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HEALTH SCIENCES

Antarctic bryophyte *Sanionia uncinata* (HEDW.) Loeske, Amblystegiaceae, antimicrobial, antioxidant, cytotoxic, and acetylcholinesterase activities

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Abstract: *Sanionia uncinata*, or Sickle-leaved-Hook-moss, is a cosmopolitan pleurocarpous moss composing the Antarctic Peninsulae biodiversity, primordially forming dense mats over rocks. The species was collected in 24 different spots located at King George Island and was processed to obtain 24 ethanolic extracts (ADS#) by a serial-24h-maceration, which were prospected for antimicrobial, cytotoxic, antioxidant, and acetylcholinesterase (AChE) inhibition activities by using *in vitro* tests. Alien material was removed from the non-sterilized plant samples before being submitted for extraction. It was observed that extracts collected in different spots showed different biological activities. Extracts ADS04(10.66±0,17mm) and ADS14(11.37±0,11mm) were active against *Staphylococcus aureus*, according to the diffusion in bioautography assay. They showed significant antioxidant activity and inhibited AChE; the cytotoxicity observed to the human breast cancer cells MCF-7 and MDA-MB-231 were higher than in normal cell line MCF-10A. ADS04 was 7.62 times more cytotoxic to MCF-7, and ADS14 was 2.03 times more cytotoxic to MDA-MB-231 than to MCF-10A. The extracts showed similar cytotoxicity between PC-3, a human prostate cancer cell line, and MCF-10A. *Sanionia uncinata* extracts are a vital potential source of biologically active compounds, particularly ADS04 and ADS14, including further prospection on eventual bryophyte's endophytic fungi.

Key words: acetylcholinesterase inhibition, antibacterial activity, bryophyte, bioautography, cytotoxicity, moss.

INTRODUCTION

Bryophytes are a group of plants originating in the Middle Ordovician about 475 million years ago (Wellman & Gray 2000). Today, they are the second-most diverse group of land plants after angiosperms, comprising estimates between 15,000 and 18,000 species worldwide (Goffinet et al. 2009). In the Antarctic Continent, they are the dominant terrestrial plants and stand out for abundance, species variety representativeness, and floristic composition, which have been the object of various studies (*e.g.*, Seppelt & Green 1998, Bednarek-Ochyra et al. 2000, Ochyra et al. 2008, Kurbatova & Ochyra 2012, Cannone et al. 2013, Bramley-Alves et al. 2014, Câmara et al. 2017, De Freitas et al. 2018, Henriques et al. 2018, Mundim et al. 2021).

Phytochemical studies were performed on Antarctic mosses. Five bioflavonoids were isolated from the redshank *Ceratodon purpureus* (Hedw.) Brid. (Ditrichaceae), and

are described as being bonded to the moss's cell walls (Waterman et al. 2017). Earlier, benzonaphthoxanthenones ohioensins A and B (Bhattarai et al. 2009) and ohioensin F and G (Seo et al. 2008) were isolated from the alpine haircap *Polytrichastrum alpinum* (Hedw.) G. L. Sm. (Polytrichaceae). *Sanionia uncinata* (Hedw.) Loeske (Amblystegiaceae Kindb.), commonly known as Sickle-leaved Hook-moss, is one of the most common species in Antarctica, despite a wide distribution worldwide (Smith 1996, Park et al. 2018), including the Arctic (Torres-Mellado et al. 2011, Zúñiga-González et al. 2016).

Previous studies carried out with *S. uncinata* demonstrated that sanionins A and B (Ivanova et al. 2007) found in the aqueous fraction obtained from the hydroethanolic plant extract showed photoprotective activity and low toxicity to zebrafish and cell culture (Fernandes et al. 2019). The extract has also shown genotoxicity and the indicative presence of phenolic acids, catechins, caffeic and ferulic acids, orientin, homoorientin, rutin, quercetin, flavanones, and flavones in hydroalcoholic extract (Fernandes et al. 2015, 2017), as well as the presence of carotenoids (Fernandes et al. 2011), and antioxidant activity (Bhattarai et al. 2008).

Nature is a source of potential new human and veterinary drugs. Previous works have shown that plant extract large-scale screening can lead to the selection of antimicrobial (Suffredini et al. 2004, 2023), cytotoxic (Suffredini et al. 2006a, b, 2007a, b, Ozi et al. 2011, Dutra-Correa et al. 2018), and antioxidant active natural products (Cavarsan et al. 2017; Marin et al. 2018). Therefore, the present work aims to verify the therapeutic potential of plant extracts obtained from *S. uncinata* against biological models of cytotoxicity, antimicrobial and antioxidant activity, and enzyme inhibitory activity of the species collected in 24 different sites in the Antarctica Peninsula.

MATERIALS AND METHODS

Sampling

Fieldwork was carried out with the support of the Brazilian Navy with the Polar Ship Almirante Maximiano during the 38th Brazilian Antarctic Operation (OPERANTAR XXXVIII) in December 2019. Samples were collected on King George Island at the Keller Peninsula, South Shetlands, at 13 different sites, whose locations were recorded by the Global Position System (GPS Garmin® E-trex 10). The collection was carried out in areas with different altitudes, subject to thawing snow, and locations close to regions with seawater spray action, for about 13 km, covered on the same day (Figure 1).

The samples were taken from the rocky or intemperized soil substrates (Table I), placed in tissue-non-tissue bags (TNT), and identified with usual data related to the collection conditions and environmental characteristics, such as kind of substrate, luminosity condition, exposure to wind, seawater influence, association with other species, among others. Herborized vouchers were deposited at the UB Herbarium, University of Brasília. The plant material was identified by Amanda Leal da Silva Teodoro and Paulo Eduardo Aguiar Saraiva Câmara, specialists in Bryophytes. The collected material was cleaned from soil residues and other alien material. The samples destined for extraction were stored in a freezer at -80 °C until being processed, and the vouchers were dried at the laboratory's temperature.

