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Assessment of *In vitro* Antioxidant, Antidiabetic and Cytotoxic Activities of *Sphaeranthus africanus* Extracts

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Abstract – *Sphaeranthus africanus* is commonly used as a traditional remedy for sore throats and pain treatment in Vietnam. The aerial parts have been studied for its anti-inflammatory and anti-proliferative properties. However, the antioxidant and antidiabetic potential of the plant has not been explored. In this work, hydrophilic extracts of the plant's aerial parts were prepared in order to investigate its antioxidant and anti-diabetic properties. Also, the cytotoxicity of the root was evaluated and compared to that of the aerial parts. All of the extracts inhibited lipid peroxidation with IC_{50} values ranging from 2.05 to 3.56 μg/mL, indicating substantial antioxidant activity. At an IC_{50} value of 4.80 μg/mL, the 50% ethanol extract exhibited the most potent inhibition of α-glucosidase. The cytotoxic activity of root extracts is 2 to 5-fold less than that of the aerial parts. Nevertheless, dichloromethane and ethyl acetate extracts of the root demonstrated a selective effect on leukemia cells, with no harm towards the normal HEK-293 cell line. This work provides a scientific support for the antioxidant and antidiabetic activity of the plant. Hence, it may find a promising material for the development of novel antioxidant and antidiabetic agents. More research can be conducted on the phytochemistry and anticancer activities of the plant's root.
Keywords – *Sphaeranthus africanus*, Antioxidant, Antidiabetic, Anti-proliferative activities

Introduction

Nowadays, despite the fact that many antidiabetic and anti-cancer agents have been introduced to the market, these serious diseases remain major global public health concerns in both developed and developing countries.[1,2] Oxidative stress has been implicated in the progression of diabetes mellitus and long-term diabetes complications.[3] In addition, it has been proposed as the root cause of neurodegenerative disease, cardiovascular disease, inflammatory disease, and other pathologies.[4,5] Specifically, oxidative stress is notably known to damage the DNA molecule and regulate the progression of various cancers.[6]

Nature has endowed Vietnam with an extremely diverse natural heritage. According to the National Institute of Medicinal Materials, approximately 3950 of 12000 vascular plant species are known to be medicinal plants in Vietnam,[7] belonging to over 2256 genera and 305 families.[8] Many plants have been used in Asian traditional medicine. *Sphaeranthus africanus*, from the Asteraceae family, grows in Tanzania, Madagascar, the Indian Subcontinent, and Southeast Asia.[9] This plant has been used in traditional Vietnamese medicine to alleviate swelling and as a sedative to relieve pain. Pressed juice from fresh leaves of *S. africanus* was used to treat sore throats and for mouth washes. The decoction also has antitussive and expectorant roles.[10] Several studies on the plant have previously been conducted. Isolated compounds from the plant showed antiproliferative, anti-inflammatory, and anti-bacterial activities.[11-13] In order to explore other bioactivities of this interesting plant, the antioxidant and anti-diabetic pro-

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perties of extracts from the aerial portions of *S. africanus* were investigated. In addition, a study of the cytotoxicity of extracts derived from the root and aerial parts of the plant was conducted.

Experimental

Plant materials – *S. africanus* was collected in Quang Nam Province, in the central region of Vietnam. The collected plant was identified by Dr. Huynh Loi and authenticated at the School of Medicine, Vietnam National University, HCM City, Vietnam. A voucher specimen has been deposited at the School of Medicine, Vietnam National University. The roots and aerial parts of the plant were washed, dried at room temperature from 48 to 92 h, ground into powder, and then stored in glass bottles preserved from light and moisture until use.

Plant extraction – To prepare the extracts for antioxidant and antidiabetic testing, the powder of the aerial parts (10 g) was extracted by the refluxing method with 96% ethanol, 50% ethanol, and distilled water. Then, evaporation allowed for condensed extracts, which were symbolized as SaE-96, SaE-50, and SaW. This yielded 22.3%, 26.4%, and 37.6% of ethanolic 96%, ethanolic 50%, and aqueous extracts, respectively. For the antiproliferative activity, the root and aerial parts powder (10 g) were extracted successively with 100 mL each of *n*-hexane, dichloromethane, ethyl acetate, and methanol by a Soxhlet extractor. All the extracts were then evaporated to dryness by a rotary evaporator at 50ºC, affording SaH (60 mg), SaD (40 mg), SaA (70 mg), and SaM (90 mg). The obtained extracts were kept at 5ºC prior to further experimental analysis.

