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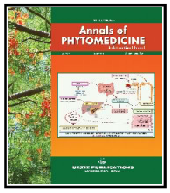

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The anti-neuroinflammatory potential of himalayan high-altitude medicinal plant secondary metabolites using SK-N-SH neuronal cell lines

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Abstract

In order to control the neurological complications associated with HIV, it is pertinent to consider the development of an effective antioxidant therapy. The current study was targeted to evaluate the antioxidant capacities of secondary metabolites isolated from Himalayan high-altitude medicinal plants using a standard oxygen radical absorbance capacity assay based on fluorescein (ORAC-Fl assay). Secondary metabolites (SMs) 1-6 were found to be effective in reducing gp120-41 and Tat-generated oxidative stress in SK-N-SH cell lines, quantitated using a fluorescent probe, 5(6)-carboxy-2,7-dichlorofluorescein diacetate (CDFH-DA). These encouraging *in vitro* preliminary findings suggest that the use of these secondary metabolites may result in the creation of an effective antioxidant therapy that could be used to prevent, delay, or even treat neurological complications linked to oxidative stress caused by HIV proteins.

1. Introduction

The prevalence of HIV and HIV associated dementia (HAD) is rapidly increasing worldwide, despite the advancement of antiretroviral therapy, particularly in the African continent (Patel *et al.,* 2010; Amalia *et al.,* 2023). HIV and its pathogenic proteins Tat1, gp120 and Nef 41 are all recognized to play a significant part in the pathophysiology of HAD (Sheng *et al.,* 2000; Stephenson *et al.,* 2018; Rempel *et al.,* 2005). One of the most important pro-inflammatory mediators activated by HIV and its pathogenic proteins is the production of reactive oxygen species (ROS) by them (Aggarwal *et al.,* 2022; Moond *et al.,* 2023; Devi *et al.,* 2023). Neurons constitutively express lower levels of manganese dependent superoxide dismutase enzyme (MnSOD) than that of astroglial cells (Mengfan *et al.,* 2022) and are therefore more prone to the oxidative damage induced by ROS.

Many researchers reported that both gp120 and HIV trans activating protein (Tat) are known to induce oxidative stress and affect the CNS health of the HIV positive individuals (Price *et al.,* 2005; Visalli *et al.,* 2007; Banerjee *et al.,* 2010; Butler *et al.,* 2011). It has been demonstrated that the HIV-lowering medication known as highly active antiretroviral therapy (HAART) causes oxidative stress and mitochondrial dysfunction in blood brain barrier endothelial cells (Manda *et al.,* 2011).

Since it is well established that pathogenic proteins of HIV and HAART generate oxidative stress, it is hence essential to design an effective antioxidant therapy to counteract the oxidative stress associated with the HIV proteins. In present study, some secondary metabolites isolated from some high altitude Indian medicinal plants belonging to the Rutaceae family were tested using SK-N-SH cell lines. SMs 1-3 were extracted from *Skimmia anquetilia* N.P.Taylor and Airy Shaw (Rutaceae), SM 4 from *Boenninghausenia albiflora* (Hook.) Meisn. (Rutaceae), and SM 5-6 were extracted from *Glycosmis arborea* (Figure 1) (Rutaceae). As far as we are aware, these specific SMs have not tested for their ability to offer neuroprotection against gp120 and Tat induced neuronal damage. These SMs were therefore also tested for their ability to offer neuro-protection against damage induced by these free radicals them (Moond *et al.,* 2023; Devi *et al.,* 2023).

2. Materials and Methods

2.1 Chemicals

2,2‘-Azobis-2-methyl-propanimidamide, dihydrochloride (AAPH; Sigma), 5(6)-carboxy-2,7-dichlorofluorescein diacetate (CDFH-DA; Sigma), Fluorescein (Sigma), Glutamine(Sigma), Glucose (Sigma), Cystine (Cys; Sigma), sodium nitroprusside (SNP; Sigma), Ferrous sulphate ($FeSO_4$; Merck), Hydrogen peroxide (30% H_2O_2; Sigma), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; Sigma), trypsin-EDTA (Highveldt Biologicals, SA), Dulbeco’s

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phosphate buffered saline with (PBS^{+}) or without (PBS^{-}) magnesium (Mg^{2+}) and calcium (Ca^{2+}) (Gibco), DMEM (powder; Highveld Biologicals, SA), Dulbecco's modified Eagle's medium (DMEM, liquid with and without phenol red, Gibco); penicillin/streptomycin (P/S; Highveld Biologicals or Gibco), Fetal calf serum (FCS; Highveld, Gibco), recombinant HIV proteins gp120-41 and Tat (Abcam), Abcam quick cell proliferation cell kit II; Abcam (ab65473), and phosphate buffer 750 mM (pH 7.4).

