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Immunomodulating polyphenols from *Sideritis scardica*

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ABSTRACT

The herb *Sideritis scardica* is used in traditional medicine in the Balkan Peninsula. Limited information exists on its health effects, such as improvement of cognitive functions. This study explores the *in vitro* anti-inflammatory effects of extracts and polyphenols of *S. scardica* with focus on neurodegenerative disease. BuOH, DCM and EtOAc fractions were isolated from the crude EtOH extract of aerial parts of *S. scardica* and further purified by chromatographic methods. Eleven phenolic compounds were isolated. The extracts and compounds were assessed for immunomodulatory, enzyme-interacting and cytotoxic properties. The crude extract, EtOAc fraction and precipitate, BuOH fraction, verbascoside and acetylallosylglucosyl-isoscutellarein 4′-methylether displayed DPPH scavenging activity. The EtOAc fraction and echinaticin displayed XO inhibiting activity. The DCM fraction reduced nitric oxide production in D2SC/1 dendritic cells and demonstrated potent activity in a human complement assay. Verbascoside and chlorogenic acid lowered NO levels without affecting cell viability in D2SC/1 dendritic cells. The results reveal a broad-spectrum activity of this herb, which might potentially serve as a multi-target system in Alzheimer's disease.

1. Introduction

Sideritis scardica Griseb. (Lamiaceae), a hairy perennial shrub with yellow flowers blooming on the stem, known as "tea of longevity", grows on rocky ground at high altitude and has been used in traditional medicine in Eastern Europe since ancient times against a wide spectrum of illnesses and diseases such as common cold, bronchitis, kidney disease and gastrointestinal disorders (Chinou, 2015). Recently, *S. scardica* has become increasingly popular in the EU (European Union), where the herb is available as food supplement marketed as herbal tea prepared by decoction or infusion, commonly known as Mountain tea, Greek mountain tea, Pirinski tea, Ironwort and Shepherd's tea depending on the area. In Greece, Bulgaria, Albania and the Republic of North Macedonia, the fragrant herb is used in the local cuisine for its aromatic properties and consumed as herbal tea (Chinou, 2015; Todorova & Trendafilova, 2014). Anti-inflammatory, cognitive enhancing and triple monoamine reuptake inhibitory effects of extracts and isolated compounds have been reported, likely attributed to the content of phenolic compounds and terpenoids (Knörle, 2012). One study has revealed anti-inflammatory and gastroprotective activities in rats, as well as cytotoxic effects to the tumor cell lines, HL60 and PBMC, of extracts and flavones obtained from *S. scardica* (Tadić et al., 2012). Recently, promising results from studies on ovarieoctomized rats have been been reported for improvement of glucose tolerance and triglyceride levels, as well as antioxidative effects of a 70 % aqueous ethanolic extract of *S. scardica*, possibly mediated through AMPK activation (Jeremic et al., 2019). The chemistry of *S. scardica* has been reported in numerous investigations (Żyżelewicz et al., 2020). However, there is limited information on the biological profile of extracts and compounds from *S. scardica* (Chinou, 2015). Recently, herbal tea made from *S. scardica* was approved by the European Medicines Agency (EMA) as a traditionally used herbal medicinal product (THMP) on the basis of its longstanding use for the relief of cough associated with cold and relief of mild gastrointestinal discomfort (European Medicines Agency, 2015). Thus, further research is needed in order to fill the knowledge gap between the information on traditional use and the potentially multifaceted effects and underlying mechanisms of bioactive compounds of *S. scardica*. This study aims to phytochemically screen a variety of extracts and explore the *in vitro* anti-

Abbreviations: 15-LOX, 15-lipoxygenase; AChE, acetylcholinesterase; AD, Alzheimer's disease; AMPK, 5′adenosine monophosphate-activated protein kinase; BuChE, butyrylcholinesterase; BuOH, *n*-butanol; DCs, Dendritic cells; DCM, dichloromethane; DPPH, diphenylpicrylhydrazyl; EtOAc, ethyl acetate; EtOH, ethanol; IFNγ, interferon-gamma; LPS, lipopolysaccharide; ROS, reactive oxygen species; TFA, Trifluoroacetic acid; XO, xanthine oxidase.