Extract and standard compound preparation

The collected materials were removed from the -80 °C freezer and subsequently dried by lyophilization (VirTis lyophilizer, Unitop FM-25 XL model), to finally be crushed with a blender before being subjected to static maceration (Brasil 2019) for 24 h, for four consecutive times, totaling 96 h, using analytical grade ethanol as

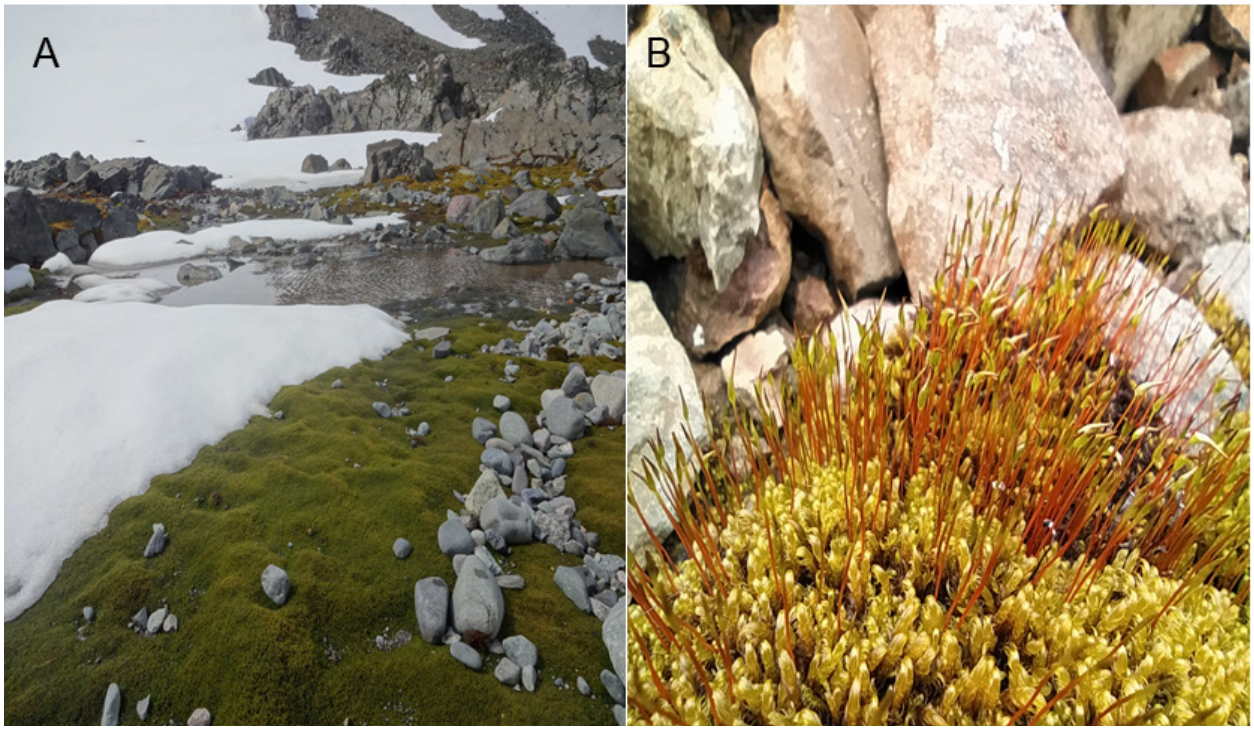

Figure 1. The fieldwork location during the 38th Brazilian Antarctic Operation (OPERANTAR XXXVIII), Keller Peninsula, Antarctic. A. Plant collection site; B. *Sanonia uncinata* detail.

extractor liquid. The plant samples were cleaned from foreign material without being sterilized before being submitted to extraction. The 24 extracts were identified as ADS02, ADS04, ADS06, ADS08, ADS09, ADS10, ADS11, ADS12, ADS13, ADS14, ADS16, ADS18, ADS20, ADS21, ADS22, ADS24, ADS26, ADS28, ADS30, ADS32, ADS34, ADS36, ADS38, and ADS40 (Table I), and were kept frozen at -20 °C until use.

To perform the bioautography assays, plant extracts were prepared at a concentration of 100 mg/mL, diluted in a solvent mixture composed of dimethylsulfoxide (DMSO) and ethanol (2:8). The extracts from the previously obtained solution were prepared at 40 mg/mL for cytotoxicity, enzyme inhibition, antioxidant activity, and total phenolic content assays (Suffredini et al. 2007a, b).

Rutin and Trolox were prepared at 1,0 mg/mL and ½-fold diluted in ethanol for the antioxidant assays. Physostigmine was prepared at the same concentrations to be used in the acetylcholinesterase assays. Doxorubicin was prepared at 25 mM and log-fold diluted for the cytotoxicity assays. Chlorhexidine 1% was used in the antimicrobial assays.

Sanionia uncinata bioautography antimicrobial analysis

The 24 plant extracts were screened for antimicrobial activity using the diffusion in bioautography (DeB) assay, where a single drop of each extract was distributed in a thin-layer chromatography (TLC) plate before being tested by the bioautography assay by pouring the inoculated medium over the plates under sterile conditions and incubating them accordingly. Also, the active extracts were submitted to TLC elution with a mobile phase composed of chloroform (Wagner & Bladt 1996). After that, the chromatograms were analyzed by using the same microbiological technique. To improve

Table I. Field data and plant extraction information related to the different sample collections made with *Sanionia uncinata*, Amblystegiaceae. Geographic coordinates are displayed in decimal degrees. R = on rocky substrate; S = on intemperized soil substrate.

Collect #	Voucher #	Substrate	Lat (S)	Long (O)	Elevation [m]	Extract [g]/plant [g]	Yield [%]
ADS02	Silva 584	R	62.0888	58.40255	34	0.3123/5.7248	5.45
ADS04	Silva 585	R	62.0888	58.40255	34	0.3817/4.1674	9.15
ADS06	Silva 586	R	62.0888	58.4101	57	0.1119/2.1592	5.18
ADS08	Silva 587	R	62.0864	58.4065	129	2.1231/38.6857	5.48
ADS09	Silva 588	R	62.0864	58.4065	129	0.4483/7.6852	5.83
ADS10	Silva 589	R	62.0850	58.4167	39	0.5876/19.1359	3.07
ADS11	Silva 590	R	62.0864	58.4065	129	0.8144/18.9746	4.29
ADS12	Silva 591	R	62.0840	58.4216	3	1.2312/37.1252	3.31
ADS13	Silva 592	R	62.0858	58.413	94	1.0322/26.8820	3.83
ADS14	Silva 594	R	62.0775	58.4216	13	0.1969/15.8036	1.24
ADS16	Silva 596	R	62.0752	58.4210	1	0.2418/14.4667	1.67
ADS18	Silva 593	R	62.0812	58.4239	6	0.5156/13.9309	3.7
ADS20	Silva 595	R	62.0812	58.4239	6	0.4708/13.0956	3.59
ADS21	Silva 597	R	62.0812	58.4239	6	0.6408/9.6567	6.63
ADS22	Silva 598	R	62.0812	58.4239	6	0.2693/8.5079	3.16
ADS24	Silva 599	R	62.0855	58.4197	0	1.0812/28.5896	3.78
ADS26	Silva 600	S	68.0850	58.4193	1	0.8416/26.8887	3.12
ADS28	Silva 601	R	62.0891	58.4115	4	0.5031/16.8796	2.98
ADS30	Silva 602	R	62.0891	58.4115	4	0.5889/10.1268	5.81
ADS32	Silva 603	R	62.0891	58.4115	4	0.2529/6.6319	3.81
ADS34	Silva 604	R	62.0891	58.4115	4	0.1668/2.8628	5.82
ADS36	Silva 605	S	62.0905	58.4045	11	1.261/31.2129	4.03
ADS38	Silva 606	S	62.0905	58.4045	11	0.3254/7.8350	4.15
ADS40	Silva 607	S	62.0905	58.4045	11	0.9272/14.4971	6.39