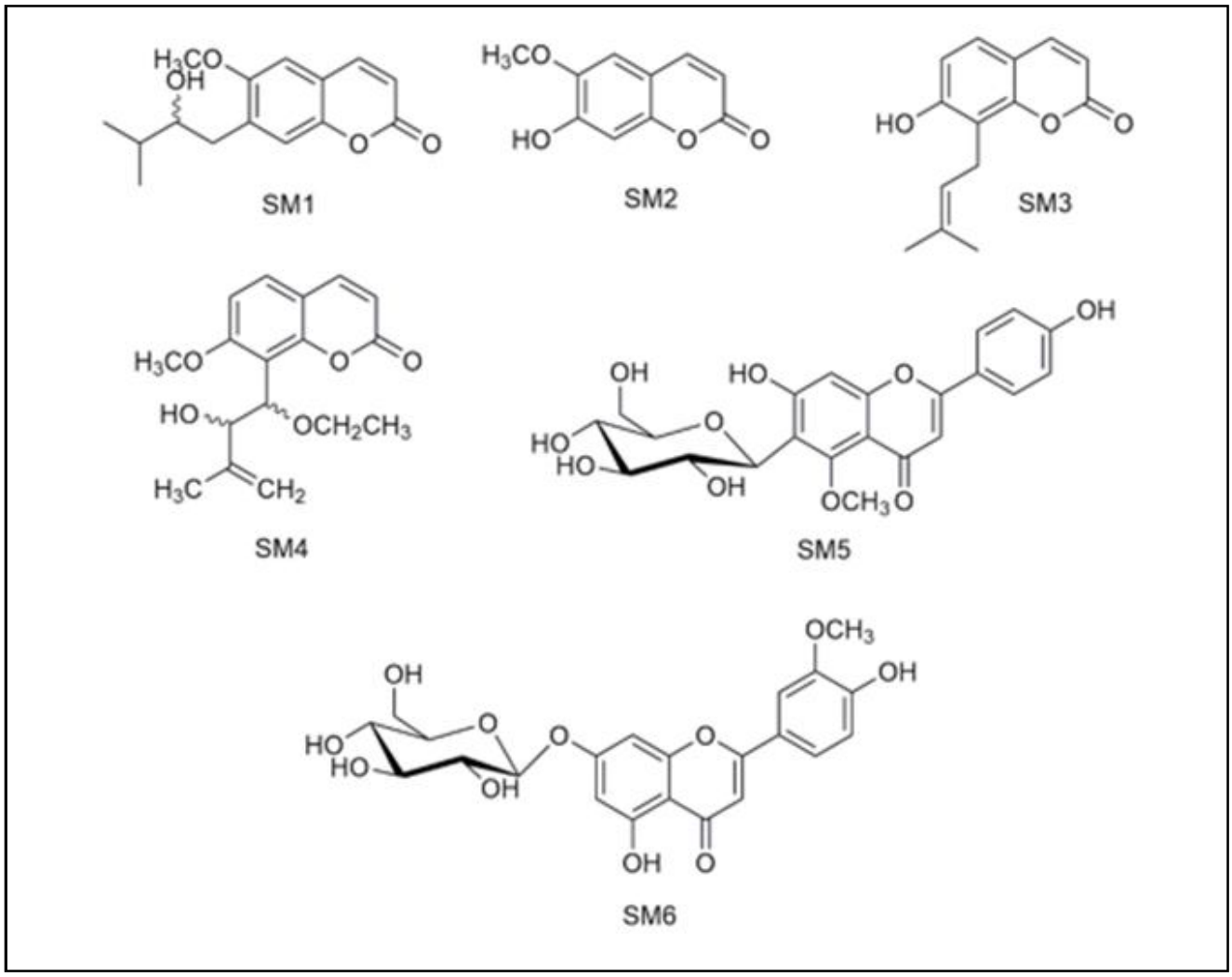

Figure 1: Structures of SMs from *Skimmia anquetelia* (1, 2, 3), *Boenninghausenia albiflora* (4), and *Glycosmis arborea* (5, 6).