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inflammatory effects of extracts and isolated polyphenols of *S. scardica* with emphasis on the neurodegenerative disease Alzheimer's disease (AD) in order to discover new bioactive compounds that may contribute to improvement of the global health challenges related to AD.

2. Materials and methods

2.1. General experimental procedures

1D and 2D nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVII 400, AVIII 400 or AVII 600 instrument (Bruker, Rheinstetten, Germany), using CD_3OD or $CDCl_3$ (Sigma-Aldrich, St. Louis, MO, USA) as solvents with tetramethylsilane (TMS) as reference. Column chromatography (CC) was performed with a VersaFlash chromatography system (Supelco, Bellefonte, PA, USA) with VersaPak C18 (4 cm × 15 cm and 2.3 cm × 11 cm, 45–75 μm) cartridges (Sigma–Aldrich), or with laboratory-packed columns containing Sephadex LH-20 gel (Pharmacia, Uppsala, Sweden). Fractions from CC were combined as indicated by thin-layer chromatography (TLC). Analytical TLC was performed using reverse phase silica gel, 60 RP-18 F_{254S}, 0.2 mm thickness on aluminium foils (Merck, Darmstadt, Germany), and spots were visualized by UV irradiation (254 and 366 nm) by spraying with $Ce(SO_4)_2$ (1 % in 10 % aqueous H_2SO_4) followed by heating (105 °C, 5 min) or by spraying with diphenylpicrylhydrazyl (DPPH) (0.04 % (w/v) solution in MeOH). Preparative HPLC was carried out on a ProStar Polaris system (Varian, Palo Alto, CA, USA) equipped with a Kinetex C18 100A (150 mm × 21.2 mm, 5 μm) column (Phenomenex, Torrance, CA, USA) with a mobile phase gradient of water (0.1 % TFA) (A) and methanol (B): 20–80 % B, 0–20 min; 80 % B, 20–25 min, 320 nm UV detection. Solvents were removed on a rotary evaporator (IKA model RV10/HB10, Staufen, Germany) followed by oil pump (Edwards E-LAB2 High Vacuum, Crawley, England) and/or a miVac vacuum centrifuge (Genevac, Ipswich, England).

2.2. Plant material

Aerial parts of *Sideritis scardica* Griseb. were obtained from Greuther Teeladen GmbH & Co. KG, Vestenbergsgreuth, Germany; "Griechischer Bergtee" charge nummer 121402843. The plant name is in accordance with https://powo.science.kew.org (checked 12.05.2022). A voucher sample is kept in the Section of Pharmacognosy, Department of Pharmacy, University of Oslo (registry number SS-A-444).

2.3. Extraction and isolation of compounds

The dried herbal material was powdered in a RAW Pro X1500 blender, then the powdered herb (400 g) was mixed with diatomaceous earth (Dionex, Sunnyvale, CA, USA) (4:1 v/v), loaded in 100-mL steel cartridges and extracted on an Accelerated Solvent Extraction system (ASE 350; Dionex) with 80 % ethanol (EtOH). Preheating time was 5–7 min, static extraction per cycle was 5 min, and the extraction was carried out at 60° C under a pressure of ca 1600 PSI. The extraction was performed three times. The extract was dried on a rotavapor. A dark green friable mass, 87 g (22 %) was obtained.

The EtOH extract (84 g) was suspended in 400 mL distilled water and partitioned successively with 4 × 200 mL dichloromethane (DCM), 4 × 200 mL ethyl acetate (EtOAC) and 4 × 200 mL butanol (BuOH), yielding 9.9 g (11.8 %) of DCM fraction, 3 g (3.6 %) of EtOAc fraction, 21.3 g (25.4 %) of BuOH fraction and 42 g (50 %) of an aqueous residue.