visualization, MTT viability dye, 3-4,5-dimethyl-thiazol-2-yl-2,5-diphenyltetrazolium bromide, a tetrazolium salt, was used. A detailed description can be assessed elsewhere (Suffredini et al. 2023).

The culture conditions of each microorganisms used were: *Candida albicans* (ATCC10231) was prepared at a concentration of $1.5x10^5$ CFU/mL in Sabouraud Dextrose agar medium (McCleary et al. 1960), whereas *Escherichia coli* (ATCC 29212; concentration of 1.5×10^7 CFU/mL) and *Staphylococcus aureus* (ATCC29213; concentration of 1.5×10^8 CFU/mL) (Basile et al. 1998) were both prepared in Mueller-Hinton agar medium; *Streptococcus mutans* (ATCC25175), in turn, was prepared at a concentration of 1.5×10^7 CFU/mL (Basile et al. 1998), in Brain Heart Infusion agar medium. Regarding temperature and cultivation time, *C. albicans*, *E. coli*, and *S. aureus* were kept in the

incubator at 36 °C for 24 h, whereas *S. mutans* remained at 36 °C for 48 h.

Cytotoxicity test against breast and prostate tumor cells

Cell culture and samples –Plant extracts were tested against one human prostate cancer cell line, PC-3, and against three human breast cell lines, two of which cancer cell lines, MCF-7 (non-metastatic) and MDA-MB-231 (metastatic), and one of them a normal cell line, named MCF-10A. The cytotoxicity test was performed as described elsewhere (Monks et al. 1991). Tumor growth was quantified by the sulforhodamine B (SRB) method. The results, analyzed as the percentage of growth inhibition, were used to calculate each extract's 50% inhibitory concentration, or IC50.

Antioxidant activity for *Sanionia uncinata* and total phenolic compound evaluation

Beta-carotene (β-carotene) and diphenyl-picryl-hydrazyl (DPPH) antioxidant activity tests were performed according to classic techniques described in the literature (Duarte-Almeida et al. 2006, Sherma 2018). Drop autography and autography after elution were used in the qualitative analyses, as well as the quantification of the antioxidant capacity was assessed by the usual tests performed in an aqueous medium for both β-carotene and DPPH tests (Duarte-Almeida et al. 2006, Sherma 2018). Rutin and Trolox were used as standard drugs.

The total phenolic compound amount in each extract was tested by the Folin-Ciocalteu (FC) reagent test described elsewhere (Zhang et al. 2006, Vasconcelos et al. 2017). Trolox and rutin were used as reference compounds

Analysis of acetylcholinesterase inhibitory activity

S. uncinata plant extracts were qualitatively tested (Rhee et al. 2001), with adaptations to run an autography assay on a silica gel GF254 chromatographic plate. The standard substance used was physostigmine, diluted in methanol at a 1.5 µg/mL concentration. Also, the quantification of the enzyme inhibitory activity 50% related to each of the 24 extracts was assessed (Ellman et al. 1961).

Statistics and Experimental Design

For all the analyses, the Shapiro-Wilk normality test (Zar 1999) was performed to ensure parametric tests whenever possible, and the outlier identification was made by the ROUT method (Q-5%). An alpha = 0.05 is the significance level established to reject the null hypothesis: the absence of significant differences between the plant extract and reference substance groups. The statistical package GraphPad Prism10.0 software, plus the RealStats, an Excel supplement, were used for all analyses (GraphPad Software, www.graphpad.com). Figure 2 represents the experimental design of the present study and resumes the statistical approaches. The following tests were applied in the analyses: A simple linear correlation (according to Table I) to evaluate extract yields; one-way ANOVA followed by Tukey's test for mean comparisons (Zar 1999) to evaluate inhibition growth diameter in the diffusion in bioautography assay; the percentage of cell growth, based on the formula %growth = [T-T0]/[C100-T0]*100, where T = optical density obtained from wells with treatment; T0 = optical density of wells containing growing cells up to the moment immediately before adding treatment; C = 100% growth control (Monks et al. 1991) was used to obtain the inhibitory concentration 50 (IC50), calculated using the "Dose-response – EC50 shift by global fitting", followed by a Kruskal-Wallis test and Dunn's post-test to compare cell susceptibility in comparison to MCF-10A, a normal human breast cell line; the "Dose-response – EC50 shift by global fitting"