2.2 Extraction of SMs

The SMs 1, 2 and 3 were extracted from methanol (CH_3OH) soluble part of 90% ethanolic extract of air dried leaves of *Skimmia anquetelia* using chloroform: methanol ($CHCl_3$:CH_3OH; 9:1) then at C_6H_{12}:EtOAc (85:15) as solvent system (Sharma *et al.,* 2008; Sharma *et al.,* 2008). The SM 4 was isolated from silica gel CC from 90% ethanolic extract of the leave part of *Boenninghausenia albiflora* using $CHCl_3$:CH_3OH (99:1), followed by C_6H_{12}:EtOAc (7:3) as solvent systems (Sharma *et al.,* 2006). SMs 5 and 6 were isolated as white solid powder from silica gel CC of MeOH soluble part of 90% ethanolic extract of leaves of *Glycosmis arborea* using $CHCl_3$:CH_3OH (95:5) as eluent (Sharma *et al.,* 2010; Khan *et al.,* 2013).

2.3 ORAC-Fl Assay

The methodology employed by Ou *et al.* (2001), and modified by Marnevick *et al.* (2011) was followed with further modifications. The SMs solutions 1/64 dilution of the stock solutions of 1.2 mg/ml, and Trolox standard at 8 different dilutions were added to 96 well white fluorimetry plates. 125 µl of the working solution of fluorescein (95.7 nM) was added to the control and SMs solutions. The plate was then gently shaken and immediately put into the fluorimeter to measure the fluorescence values (λ_{ex} = 485 nm and λ_{em}=520 nm). The fluorescence values at this time point were recorded as t_0. Fresh AAPH solution was made with warm phosphate buffer (37 ^{O}C), and 25 µl was added to each microplate well. The plate was once more softly shaken and put back into the fluorescence plate reader after the addition of AAPH. These fluorescence readings matched t_1 time point. Following this, the fluorescence values were recorded for various time points at regular intervals from t_1 to t_{120}: for first 30 min, every 5 min, for the next 30 min, and for the next 60 min, at intervals of 20 min each. For every assay, all reaction mixtures were made in triplicate, and each sample was subjected to at least two

separate assays. The antioxidant capacities were expressed as trolox equivalents (TE; µg/ml).

2.4 Cell Culture

Human neuroblastoma (SK-N-SH) cell lines were regularly grown in DMEM with 10% FCS, glutamine, high glucose (4.5 g/l), and sodium bicarbonate (3.7 g/l) added. Antibiotics penicillin/streptomycin (P/S) (100 U/ml) were added. Regular tests for mycoplasma and microbial contamination of the medium were performed. The cells were cultivated in 10 cm flat-bottom tissue culture petri dishes and incubated at 37ºC in a humidified atmosphere with 5% CO_2 until the required confluency was reached. Standard culture conditions were followed during this time. After treatment with trypsin-EDTA (TE) solution, cells were counted manually using a haemocytometer to determine the seeding concentration.

2.5 Treatments for the cell culture assays

The cells were seeded (2×10^4 / well; n=3) in two different 96 well tissue culture plates for 24 h. After washing with PBS^+, the cells were treated approximately for 1h with the 8 µl of 1: 64 concentration of the of SMs diluted to 100 µl in PBS^+. 8 µl of HIV proteins (gp120-41 and Tat) and the free radical generators [AAPH (200 µM), H_2O_2 (200 µM and 1000 µM), SNP (500 µM), SNP-Cys (200 µM each) and $FeSO_4$ (200 µM)] were then added. In each well, 100 µl of DMEM without FCS and phenol red were added two h after the incubation. The solution was allowed to incubate overnight; one of the plates was used for CDFH-DA assay and the other one for the WST-1 assay.