DPPH radical scavenging assay – The extracts' scavenging activity was determined using published methods.[14-18] Briefly, 0.5 mL of various concentrations of each sample (5, 10, 25, 50, 100, 250, 500, 1000, and 2000 μg/mL) or ascorbic acid (Merck, Germany) (0.01, 0.05, 0.1, 0.25, 0.5, and 1 mM) was added into a tube containing 0.5 mL of 0.6 mM DPPH (Sigma Aldrich, USA) solution dissolved in methanol, and the volume was made uniformly to 4 mL using methanol. The solution was mixed and then allowed to stand in the dark at room temperature for 30 minutes. Absorbance was measured at 515 nm using methanol as a blank on a UV-visible spectrometer (Thermo Fisher Scientific, US). 0.5 mL of DPPH was added to 3.5 mL of methanol, and the absorbance was taken for the control reading. The ascorbic acid was used as a positive control to assess the potential scavenging abilities of the samples. All analyses were processed in triplicate.

Lipid peroxidation inhibition assay (Malondialdehyde assay) – This assay was carried out according to the method described by Chang et al.[19] In brief, after collecting mouse brain tissue, it was immediately put in an ice-cold 5 mM phosphate buffer (PBS, pH 7.4) at a ratio of 1:10 (w/v) brain:phosphate buffer. The tissue was then homogenized at 13000 rpm in an ice-cold After collecting mouse brain tissue, it was immediately put in ice-cold 5 mM phosphate buffer (PBS, pH 7.4) at a ratio of 1:10 (w/w) brain:phosphate buffer. The tissue was then homogenized at 13000 revolutions per minute in an ice-cold state. 0.1 mL of different concentrations of each sample (10, 25, 50, 100, 250, 500, and 1000 g/mL) or Trolox (Sigma Aldrich, USA) (25, 125, 250, 1250, and 2500 g/mL) was added to 0.5 mL of brain homogenate, and the volume was adjusted to 2 mL with 50 mM phosphate buffer. The mixture was then incubated for 15 minutes at 37ºC to allow for lipid peroxidation and MDA generation. The reaction was halted using 1 mL of trichloroacetic acid at 10% concentration. The tube was then centrifuged for 5 minutes at 10000 rpm and 5ºC. The supernatant was transferred to a separate tube (2 mL) and allowed to react at 95ºC for 15 minutes with a 0.8% TBA solution (1 mL). The control was made as described above, excluding the test sample. As the tubes reached room temperature, the absorbance of the mixture was measured at 532 nm against a blank solution without a sample and TBA. All tests were conducted in triplicate. Trolox, a water-soluble analog of vitamin E, was used as positive control.

α-Amylase inhibitory assay – The α-amylase inhibition assay was performed using the 3,5-dinitrosalicylic acid method as previously described with slight modifications.[20] In brief, 250 μL of each sample (500, 1000, 1500, 2000, 2500, 3000 μg/mL) in 0.02 M sodium phosphate buffer (pH 6.9) or acarbose (Sigma Aldrich, USA) (100, 150, 200, 250, 300 μg/mL) in 0.02 M sodium phosphate buffer (pH 6.9) containing 6 mM NaCl were incubated with 250 μL of α-amylase (HIMEDIA, India) (2 U/mL) at 37ºC for 15 min. Then, 250 μL of 1% soluble starch was added as a substrate and incubated further at 37ºC for 20 min; 500 μL of the DNSA (DNSA, China) color reagent (12 g of sodium potassium tartrate tetrahydrate in 8.0 mL of 2 M NaOH and 20 mL of 96 mM of 3,5-dinitrosalicylic acid solution) was then added, boiled for 10 min, cooled to room temperature, and the absorbance was measured at 540 nm. The measurements were performed in triplicate. Acarbose was used as positive control.

α-Glucosidase inhibitory assay – The α-glucosidase

inhibitory assay was conducted with minor modifications to the method described by Hua-Qiang Dong et al. in 2011.[21] The reaction mixture containing 60 μL of varying concentrations of test extracts (10, 25, 50, 75, 100, 250, 500 and 2000 μg/mL) or acarbose (50, 100, 250, 500, 1000, 1500, 2000 μg/mL), 50 μL of 0.1 M phosphate buffer (pH 6.8) containing α-glucosidase (Sigma Aldrich, USA) (0.2 U/mL) was incubated in 96-well plates at 37ºC for 10 min. Then, 50 μL of soluble *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG) was added and incubated at 37ºC for another 20 min. After incubation, absorbance was taken at 405 nm using a plate reader (Biotek, USA). Acarbose (Sigma Aldrich, USA) was used as a positive control. The measurements were performed in triplicate.