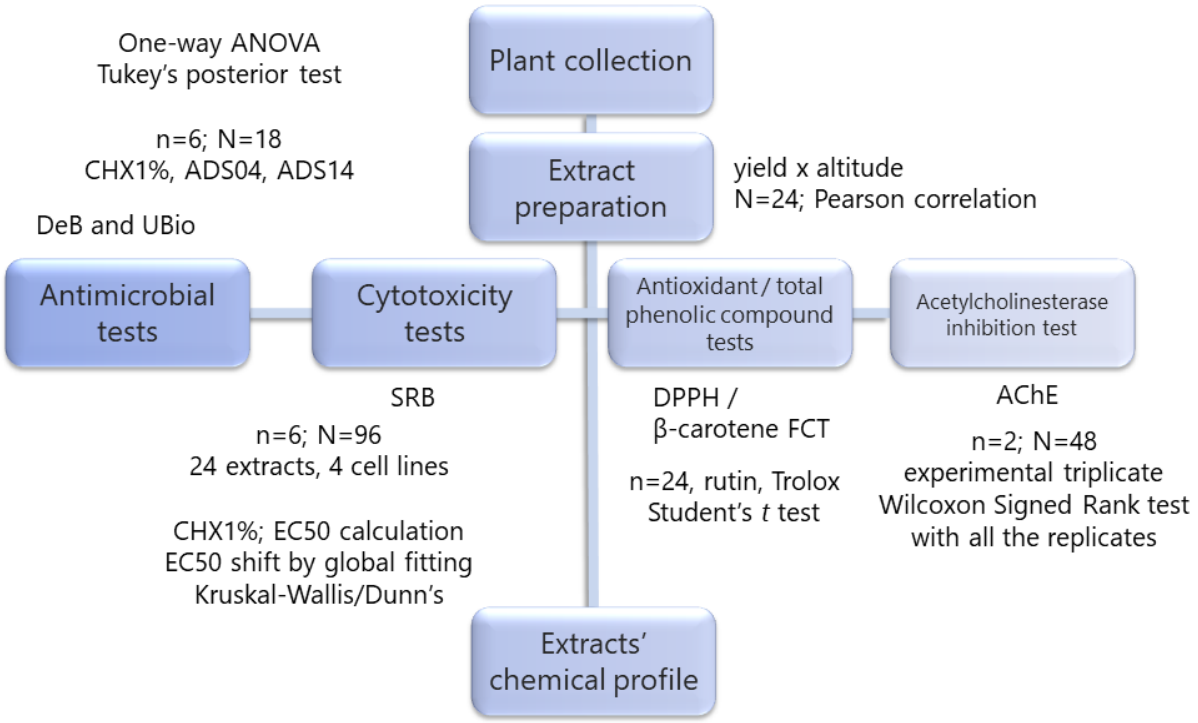

Figure 2. Experimental design developed for the present study.

test was used to access the β-carotene-test antioxidant activity 50% (AA50) and the DPPH-test radical scavenge activity 50% (ASRL50); AA50 data and ASRL50 data were compared to that obtained for rutin using the one-sample t-test (Duarte-Almeida et al. 2006); results from the Folin-Ciocalteu test, given in mg/g, were obtained by considering rutin and Trolox as reference phenolic compounds (Vasconcelos et al. 2017); acetylcholinesterase inhibition results were tested by a one-sample Wilcoxon signed rank test considering the theoretical median those obtained for physostigmine as reference substance (significance if $p<0.05$). More specifications for the statistical analyses can be assessed in the Supplementary Material.

RESULTS

Twenty-four samples were obtained from 13 sites located at different altitudes, relatively close to each other; extracts were obtained without considering the plant's previous sterilization. Collections were made without a prior pairing or altitudinal design. So, a Pearson correlation was assessed to infer the relationship between the extract yield (weight of dry extract/weight of crude plant material * 100) and altitude (given in meters), showing non-influence of terrain elevation on the samples (r=0.2401; p=0.2585). Figure 3a shows how the extract yield (weight of dry extract/weight of crude plant material * 100) and altitude (given in meters) are correlated, showing no dependence between the two variables (F=1.346; DFn, DFd=1, 22; p=0.2585; R squared=0.05764; slope=0.009465).

A general table with the bioprospection results (Table II) is given, i.e., data related to the diffusion in bioautography assay, cytotoxicity, total phenolic compounds, antioxidant and radical scavenge assays, and acetylcholinesterase inhibition assay.