2.6 CDFH-DA assay

CDFH-DA is taken up by the cells, and metabolized by the mitochondrial enzymes to CDFH, another non-fluorescent analogue which reacts with the intracellular ROS to form a fluorescent compound DCF which is then quantitated. The CDFH-DA assay was carried out as described elsewhere (Fujisawa *et al.*, 2004; Qu *et al.*, 2001; Atsumi *et al.*, 2005) with a few modifications. Briefly, one of the 96 well plates with appropriate treatment and control groups were first washed with PBS^+ and incubated with 200 µl of 12 µM CDFH-DA solution for 1 to 4 h in an incubator under standard conditions.

Next, 100 µl of the solution was transferred to a 96-well white fluorimetry plate after the plates were thoroughly but gently shaken. Using a Varian Eclipse fluorescence spectrophotometer provided by SMM Instruments, Sea Point Technology, South Africa, the fluorescence values (λ_{ex} = 485 nm and λ_{em} = 530 nm) were measured.

2.7 WST-1 assay (Abcam)

The method described by Abcam (www.abcam.com) was followed with a few modifications. Briefly, SK-N-SH cells were grown in DMEM containing FCS and P/S, until the required confluency was obtained. After that, the cells were seeded into 96-well cell culture plates (2 x 104 cells/well) and left for a full day (24 h) of incubation. After this, the media was removed and the wells were washed (× 2) with 100 µl PBS^+. 1000 µl of WST-1 reagent was dissolved in 10 ml of media as per the manufacturer's instruction (AbCAM) and 110 µl of this freshly prepared solution was added to each well. Appropriate controls were included as per the manufacturer's instructions. The plate was then incubated for 1-4 h under standard aseptic condition. The absorbance values for each well were read at 430 nm (optimized in our lab) in a 96 well microplate reader. The reference wavelength was set at 650 nm.

2.8 Statistical analysis

Mean +/- SEM is used to express data. Graphpad PRISM (version 3, San Diego, USA) was the software used for all statistical analyses. When appropriate, a one-way ANOVA test was used to analyse the data. Using the Dunnet test, post hoc analyses were performed by comparing the SMs' values with vehicle control. The fluorescence decay curves for the trolox assay were first created using the mean fluorescence values at various time points (Figure 2). Using the GraphPad Software provided by Prism, the area under the curve (AUC) was computed from these fluorescence decay curves. ORAC values were reported as TE (µM/mg), and table 1 displays the tabulated results. Strong, moderate, and weak antioxidants were assigned to SMs based on their antioxidant capacities.

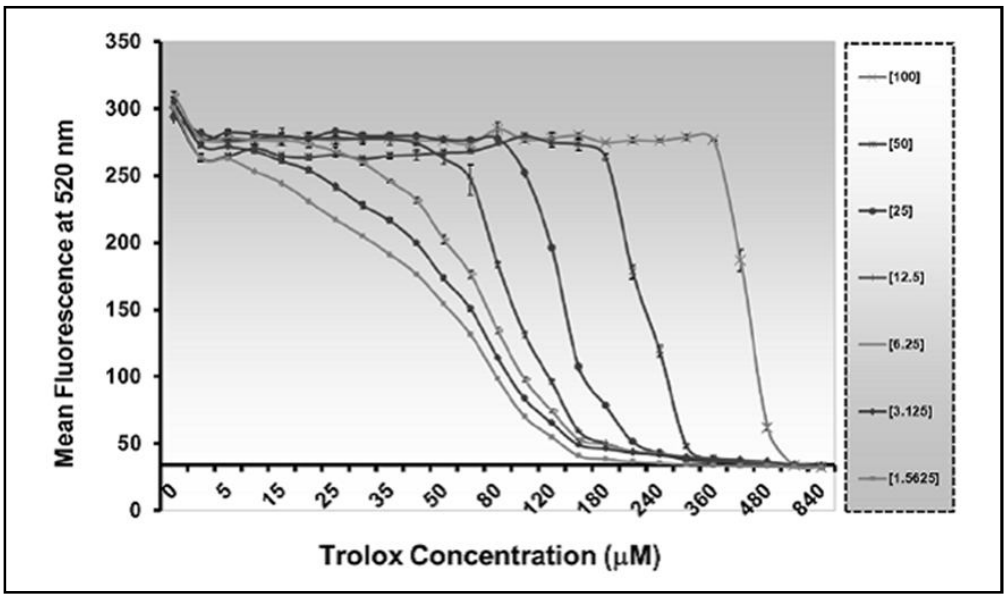

Figure 2: Fluorescence decay curve for the ORAC-Fl assay.