Cell culture – Human acute lymphoblastic leukemia CCRF-CEM cells were kept in RPMI 1640 medium (Gibco®, Invitrogen, Darmstadt, Germany) supplemented with 2 mM L-glutamine (Sigma-Aldrich®, MO, USA), 10% heat-inactivated fetal bovine serum (FBS) (PAA laboratories, Pasching, Austria), 100 units/ml penicillin and 100 μg/ml streptomycin (1% penicillin/streptomycin) (PAA laboratories). Human embryonic kidney HEK-293 cells were cultivated in DMEM:Ham′s F12 (1:1 mixture) supplemented with 2 mM L-glutamine, 10% FBS and 1% penicillin/streptomycin. All cells were kept in a humidified 5% CO_2 atmosphere at 37ºC and passaged at 90% confluence.

Cytotoxicity assay – The anti-proliferative activity was measured using the XTT viability assay, which was developed to improve existing tetrazolium assays and was first described in 1988 by Scudiero et al.[22] For HEK-293 cells, 100 μL of a suspension of 5×10^4 cells/mL was seeded in 96-well microplates and grown over night in a humidified atmosphere containing 5% CO_2 at 37ºC before extracts were added. For CCRF-CEM leukemia cells, 100 μL of a suspension containing 1×10^5 cells/mL was treated immediately after seeding. Each sample was tested in at least two different cell passages and three independent wells per plate. Since all test samples were dissolved in DMSO (dimethyl sulfoxide), control cells correspond to vehicle-treated cells (0.5% DMSO final concentration). After a sufficient incubation period, 50 μL of XTT solution was added. This solution consisted of an XTT labeling reagent and an electron-coupling reagent. After incubation with XTT for 1.5 h (HEK-293 cells) or 4 h (CCRF-CEM cells) at 37ºC and 5% CO_2, the absorbance of each well was measured at 490 nm with a reference wavelength of 650 nm using a Hidex Sense microplate reader (Hidex, Turku, Finland). Vinblastine served as a positive control.

Result and Discussion

The importance of controlling postprandial hyperglycemia in the early prevention of type 2 diabetic complications has been established for a long time.[23] Inhibiting α-glucosidase and α-amylase is an interesting way to prevent fasting blood glucose elevation.[24-26] Furthermore, scientists have given much more attention to the antidiabetic activity of a compound when its antioxidant activity is also evaluated.[21] Indeed, oxidative stresses are involved in initiating beta cell damage and insulin resistance. Antioxidants also have the capacity to prevent the progressive impairment of pancreatic beta-cell function and thus reduce the occurrence of type 2 diabetes.[27] In the present study, hydrophilic extracts were obtained to investigate their antioxidant and anti-diabetic activities. The DPPH radical scavenging and lipid peroxydation inhibitory tests were used to assess anti-oxidant activity. The α-amylase inhibitory together with α- glucosidase assay were conducted to determine the antidiabetic property. Additionally, the cytotoxicity potential of extracts from the root was evaluated and compared to the aerial parts.

Table 1 summarizes the complete antioxidant activity results of the tested plant extracts. The activity was intermediate on the DPPH test for all extracts; the IC_{50} values of 14.56, 16.35, and 14.83 μg/mL were found for SaE-96, SaE-50, and SaW, respectively. However, the activity was strong on the lipid peroxidation inhibition assay. The IC_{50} values of 2.79, 2.05, and 3.56 μg/mL were observed for SaE-96, SaE-50, and SaW, respectively. The results indicated that antioxidant activity was detected for all extracts, with a higher inhibitory activity against lipid peroxidation.

