the yellow colour of β-carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion (Wannes *et al.*, 2010). The rate of β-carotene bleaching can be slowed down in the presence of antioxidants. β-carotene (2 mg) was dissolved in 10 ml chloroform and to 2 ml of this solution, linoleic acid (40 mg) and Tween 40 (400 mg) were added. Chloroform was evaporated under vacuum at 40 ºC and 100 ml of distilled water was added, then the emulsion was vigorously shaken during two minutes. Reference compounds (ascorbic acid, BHA and BHT) and sample extracts were prepared in ethanol. The emulsion (3.0 ml) was added to a tube containing 0.12 ml of solutions 1 mg/ml of reference compounds and sample extracts.

The absorbance was immediately measured at 470 nm and the test emulsion was incubated in a water bath at 50 ºC for 120 min, when the absorbance was measured again. Ascorbic acid, BHA and BHT were used as positive control. In the negative control, the extracts were substituted with an equal volume of ethanol. The antioxidant activity (%) was evaluated in terms of the bleaching of the β-carotene using the following formula: % Antioxidant activity = $[1 - (A_0 – A_t) / (A_0^0 – A_t^0)] \times 100$; where A_0 is the initial absorbance and A_t is the final absorbance measured for the test sample, A_0^0 is the initial absorbance and A_t^0 is the final absorbance measured for the negative control (blank). The results are expressed as percentage of antioxidant activity (% AA). Tests were carried out in triplicate.

Determination of the maximum absorption wavelength and Sun Protection Factor (SPF) *in vitro*

For determining of the maximum absorption wavelength (λ_{max}), the dried extracts were diluted in absolute ethanol, obtaining concentrations of 5, 25, 50 and 100 mg/l. Subsequently, was performed spectrophotometric scanning at wavelengths between 260-400 nm, with intervals of 5 nm. The readings were performed using 1 cm quartz cell, and ethanol used as blank (Violante *et al.*, 2009). Calculation of SPF was obtained according to the equation developed by Mansur et al. (1986):

$$SPF_{spectrophotometric} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

Where: EE (λ) – erythemal effect spectrum; I (λ) – solar intensity spectrum; Abs (λ) – absorbance of sunscreen product; CF – correction factor (= 10). The values of EE x I are constants. They were determined by Sayre et al. (1979), and are showed in Table 2.

Table. 2: Normalized product function used in the calculation of SPF.

Wavelength (nm)	EE x I (normalized)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180
Total	1.0000

EE – erythemal effect spectrum; I – Solar intensity spectrum.

Statistical analysis

The data obtained were analyzed using the GraphPad Prism® version 5.0 and expressed as mean ± S.D. The IC_{50} values were calculated by linear regression. Statistically significant differences were calculated by the application of Student's *t*-test. Values were considered significantly different at $p < 0.05$.

RESULTS AND DISCUSSION

The preliminary phytochemical analysis demonstrated that all the extracts were positive for the presence of flavonoids and tannins. The Nv-EtOH, Nv-Hex and Nv-AcOEt also showed positive reaction for the presence of anthracene derivatives, mono and diterpenes, triterpenes and steroids. The Nv-$CHCl_3$ and Nv-AcOEt extracts were positive for the presence of coumarins and naphthoquinones. All extracts were negative for the presence of alkaloids and lignans (Table 3). As expected, phenols were found in extracts of leaves. Among the phenolic compounds, flavonoids and tannins occurred more frequently. These results are in agreement with the major classes of secondary metabolites found in the family Bromeliaceae (Manetti *et al.*, 2009).

Table 4 summarizes the results from the quantitative determination of phenolic and flavonoids as well as the effect of extracts from *N. variegata*, ascorbic acid, BHA and BHT on the DPPH free radical scavenging and β-carotene-linoleic acid bleaching test.

The total phenolics content of the plant extracts was determined by the Folin-Ciocalteu method. This method for total phenol is useful in order to know the efficiency of extraction of phenolic in solvents. The most significant total phenolic content was of 608.5 ± 14.74 mg of gallic acid equivalent/g for Nv-AcOEt. The level of flavonoids, expressed in catechin equivalents (CE) in mg/g of plant extract was of 272.50 ± 9.25 for the Nv-AcOEt.

Assays based on the scavenging of DPPH have been widely used to measure the antioxidant activity of different phenolic compounds.

Table. 3: Phytochemical characterization of dried extracts from the leaves of *Neoglaziovia variegata*.