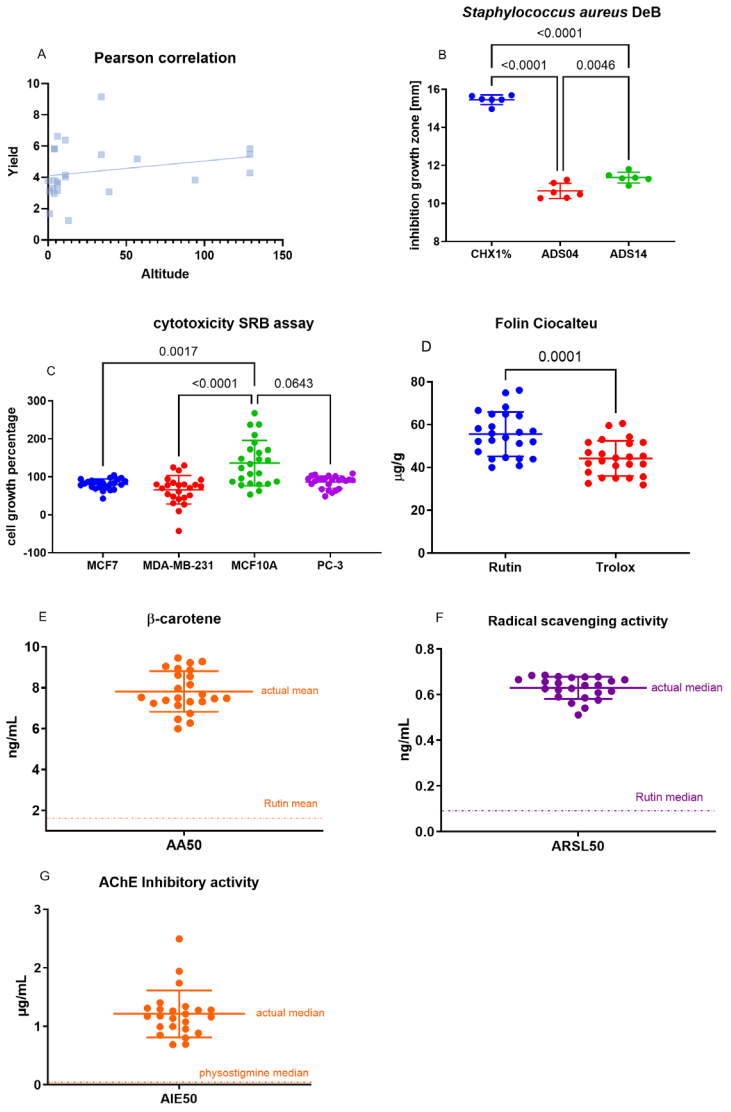

Figure 3: Statistical analyses. **A.** Pearson correlation analysis performed with the *Sanionia uncinata* extracts, yields, and altitudes related to the collection sites. Pearson r=0.2401; *p*=0.2585; Y=0.009465*X+4.104. **B.** Comparison of the growth inhibition zone diameters among 1% chlorhexidine, ADS04, and ADS14 against *Staphylococcus aureus* in the diffusion in bioautography assay. $F_{(2,15)}$=389.9. **C.** *Sanionia uncinata* extracts' cytotoxicity to MCF-7 and MDA-MB-231 breast cancer cell lines, MCF-10A normal breast cell line, and PC-3 prostate cancer cell line, expressed in 50% inhibition concentration (IC50), in µg/mL. Kruskal-Wallis and Dunn's post-test, obtained by comparing cell growth percentages for the different breast cells tested. K-S normality test=0.09058, 0.1252, 0.1334, 0.2161; breast cells: *p*>0.1000, prostate cancer cell: *p*=0.0051; K-$W_{(4,96)}$=25.55; p<0.0001. **D.** Comparison of the total phenolic content obtained to the 24 *Sanionia uncinata* extracts by the Folin Ciocalteu test based on rutin and Trolox (t=4.196; df=46; p<0.0001). **E.** *Sanionia uncinata* extracts 50% antioxidant activity obtained by the β-carotene method compared to rutin. One-sample t-test, considering 24 extracts (t=30.66; df=23; p<0.0001). **F.** *Sanionia uncinata* extracts' 50% free radical scavenging potential compared to rutin, performed in a single technical assay, in duplicates. Rutin was used as the control substance, and its 50% radical scavenging activity was used as the theoretical mean. Rutin median=0.093 ng/mL; extracts median=0.6410 ng/mL, *n*=24. W=0.9131; *p*=0.0412; WSRt=300.0 (300.0, 0.00); p<0.0001. **G.** Comparison of the extracts' acetylcholinesterase inhibitory activity with physostigmine by the Wilcoxon signed rank test (sum of signed rank=300.0; sum of positive ranks=300.0; sum of negative ranks=0.000; *p*<0.0001; rutin theoretical median=0.040 ng/mL; extracts median=0.6410 ng/mL). The physostigmine acetylcholinesterase inhibitory activity concentration was given as the theoretical median.

Bioautography analyses

Diffusion in bioautography (DeB)

Only *S. aureus* out of all the microorganisms used in the assay was sensitive to the extracts, and only the extracts ADS04 (Figure 4b) and ADS14 (Figure 4c) have shown growth inhibition activity against the bacteria (Figure 4a). Statistical differences were observed in the growth inhibition diameter zones obtained in the DeB for 1% chlorhexidine, ADS14, and ADS04 ($F_{(2,15)}$=389.9; p<0.0001; Figure 3b). A *posteriori* comparison revealed that 1% chlorhexidine samples were more active than the two plant extracts (15.45±0.26 mm *vs.* 10.66±0.41 mm and 11.37±0.28 mm; p<0.0001 for CHX1% *vs.* ADS04 and CHX1% *vs.* ADS14). Furthermore, significant differences were shown between the two

Table II. Data obtained from the biological assays for the 24 extracts obtained from *Sanionia uncinata*, Amblystegiaceae.

	DeB-inhibition zone diameter [mm]	Cytotoxicity MCF-10A [µg/mL]	Cytotoxicity MCF-7 [µg/mL]	Cytotoxicity MDA-MB-231 [µg/mL]	Cytotoxicity PC-3 [µg/mL]	Total phenolic compound rutin [mg/g]	Total phenolic compound Trolox [mg/g]	β-carotene [ng/mL]*	DPPH [ng/mL]	AChE [µg/mL]**
	mean±S.E.	IC50	IC50	IC50	IC50	individual result	individual result	mean±S.E.	mean±S.E.	median
ADS02	-	267.40	93.29	41.50	95.71	68.22	54.32	8.62	0.640	1.293
ADS04	10.66±0.17	209.82	68.83	27.54	80.59	56.41	44.94	9.04	0.650	0.800
ADS06	-	170.54	64.80	30.52	67.34	52.65	41.95	9.28	0.615	0.693
ADS08	-	123.00	97.68	94.02	102.80	52.25	41.64	9.45	0.621	0.850
ADS09	-	82.24	72.04	79.10	48.86	53.96	43.00	8.55	0.665	1.263
ADS10	-	95.83	90.44	69.79	68.64	52.05	41.48	8.94	0.678	1.077
ADS11	-	53.60	83.67	84.59	58.00	40.82	32.57	9.22	0.608	1.340
ADS12	-	80.18	82.17	79.70	63.60	58.18	46.34	8.87	0.541	1.280
ADS13	-	78.11	86.77	129.70	89.54	59.07	47.06	7.68	0.590	1.173
ADS14	11.37±0.11	237.77	42.91	116.90	63.47	64.92	51.70	7.24	0.626	1.170
ADS16	-	237.16	66.58	48.12	81.11	55.88	44.52	7.46	0.642	1.943
ADS18	-	143.27	62.32	78.14	92.42	64.15	51.09	8.15	0.627	1.273
ADS20	-	163.15	88.67	54.39	91.62	66.64	53.06	7.14	0.511	0.687
ADS21	-	145.72	78.20	61.31	94.91	40.00	31.92	7.32	0.576	1.213
ADS22	-	109.02	88.36	92.63	103.22	74.86	59.59	7.49	0.562	0.953
ADS24	-	87.31	104.12	74.88	101.64	44.65	35.61	7.31	0.586	0.997
ADS26	-	62.84	97.25	70.96	88.41	51.26	40.86	6.46	0.656	0.883
ADS28	-	87.61	87.33	80.23	91.22	43.89	35.00	5.99	0.679	1.160
ADS30	-	107.49	74.37	124.73	93.38	47.35	37.75	7.53	0.677	1.403
ADS32	-	133.96	72.52	-42.59	95.31	64.89	51.67	7.48	0.659	1.740
ADS34	-	147.78	81.08	51.64	94.66	56.98	45.39	6.27	0.666	1.137
ADS36	-	81.51	94.03	78.10	108.99	44.07	35.15	6.74	0.675	1.310
ADS38	-	190.18	83.19	9.71	91.76	76.11	60.58	7.39	0.684	0.993
ADS40	-	172.60	79.33	44.68	105.82	43.90	35.01	7.96	0.685	2.497

(-) no inhibition growth; * mean±S.E. of two independent experiments; **median of three independent experiments.

extracts (p=0.0046) despite their closest mean values (ADS04 *vs.* ADS14).

Unidimensional bioautography (UBio)

The UBio results for 1% chlorhexidine (Figure 4d) and ADS14 and ADS04 (Figure 4e) showed the growth inhibition zones in the application point and the solvent line, respectively, for the control substances and the extracts. The U.V. 365 nm visualization of the extracts' chromatograms (Figure 4f) indicates that the growth inhibition zones coincide with the blue compounds observed at Rf 0.66, which may indicate the presence of phenolic acids yet to be thoroughly determined.