S. africanus extracts exhibited inhibitory effects on both enzymes tested, as presented in Table 2. In particular, a moderate activity was found for extracts against α-amylase with IC_{50} values of 0.47, 0.16, and 0.38 μg/mL

Table 1. IC_{50} values (μg/mL) of *S. africanus* extracts on DPPH and lipid peroxidation inhibition activity

Sample	DPPH scavenging activity	Lipid peroxidation inhibition activity
SaE-96	14.56 ± 1.47^{c}	2.79 ± 0.07
SaE-50	16.35 ± 1.99^{b}	2.05 ± 0.06
SaW	14.83 ± 0.89^{a}	3.56 ± 0.33
Ascorbic acid	5.09 ± 0.01^{d}	-
Trolox	-	27.88 ± 0.02^{e}

The values are the mean of three determinations ± SD; Different superscript letters (^{a,b,c,d,e}) in a column indicate significant differences at $p < 0.001$; SaE-96, 96% ethanol extract; SaE-50, 50% ethanol extract; SaW, water extract.

Table 2. IC_{50} values (μg/mL) of *S. africanus* extracts on α-amylase and α-glucosidase inhibitory activities

Sample	α-amylase inhibition	α-glucosidase inhibitory
SaE-96	0.47 ± 0.02[c]	65.86 ± 1.39[c]
SaE-50	0.16 ± 0.01[b]	4.80 ± 0.27[b]
SaW	0.38 ± 0.02[a]	15.45 ± 0.84[a]
Acarbose	0.047 ± 0.01[d]	133.47 ± 3.64[d]

Data express means ± SD of three independent experiments. Different superscript letters ([a,b,c,d,e]) in a column indicate significant difference at $p < 0.001$

for SaE-96, SaE-50, and SaW, respectively. All extracts showed a potent inhibitory capacity against α-glucosidase, with IC_{50} values of 65.86, 4.80, and 15.45 g/mL for SaE-96, SaE-50, and SaW, respectively. SaE-50 extract was found to be the most active in inhibiting the α-amylase and α-glucosidase with IC_{50} values of 0.16 and 4.80 μg/mL.

This combination of strong α-glucosidase inhibitory activity and mild α-amylase inhibitory activity is very interesting and beneficial. Indeed, inhibiting both α-glucosidase and α-amylase enzymes is very effective, but it can also cause side effects due to abnormal bacterial fermentation caused by excessive α-amylase inhibition. Finding a compound with strong anti α-glucosidase activity and mild anti α-amylase activity has long been a challenge for researchers. Several studies observed these properties for phenolic compounds,[25,26,28,29] and Hua-Qiang Dong also describes these particularities for trilobatin from *Lithocarpus polystachyus* Rehd in 2012.[21]

On the other hand, the *S. africanus* samples showed intermediate radical scavenging activity and a strong lipid peroxidation inhibitory action. This last property is beneficial because lipid peroxidation deconstructs essential polyunsaturated fatty acids such as omega-3, omega-6, and omega-9;[30] and this lipid peroxidation is also toxic due to the generation of free radicals.

These positive antidiabetic and antioxidant results make the method of extraction of the material interesting. The activity of the samples has been evaluated for each assay based on their IC_{50} on the equation below to facilitate comparison of the materials and methods of extraction:

For antioxidant assays

DPPH Radical Scavenging Activity: Ascorbic Acid >> SAE-96% > SAW > SAE-50%

Lipid Peroxidation Inhibitory Activity: SAE-50% > SAE-96% > SAW >> Trolox

For antidiabetic assays

α- Amylase Inhibitory Activity: Acarbose >> SAE-50% > SAW > SAE-96%

α- Glucosidase Inhibitory Activity: SAE-50% > SAW > SAE-96% >> Acarbose

In the malondialdehyde test, the α-amylase inhibitory test, and the α-glucosidase inhibitory test, SAE-50% had lower IC_{50} than SAW and SAE-96%. The IC_{50} of SAE-50% was higher than that of SAE-96% and SAW only on the first DDPH test, but their IC_{50} were relatively close (16,35 μg/mL for SAE-50%, 14,56 μg/mL and 14,83 μg/mL for SAE-96% and SAW, respectively), while the IC_{50} difference was more important between these three samples in the three last tests. Therefore, the material extraction by the refluxing method with 50% ethanol seems to potentialize the activity of *S. africanus* in comparison to the refluxing method with water or with 96% ethanol. This potentialization by ethanol 50% can be explained by the balanced polarity of ethanol 50% between the very high polarity of water and the low polarity of ethanol 96%.