3. Results

HIV and its pathogenic proteins are known to generate ROS on their own as well as aggravate Therefore, the primary goal of the current study was to examine the SMs (1-6) that could lower the ROS produced in SK-N-SH cells by HIV proteins gp120 and Tat, and to compare the results to other sources of free radicals.

In the first step, the antioxidant capacities of SMs were evaluated by using ORAC-Fl assay. On the basis of their TE, SMs were classified as strong (TE>150), moderate (TE=100-150) and weak (TE=10-50) antioxidants. It was found that most of the SMs showed some antioxidant capacities and two of among them SM1 and SM 6 showed strong antioxidant capacity (Table 1).

Table 1: Comparison of the calculated ORAC, total ORAC and TE values of SMs 1-6

SM No.	ORAC	Total ORAC*	TE (ig/ml)**	Antioxidant capacities
1.	74.3	4755.2	190.4	Strong
2.	24.4	1561.6	62.7	Moderate
3.	24.9	1593.6	63.7	Moderate
4.	21.7	1388.8	55.6	Moderate
5.	19.8	1267.2	50.7	Moderate
6.	67.1	4294.4	171.8	Strong

* Total ORAC (ORAC x Dilution factor)
**TE values (Total ORAC/Volume of SM in µl)

All SMs were selected for further investigation of their ability to protect SK-N-SH neuronal cell lines. The effect of various free radical generators and HIV proteins on SK-N-SH cell proliferation was first studied using WST-1 assay. When the free radicals were tested for their effect on the SK-N-SH cell proliferation using this assay, Tat and SNP were found to increase the cell proliferation, whereas gp120-41 and SNP-Cys, were found to be cytotoxic (Figure 3).

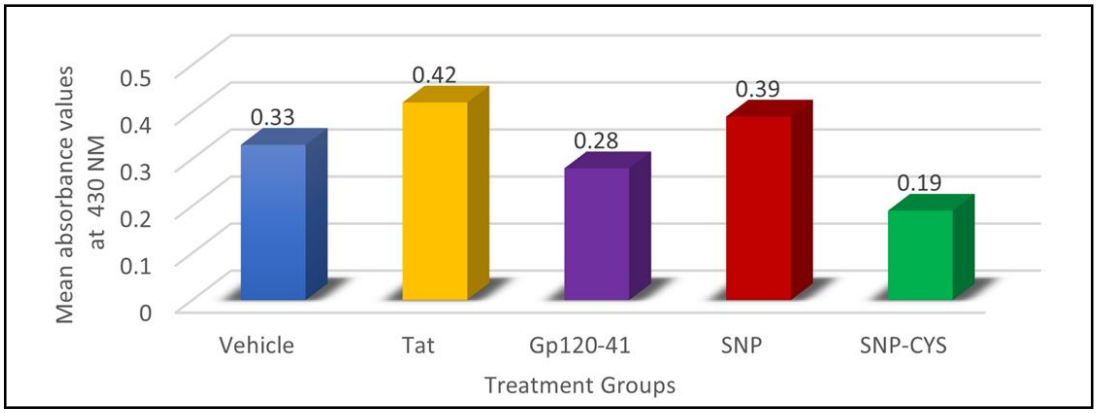

Figure 3: Effect of HIV Tat, gp120-41, SNP and SNP-CYS on SK-N-SH cell proliferation using WST-1 assay.

The absorbance values after SNP-Cys and gp120-41 treatment were less than that of the control which could therefore be attributed to the suppression of the mitochondrial activity by these agents.

For the current investigation, CDFH-DA method was used to quantitate the intracellular oxidative stress induced by HIV proteins, other free radical generators and modulation of ROS by the SMs. 12 M CDFH-Da was used for the measurement of intracellular ROS levels of SK-N-SH cells. Using this assay, it was found that gp120-41 used in the current investigation generates intracellular oxidative stress (Figure 4). SMs 1, 2, 4, 5 and 6 were found to be effective in controlling ROS levels generated by gp120-41.