The EtOAc extract (1.4 g) was applied to a Sephadex LH20 column (4.5 × 20 cm) and eluted with a stepwise gradient of methanol-H_2O (2:3, 324 mL; 3:2, 140 mL; 1:0, 235 mL) and acetone-H_2O (7:3, 250 mL) to yield 9 fractions (EtOAc S1-S9). EtOAc S4 (169 mg) was purified on a VersaPak C18 cartridge (2.3 × 11 cm) with methanol-H_2O (1:4, 90 mL; 2:3, 120 mL; 3:2, 90 mL; 7:3, 90 mL; 4:1, 120; 1:0, 150 mL) to give ten subfractions (EtOAc S4V1-V10). EtOAc S4V4 (42 mg) and EtOAc S4V8 (22 mg) were purified by preparative HPLC to obtain compound **5** (EtOAc S4V8H8, 5.3 mg), compound **7** (EtOAc S4V8H5 + EtOAc S4V8H9-10, 1.5 mg + 2 mg + 1.5 mg) and compound **8** (EtOAc S4V4H4, 6.2 mg). EtOAc S9 (21.9 mg) was identified as compound **3**. All compounds were identified by ^1H, ^{13}C and 2D NMR spectroscopy.

The BuOH extract (10.0 g) was applied to a Sephadex LH20 column (4.5 × 30 cm) and eluted with a stepwise gradient of methanol-H_2O (1:4, 1140 mL; 3:7, 400 mL; 2:3, 520 mL; 1:1, 175 mL; 7:3, 450 mL; 1:0, 375 mL) and acetone-H_2O (7:3, 450 mL) to yield 7 fractions (BuOH S1-S7). BuOH S5 (1240 mg) was applied on a VersaPak C18 cartridge (4 × 15 cm) and eluted with a stepwise gradient of methanol-H_2O (1:9, 60 mL; 1:4, 60 mL; 2:3, 150 mL; 3:2, 180 mL; 7:3, 180 mL; 1:0, 180 mL) to give 10 subfractions (BuOH S5V1-10). BuOH S5V1 (19 mg) was identified as compound **2** and S5V4 (508 mg) as compound **1** as described above. BuOH S5V8 (88 mg) was reapplied on a VersaPak C18 cartridge, rechromatographed with MeOH-H_2O (1:1, 210 mL; 3:2, 210 mL; 7:3, 150 mL; 1:0, 120 mL) as mobile phase which yielded 5 fractions (BuOH S5V8V1-5). BuOH S5V8V2 (63 mg) was purified with preparative HPLC and yielded compound **4** (BuOH S5V8V2H2 + H5, 14.5 mg) and compound **6** (BuOH S5V8V2H3, 4 mg), compound **9** (BuOH S5V8V2H6H1, acetylallosyl-glucosyl)-4'-O-methylhypolaetin, 3.1 mg), compound **10** (BuOH S5V9H8, acetylallosyl-acetylglucosyl)-4′-O-methylhypolaetin) and compound **11** (BuOH S5V8V2H2, allosyl-6-O-acetylglucosyl)-isoscutellarein), identified as above. Further investigations of compounds **9**–**11** in the different bioassays were not performed due to low yields.

2.4. Bioassays

2.4.1. Inhibition of xanthine oxidase (XO)

Assay for inhibition of xanthine oxidase (XO) was performed as previously described (Bräunlich et al., 2013; Wold et al., 2020). The enzyme inhibition was calculated by measuring the increase in absorbance at 290 nm over a period of 5 min relative to the untreated enzyme control. Quercetin was used as a positive control. The experiment was performed once with four technical replicates for each concentration.

2.4.2. Inhibition of 15-lipoxygenase (15-LOX)

The inhibitory activity towards 15-lipoxygenase (from soybean, Sigma) was investigated according to the method previously described (Bräunlich et al., 2013). The enzyme inhibition was calculated by measuring the increase in absorbance at 234 nm (A_{234}) from 30 to 90 s after addition of enzyme relative to the untreated enzyme control. Quercetin was used as a positive control. The experiment was conducted once with three technical replicates for each concentration.

2.4.3. Inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChe)

The inhibitory activity of extracts and isolated compounds on acetylcholinesterase (from *Electrophorus electricus*, Sigma) and butyrylcholinesterase (from equine serum, Sigma) was investigated according to a modified Ellman's method (Wold et al., 2020). The enzyme inhibition was calculated by measuring the absorbance at 415 nm after 6 min relative to the untreated enzyme control. Tacrine was used as a positive control. The experiment was conducted once with technical triplicates for each concentration.