Cytotoxicity against breast and prostate cancer cell lines

Sanionia extracts were evaluated for cytotoxicity against breast and prostate cancer cells, and the results were expressed as cell growth percentage (%) medians. Figure 3c shows the treatments that resulted in susceptibility differences among the cancer cell lines when compared to the normal cell line ($X^2_{(4, 96)}$=25.55; p<0.0001). MCF-10A was less susceptible to the treatments' cytotoxicity [128.5

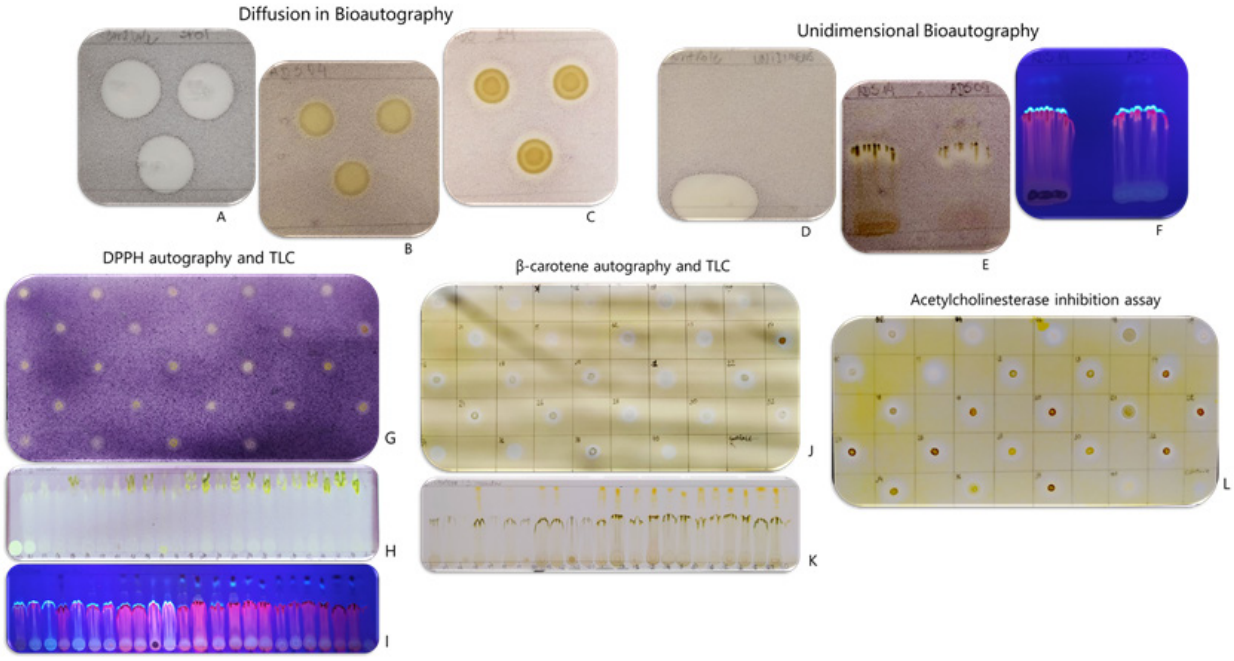

Figure 4. Thin-layer chromatography-based assays. Diffusion in bioautography analysis for A. 1% Chlorhexidine; B. *Sanonia uncinata* extract ADS04; C. *Sanionia uncinata* extract ADS14. Unidimensional bioautography assay Chromatograms for D. 1% chlorhexidine; E. *Sanionia uncinata* extracts ADS14 and ADS04; F. *Sanionia uncinata* extracts ADS14 and ADS04 chromatograms visualized under 365 nm U.V. light; G. Diphenyl-picryl-hydrazyl radical scavenger autography; each spot represents an extract; H. Diphenyl-picryl-hydrazyl radical scavenger evaluation after thin layer chromatography development; each spot represents an extract. Rutin was used as control; I. Thin layer chromatography development of *Sanionia uncinata* extracts revealed under U.V. light at 365 nm. Rutin was used as control; J. β-carotene antioxidant evaluation where each spot represents one *Sanionia uncinata* extract. Rutin was used as control; K. Thin layer chromatography development of *Sanionia uncinata* extracts revealed with β-carotene. Rutin was used as control; L. Acetylcholinesterase inhibition activity by extracts from *Sanionia uncinata*. Chromatogram resulting from autography performed to evaluate the inhibition potential of the enzyme acetylcholinesterase of *Sanionia uncinata* extracts by the Ellman test. Physostigmine was used as a control.

(53.6, 267.4)] in comparison to MCF-7 [82.68 (42.91, 104,1); p=0.0017] and MDA-MB-231 [72.92 (-42.59, 129.7); p<0.0001], but no statistical differences between the normal cell and the prostate cancer cell line [91.69 (48.86, 109.0); p=0.0643].

Total phenolic compounds by the Folin-Ciocalteu method

Quantification measures of total phenolic compounds in *S. uncinata* extracts were calculated using the Folin-Ciocalteu method based on rutin and Trolox. Rutin group (W=0.9578; p=0.3955) and Trolox group (W=0.9577; p=0.3948). Results, displayed as mean±standard deviation, showed that there is a difference in the phenolic compound amount in the extracts when calculated based on rutin (55.55±10.32 µg/g) or Trolox (44.26±8.19 µg/g) and that the methodology using rutin resulted in higher values (t=4.196, df=46, p=0.0001; Figure 3d).

Antioxidant activity of the extracts

The antioxidant activity of the extracts was assessed by methods that combined visual (qualitative; Figure 4l) and metric (quantitative; Figure 3g) evaluations, as shown in Figure 4.

β-carotene test in autography

Figure 4l displays the antioxidant activity results obtained from the β-carotene test in autography. The extract ADS14 has shown the most evident antioxidant activity, visualized by an orange spot on the extract's application point. ADS12, ADS16, ADS18, ADS20, ADS22, ADS24, ADS26, ADS32, and ADS38 have also shown a slight antioxidant potential, observed by less intense orange spots. Thus, figure 4h shows the TLC chromatogram obtained for the extracts visualized under 354 nm U.V. light, where blueish spots are observed in the solvent line, suggesting the presence of phenolic compounds. Lastly, figure 4i shows the same chromatogram visualized after spraying with β-carotene/linoleic acid reagents, revealing that the active orange spots observed in the chromatogram solvent line coincide with the blueish spots previously observed, suggesting the relationship between the phenolic acids and the antioxidant activity.

β-carotene test to obtain antioxidant activity 50%

The one-sample t-test calculation accessed the antioxidant activity (AA) of the extracts and rutin. All the extracts proved to present an extremely significant higher 50% AA concerning that observed for rutin (t=30.66; df=23; p<0.0001; rutin mean=1.61 ng/mL; extracts mean=7.82 ng/mL) (Figure 3e).