Phytochemicals	Nv-EtOH	Nv-Hex	Nv-$CHCl_3$	Nv-AcOEt
Alkaloids	-	-	-	-
Anthracene derivatives	++	++	+	-
Coumarins	-	-	++	++
Flavonoids and tannins	++	++	++	+++
Lignans	-	-	-	-
Mono and diterpenes	+++	+++	++	-
Naphthoquinones	-	-	+	+
Triterpenes and steroids	++	++	+++	-

(-) not detected; (+) low presence; (++) moderate presence; (+++) strong presence.

Table. 4: Total phenolics (TP), total flavonoids (TF) and antioxidant activity of dried extracts from the leaves of *Neoglaziovia variegata*.

Sample	TP (mg GAE/g)	TF (mg CE/g)	DPPH (IC_{50}, µg/ml)	β-carotene (% AA)
Nv-EtOH	84.33 ± 3.82	53.53 ± 1.04	117.00 ± 3.26	42.35 ± 6.26
Nv-Hex	204.04 ± 2.12	65.70 ± 1.80	194.00 ± 41.02	55.19 ± 2.45
Nv-$CHCl_3$	126.80 ± 6.16	67.87 ± 4.93	55.81 ± 4.62	63.95 ± 1.87
Nv-AcOEt	608.50 ± 14.74	272.50 ± 9.25	3.51 ± 0.18	52.77 ± 6.50
Ascorbic acid	---	---	1.91 ± 0.04	1.48 ± 0.73
BHA	---	---	3.12 ± 0.43	75.03 ± 1.93
BHT	---	---	37.51 ± 2.74	79.04 ± 2.10

The IC_{50} values were obtained by interpolation from linear regression analysis with 95% of confidence level. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum effect estimate in 100%. Values are given as mean ± SD (n=3).

This is the most widely reported method for screening of antioxidant activity of many plant drugs (Onkar *et al.,* 2012). DPPH is one of a few stable available organic nitrogen radicals and has a UV-Vis absorption maximum at 515-518 nm. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, the reduced form of the radical is generated accompanied by loss of color (Huang *et al.,* 2005). The data showed that the Nv-AcOEt extract exhibited excellent free radical scavenging activity, with a value of IC_{50} of 3.51 ± 0.18 µg/ml. Ascorbic acid was the most effective antioxidant, with a value of IC_{50} of 1.91 ± 0.04 µg/ml.

The antioxidant activity of extracts was also evaluated by the β-carotene-linoleic acid bleaching method. This method is based on the loss of the yellow color of β-carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion. β-carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. The rate of the β-carotene bleaching can be slowed down in the presence of antioxidants (Kulisic *et al.,* 2004). In this model, the extracts showed moderate antioxidant activity (42.35 – 63.95%), and the most active extract was the Nv-$CHCl_3$ with percentage of antioxidant activity of 64.95 ± 1.87. BHT was as effective as BHA, and much more effective than ascorbic acid.

According to these results, it was concluded that dried extracts from *Neoglaziovia variegata* have potent antioxidant activity, mainly by scavenging abilities observed against DPPH radical. These results corroborate studies with extracts of flowers of *N. variegata* that also showed significant antioxidant activity (Oliveira-Júnior *et al.,* 2012). Furthermore, *Encholirium spectabile*, other species belongs to the family Bromeliaceae, also exhibited antioxidant activity and total phenols content relevant (Santana *et al.,* 2012). The existing data give new information for the antioxidant potential and polyphenolic content of plant species that have not been traditionally used as medicinal plant.

Figure 1 shows the spectrophotometric absorption profile of the dried extracts of *N. variegata*. Analyzing the data can be observed that Nv-$CHCl_3$ and Nv-AcOEt showed characteristic absorption bands in regions UVB and UVA in a concentration-dependent manner, suggesting a possible photoprotective potential. The maximum absorption wavelength (λ_{max}) for Nv-$CHCl_3$ and Nv-AcOEt was 325 and 345 nm, respectively (Table 5). Nv-EtOH and Nv-Hex didn't demonstrate satisfactory absorption. These results can be justified by the high content of flavonoids present mainly in Nv-AcOEt. According to the literature, the content of flavonoids produced by a plant is considered an important factor for protecting plants against ultraviolet radiation (Souza *et al.,* 2005). The ultraviolet absorption spectrum of the flavonoids shows in general two peaks of maximum absorption (240-280 nm and 300-550 nm) (Bobin *et al.,* 1995).