This study was the first exploration of the antioxidant and antidiabetic activities of *S. africanus*. The results obtained are encouraging and promising, however, the isolation of the compounds on the three extracts should be continued. The isolation and structural study by chromatographic and spectrometric methods will allow us to know if there are any new carvotacetones in our samples and will allow us to understand more precisely their effects. Indeed, it will be possible to compare hydrophilic and lipophilic extraction methods on *S. africanus* and determine which solvent polarity is best for maximizing extraction and effects.

The cytotoxic effects of plant extracts were evaluated on CCRF-CEM and HEK-293 cells; the results are given in Table 3. In general, significant cytotoxicity was observed for all extracts except the methanolic extract. However, a massive difference is detectable between the extracts of the aerial and the root parts. The cytotoxic activity of extracts obtained from the aerial parts is 2 to 5-fold stronger than that of the root part. The IC_{50} values for n-hexane extracts of the aerial parts were 2.64 and 1.51 μg/mL, respectively, against leukemia and HEK-293 cells, whereas the IC_{50} values for n-hexane extracts of the root part were 10.53 and 5.68 μg/mL against leukemia and HEK-293 cells, respectively.

For the root part, only n-hexane extract showed activity on both cell lines. Dichloromethane and ethyl acetate extracts exhibited toxic activity on the CCRF-CEM with IC_{50} values of 19.85 and 18.19, respectively, and no cytotoxicity was found on the HEK-293 cells. The findings suggest that these extracts had a selective effect on leukemia cells. Methanolic extract did not show any

Table 3. IC_{50} (μg/mL) values of cytotoxicity of plant extracts after 72 h of treatment

Plant part (s)	Extract	CCRF-CEM	HEK-293
Aerial parts	*n*-Hexane	2.64 ± 0.06	1.51 ± 0.27
	Dichloromethane	2.70 ± 0.06	3.98 ± 0.12
	Ethyl acetate	9.35 ± 0.90	10.77 ± 0.88
	Methanol	14.30 ± 1.22	NA
Root part	*n*-Hexane	10.53 ± 0.35	5.68 ± 1.09
	Dichloromethane	19.85 ± 0.46	NA
	Ethyl acetate	18.19 ± 1.67	NA
	Methanol	NA	NA
	Vinblastine	$7.6 \times 10^{-3} \pm 2.0 \times 10^{-4}$	$2.1 \times 10^{-2} \pm 4.0 \times 10^{-4}$

Results are expressed as means ± SD of six independent experiments; NA: nonactive; IC_{50} values were determined using the four-parameter logistic curve and individual values of all independent experiments. CCRF-CEM = human acute lymphoblastic leukemia; HEK-293 = human embryonic kidney cells.

effects on both cell lines.

According to the American National Cancer Institute (NCI), the criteria for cytotoxicity activity for crude extracts is $IC_{50} < 20$ μg/mL after 48 or 72 h of incubation.[31] Therefore, besides the arial parts, the root part of the plant can also be considered a promising source for the development of novel anticancer agents. Since the plant's aerial parts have been studied and a few compounds isolated,[11,12] the root part should be considered for research focusing on phytochemistry and pharmacology as well.

S. africanus has been used in ethnomedicine in Vietnam to treat sore throats. The pounded leaves are applied externally to relieve pain and swelling.[32] Several biological activities of *S. africanus* have been reported, including antiproliferative, anti-inflammatory, and antibacterial activities. In the present study, identification of antioxidant, antidiabetic and cytotoxic activities of the extracts from *S. africanus* suggests this plant may be a potential source for the developtment of anti-diabetic and anti-cancer agents.

In conclusion, the results from this in-vitro study clearly indicated that *S. africanus* had strong inhibitory activity against α-glucosidase and mild inhibitory activity against α-amylase. These properties are beneficial as they ensure efficient blood glucose level regulation without causing abnormal bacterial fermentation or other side effects. The extracts also inhibited lipid peroxidation and had a moderate radical scavenging activity. The samples extracted by the refluxing method with ethanol at a concentration of 50% showed better global efficacity than the sample extracted by the refluxing method with water and ethanol at a concentration of 96%. This study was the first investigation of the antioxidant and anti-diabetic effects of *S. africanus*. These promising results should be confirmed in the future by other clinical tests and experiments to see if they can be an alternative or complementary treatment for diabetic patients. Together with the aerial parts, the root part of the plant can be investigated for phytochemistry and pharmacology in the future.

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Conflicts of Interest

The authors declare no conflicts of interest.

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