DPPH in autography

the plant extracts showed radical scavenging activity in the drop autography assay, as seen in Figure 4j, and observed by the presence of whitish spots in contrast to the chromatogram's purple background color, which is characteristic of the DPPH. In Figure 4k, the extracts were eluted and visualized under natural light after spraying with DPPH solution. It is possible to observe the whitish spots in the chromatogram, particularly in the solvent line and in the application point.

DPPH test to obtain free radical scavenging activity 50%

The free radical scavenging potential (ASRL50) of the *S. uncinata* extracts is shown in Figure 3f. All the extracts presented an extremely significant higher 50% AA compared to that observed for rutin (rutin median=0.093 ng/mL; extracts median=0.6410 ng/mL; p<0.0001).

Enzymatic inhibitory activity on acetylcholinesterase of the extracts

All 24 *Sanionia uncinata* extracts evaluated had shown the capacity to inhibit the enzyme

acetylcholinesterase, as shown in Figure 4l. The autography analysis for the plant extracts using the Ellman test revealed the presence of the typical characteristic whitish spot related to enzymatic inhibition. The extracts ADS4, ADS6, ADS11, and AD40 have remarkably inhibited the enzyme activity, and the presence of the whitish spots was easily observed, suggesting a potentiality for the inhibitory activity. A comparison with physostigmine results corroborates the potential capability of the extracts to inhibit acetylcholinesterase. Statistical analysis has revealed that the plant extracts show a potential enzyme inhibitory activity significantly higher than the reference substance (physostigmine median=0.040 nm/mL; extracts median=1.172; $p<0.0001$). These results are reported in the Figure 3g.

The results obtained from the autography analysis for the plant extracts of *S. uncinata* can be seen in Figure 4l. All extracts showed the characteristic whitish spot related to the occurrence of enzymatic inhibition, and the extracts ADS4, ADS6, ADS11, and ADS40 showed a more significant potential for enzymatic inhibition. Figure 3g shows the potential of acetylcholinesterase inhibition activity of the extracts in comparison to that obtained for physostigmine, which was used as the theoretical median in the Wilcoxon signed-rank assay (sum of signed rank=300.0; sum of positive ranks=300.0; sum of negative ranks=0.000; $p<0.0001$; rutin theoretical median= 0.040 ng/mL; extracts median=0.6410 ng/mL). The group did not pass the Shapiro-Wilk normality test (W=0.8637; $p=0.0040$).

DISCUSSION

The present work aimed to add information about the pharmacological potential of the ethanolic extracts from *S. uncinata* collected in Antarctica. It is essential to highlight that fungi have coevolved with terrestrial plants, including bryophytes, approximately 400 million years ago in the early Devonian before roots had evolved (Brundrett 2002), meaning that a balanced association allowed the plants to share limited sources of energy and nutrients, whose mechanisms were already studied for other species (Khalid et al. 2019). The mutualistic relationship between bryophytes and endophytic fungi is observed in biochemical coregulation, nutrient exchange and defense, resulting in the capability of fungi and plants to produce natural products strategically involved in the organisms' adaptation (Stelmasiewicz et al. 2023). Even with the focus of the present work, there is a need for more information regarding the study of the endophytic fungi composition of *S. uncinata*, a study yet to be developed.

Despite the outstanding but demanding logistics of collecting the plant and determining the extraction procedure to be adopted, the amount of extract was enough to allow for biological and chemical testing without harming the local population. According to the Pearson correlation analysis performed, extract yields showed variation among the 24 samples, spanning from 1.24 to 9.15%, with no dependence on the altitude from which the plant sample occurred.

The small amount of extract has limited the range of biological assays performed in the current work, and some of the approaches had to be redirected accordingly, as described in the experimental design. Particularly concerning the antimicrobial assays, the diffusion in bioautography test (Suffredini et al. 2023) was adopted rather than using the usual sensibility tests of microdilution broth or disk diffusion assays due to the need for a lesser extract amount and to a better analysis regarding the extract fractions. Based on DeB, the bryophyte

extracts ADS04 and ADS14 showed antimicrobial activity against *Staphylococcus aureus*.

S. aureus is an important pathogen involved in skin diseases and developing aggravation of this condition. Also, it can provoke lung infections, sepsis, and human death (Cheung et al. 2021). Some strains are resistant to antibiotics and are considered a significant threat to humankind (WHO 2018). It is also related to veterinary diseases such as bovine mastitis (Pérez et al. 2020), bringing economic concerns to dairy farmers. Although the antimicrobial resistance of *S. aureus* continues to evolve, the mortality from bacteremia caused by this bacterium has decreased over the last three decades mainly due to the rise of methicillin-susceptible strains over the severe methicillin-resistant ones (Cassat & Thomsen 2021, Bai et al. 2022), whose infection considerably reduces life expectancy. For this reason, the data obtained in the present study are relevant since two of the 24 extracts at least unveiled activity against the microorganism, although a standard ATCC® strain.

The parameters employed to compare the antimicrobial potential of plant extracts are diffuse and without a straight consensus (Christchellyn et al. 2020). For bryophytes, the parameters are yet more insipid. On this matter, some authors (Basile et al. 1999) reported the antibacterial activity against *Proteus mirabilis, P. vulgaris, P. aeruginosa, Salmonella typhi, Enterobacter aerogenes, E. cloaca,* and *Klebsiella pneumoniae*, observed for apigenin, apigenin-7-O-triglycoside, barthramiaflavone, lucenin-2, luteolin-7-O-neohesperidoside, saponin, and vitexin, isolated from species of mosses such as *Bartramia pomiformis* Hedw. (Bartramiaceae), *Dicranum scoparium* Hedw. (Dicranaceae), *Plagiomnium affine* (Blandow ex Funck) T.J Kop. and *P. cuspidatum* (Hedw.) T.J. Kop. (Mniaceae), and *Hedwigia ciliata* (Hedw.) P. Beauv. (Hedwigiaceae). Notwithstanding, they observed that the natural products were more active against the Gram-positive bacteria such as *S. aureus* and *Enterococcus faecalis*. The findings described in the present work are supported by those previous results.

When studying extracts of *Orthostichella rigida* (Müll. Hal.) B.H. Allen & Magill, Neckeraceae, an ombrophilous species from Southeastern Brazil, Christchellyn et al. (2020) observed a significant anti-*Staphylococcus aureus* activity. In turn, other researchers (Novakovic et al. 2021) verified the phytochemical potential and antimicrobial, antiviral, anti-inflammatory, and cytotoxic activities of sesquiterpenes from plants belonging to the cosmopolitan genera *Fissidens* (Fissidentaceae) and *Rhodobryum* (Bryaceae), as well as liverworts.