Table. 5: Maximum absorption wavelength (λ_{max}) and absorption type of dried extracts from the leaves of *Neoglaziovia variegata*.

Extracts	λ_{max} (nm)	Absorption type
Nv-EtOH	260	UVC
Nv-Hex	260	UVC
Nv-$CHCl_3$	325	UVA
Nv-AcOEt	345	UVA

UVC: 200-290 nm; UVB: 290-320 nm; UVA: 320-400 nm.

The SPF *in vitro* was determined by the spectrophotometric method developed by Mansur (1986) using the UVB region, considered to be the region of greatest incidence during the day in which people are exposed for longer (Dutra *et al.,* 2004). In Figure 2, it can be observed that the extracts Nv-$CHCl_3$ and Nv-AcOEt showed higher SPF at concentration 100 mg/l (11.46 ± 0.83 and 27.68 ± 4.03, respectively). Nv-EtOH and Nv-Hex showed low values of SPF (2.80 ± 0.21 and 2.97 ± 0.67, respectively). The results about SPF also showed standard concentration-dependent. Although the test has been carried out *in vitro*, it was demonstrated that this method correlates well with *in vivo* tests, because relates the absorbance of the substance in question with the erythematogenic effect of radiation and intensity of light at specific wavelengths between 290 and 320 nm (UVB region) (Violante *et al.,* 2009).