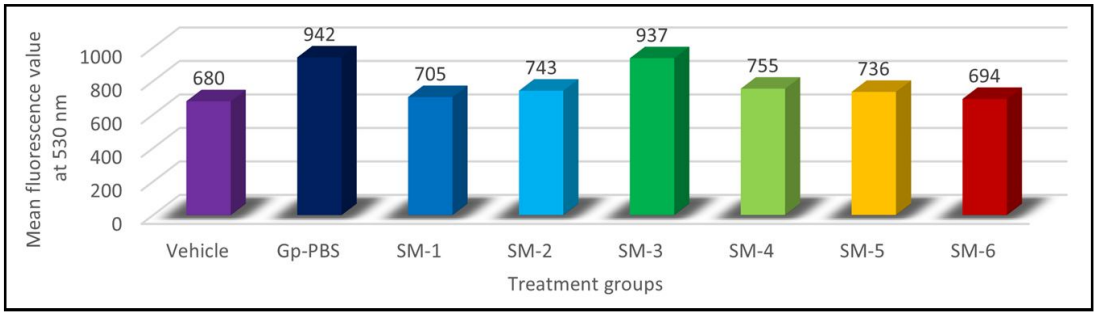

Figure 4: Modulation of gp120-41 induced oxidative stress by SMs 1-6.

When these SMs were tested for their ability to reduce Tat induced ROS inside the SK-N-SH cell lines, the SMs 1, 3, 4 and 6 were able to significantly reduce the ROS levels (Figure 5). It should be noted that SM2 did not reduce Tat induced ROS levels although they were able to effectively reduce gp120-41 induced ROS levels in these cells (Figure 4).

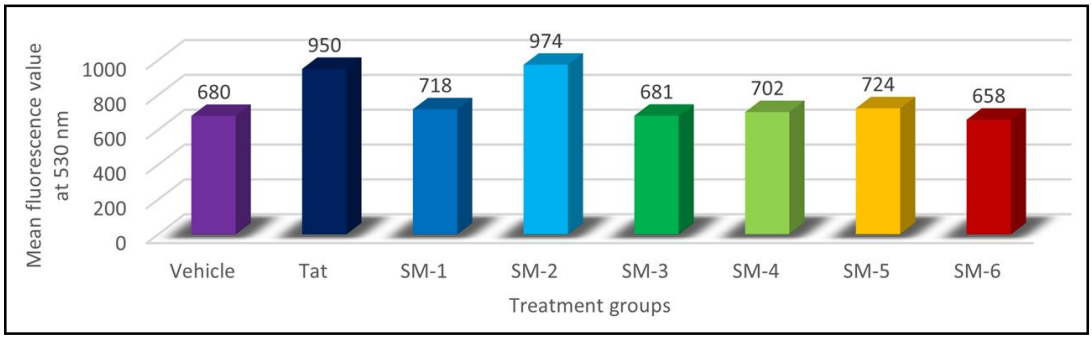

Figure 5: Modulation of Tat-induced oxidative stress by SMs 1-6.

4. Discussion

It is, therefore, appears to indicate that Tat and gp120-41 generate ROS *via* different mechanisms, and that the SMs act *via* different mechanisms. SM 3 was however not effective in controlling ROS levels induced by gp120-41. This might be due to poor bioavailability of the SM 3 inside the cells.

SMs 5 and 6 could be regarded as broad-spectrum antioxidants; whereas others could be regarded as specific for either gp120-41 (SM 3) or Tat (SM 2 and 4) induced oxidative stress and should be tested in combination with the broad-spectrum antioxidants for synergistic or additive effects. The SMs were tested at one concentration only, and therefore might show activity at higher concentration.

5. Conclusion

SMs 1-6 isolated from *S. anquetelia, B. albiflora* and *G. arborea* were found to be effective in combating intracellular oxidative stress induced by HIV proteins gp120-41 and tat in the current investigation. The SMs used in the current investigation, therefore, could prove beneficial under similar circumstances.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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