Sanionia uncinata ethanolic extracts were evaluated as cytotoxic agents aiming to prospect new potential anticancer drugs. The isolated cytotoxic molecule must present an inhibitory concentration of 50% (IC50) lower than ten µM or between 4 and 5 µg/mL (Suffness & Douros 1982). Present findings showed that extract ADS32 showed a significant level of 42.59% lethality to MDA-MB-231, a metastatic breast cancer cell line. Nonetheless, extract ADS04 inhibited metastatic cell growth by 72.46%, and ADS14 inhibited the growth of MCF-7 non-metastatic breast carcinoma by 57.09%. Interestingly, ADS04 and ADS14 stimulated the growth of MCF-10A, a normal breast cell line, by 209.82% and 237.77%, respectively. Also, ADS04 is 7.62 times more toxic to the metastatic cell than to the normal cell, while ADS14 is 2.03 times more toxic to the non-metastatic carcinoma cell line than to the normal cell line.

Plants living under extreme environmental conditions, such as those found in Antarctica, may present particularities related to the biosynthesis of secondary metabolites, resulting in chemical

diversification (Liu et al. 2013). Sanionins A and B from *Sanionia georgico-uncinata* (Müll. Hal.) Ochyra & Hedenäs (Amblystegiaceae) were reported to be slightly antibacterial (Ivanova et al. 2007) and cytotoxic, in which sanionin A was more active than sanionin B.

Sanionia uncinata extracts were also evaluated as antioxidant agents. Antioxidants can be defined as substances that delay the oxidation rate (Pietta 2000) of proteins, enzymes, lipoproteins, and other vital molecules, and the identification of such compounds may be crucial to introducing new chemical tools to be used in the prevention of chronic degenerative diseases (Duarte-Almeida et al. 2006). Several laboratory techniques have been developed to track the antioxidant capacity of natural and synthetic molecules in both *in vivo* and *in vitro* models. A broad and critical review of these models can be assessed elsewhere (Alves et al. 2010), including DPPH and β-carotene/linoleic acid co-oxidation techniques used in the present study. In the present work, autograph experiments with β-carotene showed the antioxidant activity of the *S. uncinata* extracts, which confirmed the potential to be used as antioxidant agents, as well as showed that phenolic compounds are related to an adaptative capability of the plants to that particular extreme environment.

The DPPH method is based on the capacity of an antioxidant molecule to scavenge the DPPH radical, which is purple-colored and, after reduction to hydrazine, becomes uncolored. The color decay can be observed in a spectrophotometer in visible light (Alves et al. 2010) at 520-540 nm. The DPPH radical scavenging evaluation was done in autography and in solution. All the extracts showed the capacity of scavenging radical DPPH in autography. In solution, the extracts have also shown a significant radical scavenging activity, but ADS02. Previous reports described that *S. uncinata* ethanolic and methanolic extracts containing flavonoids showed radical scavenging activity (Bhattarai et al. 2008, Fernandes et al. 2017, 2019), and that the compounds had the potential to be used in photoprotection, medicinal and cosmetics purposes. The present findings are in accordance with the available information.

To support these results, the total phenolic compound amount was analyzed using the Folin-Ciocalteu test for each extract, and results showed that all the extracts presented significant amounts of phenolic compounds, whose presence is likely related to their antioxidant capacity. Antioxidant phenolic compounds such as sanionins A and B have already been reported for *S. georgicouncinata* (Müll. Hal.) Ochyra & Hedenäs, a species also occurring in the Antarctic Continent and closely related to *S. uncinata* (Ivanova et al. 2007).

Alzheimer's disease (AD) is characterized by memory loss and learning difficulties in older adults, although young-onset dementia is diagnosed in people under 65 (Loi et al. 2023). According to the cholinergic hypothesis, acetylcholine, a neurotransmitter, declines in the synapse of neuronal cells due to a reduction in its cell biosynthesis. Also, the involvement of the acetylcholinesterase enzyme, which further stimulates the decline, is caused by the hydrolytic degradation of acetylcholine to acetic acid and choline in the cholinergic-type synapses (Saxena & Dubey 2019, David et al. 2021), which in turn increases the acetylcholine deficit and lead to the termination of synaptic transmissions in the brain. There is no treatment for AD, but drugs that indirectly inhibit acetylcholinesterase also inhibit the synapse's acetylcholinesterase decline. These medications are palliative and help to control symptoms. However, they do not prevent the progression of the disease. The drugs of choice for those cases are galantamine, rivastigmine, donepezil and derivatives, and

memantine, among others (Sharma 2018, Moreta et al. 2021, Poudel & Park 2022), and monoclonal antibodies such as aducanumab (Poudel & Park 2022). Physostigmine is also used for AD but is now in decline due to the better effectiveness of other drugs. This alkaloid was first isolated from *Physostigma poisonum* Balf. (Fabaceae), the Calabar bean, and was first synthesized in 1935 by Percy Julian and Joseph Pick (Batiha et al. 2020). In the present work, it was used as the reference substance in autography studies that were carried out. Regarding the inhibitory activity over acetylcholinesterase, *S. uncinata* extracts ADS06 and ADS20 showed the best AChE activity, although ADS04 can also be considered a promising candidate.

Several bryophytes are used in Traditional Chinese Medicine and are among the most exploited natural medicines for treating urinary tract infections, respiratory diseases, and neurological disorders (Asakawa, 1995). Studies with extracts obtained from the liverwort *Marchantia polymorpha* L. (Marchantiaceae) and *Gymnomitrion alpinum* (Gottsche ex Husn.) Schiffn. (Gymnomitriaceae) exhibit acetylcholinesterase inhibition (Asakawa et al. 2013) and may demonstrate neuroprotective properties, thus supporting the activities found in acetylcholinesterase autography tests with *S. uncinata* extracts.

Sanionia uncinata extracts ADS04 and ADS14 were shown to be a promising source of lead compounds to be used against *Staphylococcus aureus*, one of the most important human and veterinary pathogens. They also have shown cytotoxicity against non-metastatic and metastatic carcinoma breast cell lines and no cytotoxicity to a normal breast cell line. They also have the potential to inhibit the activity of the enzyme acetylcholinesterase. Present findings also have shown a significant variation in the biological activities of the extracts, depending on the area they were collected. Finally, mosses are a source of natural substances with significant pharmacological interest, which little is known. Also, the significant biological activities found in the *S. uncinata* extracts support further studies on the occurrence of bryophyte's endophytic fungi and their natural product biological activities once little is known about fungi mutualism in bryophytes. As a result, present data suggest *S. uncinata* as a potential source of new active molecules to be used as medicines.

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