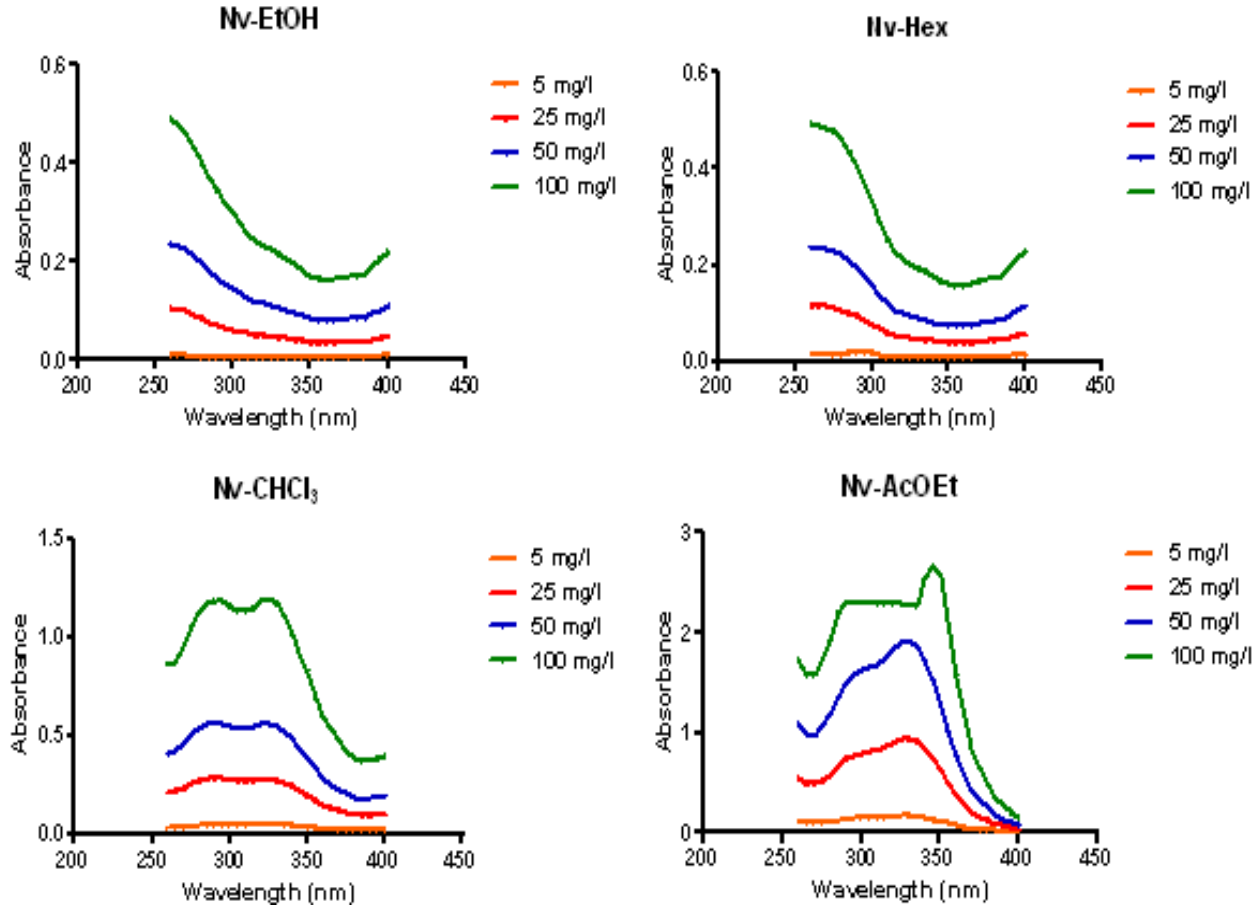

Fig. 1: Spectrophotometric absorption profile of dried extracts of *Neoglaziovia variegata* (260-400 nm).

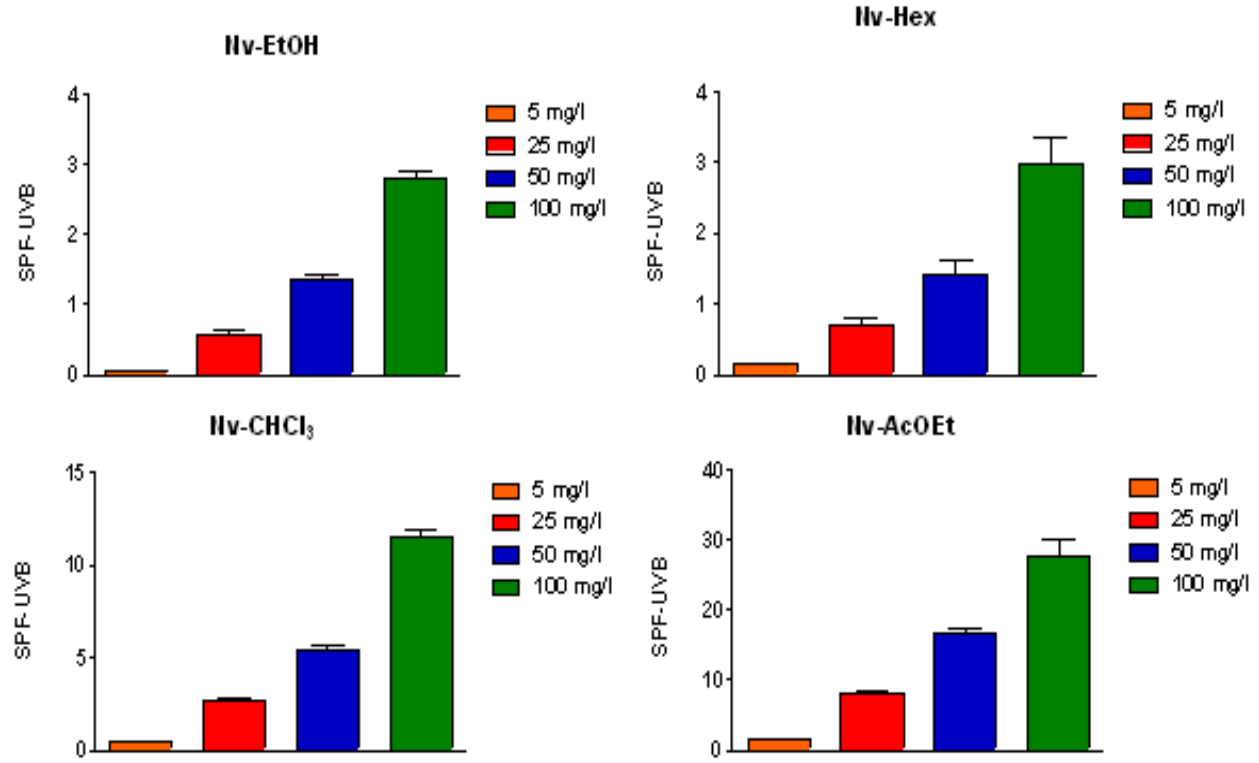

Fig. 2: Sun Protection Factor (SPF) *in vitro* of the dried extracts of *Neoglaziovia variegata*.

CONCLUSION

In summary, the present study demonstrates that *Neoglaziovia variegata* contain phenolic compounds which can serve as natural sources of antioxidants agents. The flavonoids present in the extracts could be responsible by photoprotective effect presented in this study, mainly for the Nv-AcOEt extract. It shows the possibility to use this extract as sunscreen in pharmaceutical preparations. Further research will be conducted to reach the substance responsible for antioxidant and photoprotective activities of extracts.

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