2.5. DPPH radical scavenging

Scavenging activity towards the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was measured as described previously (Wold et al., 2020) using UV-transparent 96-well plates (Corning, NY, USA). Percentage radical scavenging activity was calculated by comparing the absorbance of samples at 517 nm after 5 min to untreated DPPH control. Quercetin was used as a positive control. The experiment was conducted once with four technical replicates for each concentration.

2.6. Complement fixation assay

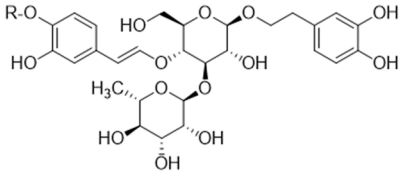
The complement fixation assay is based on inhibition of hemolysis of antibody sensitized sheep red blood cells (SRBC) by human sera, as described previously (Michaelsen et al., 2000). Percentage inhibition of hemolysis was calculated by measuring the absorbance at 415 nm by comparing with the untreated controls that indicated ~50 % hemolysis. As a positive control, a pectic polysaccharide from *Biophytum umbraculum* (syn. *B. petersianum*) (BPII) was used (Inngjerdingen et al., 2006). The assay was performed in triplicate and repeated three times for each concentration.

2.7. NO-reduction of LPS + IFNγ-activated dendritic cells

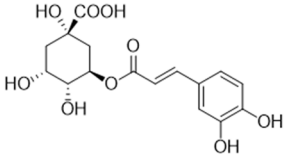
The extracts and isolated compounds were investigated by measuring the inhibition of nitric oxide (NO) release from D2SC/1 dendritic cells, induced by lipopolysaccharide (LPS) and the co-stimulator interferon gamma (IFNγ). The colorimetric assay was performed using the Griess reagent as previously described (Ho et al., 2017), with minor modifications. Dendritic cells were cultured in 96-wells plates at a density of 5×10^4/well and pre-incubated with samples for one hour (37 °C, 5 % CO_2) prior to addition of LPS (500 ng/mL, from *Escherichia coli* O55:B5, Sigma) and murine IFNγ (20 ng/mL, Peprotech, Cranbury, NJ, USA). After 24 h of incubation, Griess reagents A and B were added to the cells. The levels of nitrite in the cell supernatants were calculated by measuring the absorbance at 540 nm (A_{540}). Inhibition of NO-release was expressed as the percentage decrease in NO levels compared to the LPS + IFNγ (containing 0.5 % v/v DMSO) treated samples. Quercetin was used as a positive control. The assay was carried out in three independent experiments.

2.8. Cell viability assay

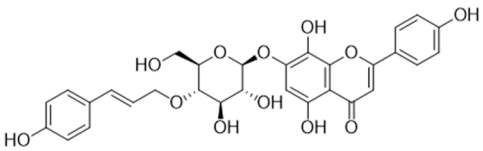
Cell viability was measured by the formation of formazan salt in the cell media (after 24 h), utilizing the MTT Assay (Cell Proliferation Kit I, Protocol 2016, Roche, Basel, Switzerland) according to the manufacturers protocol. As a control for complete reduction in cell viability, DMSO (20 % v/v) was applied. The results were calculated by subtracting A_{690} from A_{570} values and comparing to the untreated control (containing LPS, IFNγ and 0.5 % v/v DMSO).



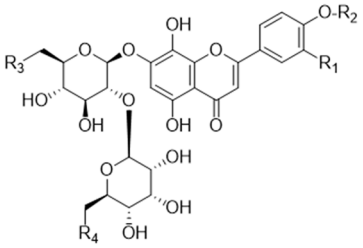
R = H, Verbascoside (**1**)
R = CH_3, Leucoseptoside A (**8**)



Chlorogenic acid (**2**)



Echinaticin (**3**)



(**4**), (**5**), (**6**), (**7**), (**8**), (**9**), (**10**) or (**11**)

Compound	R_1	R_2	R_3	R_4
Acetylallosylglucosyl-isoscutellarein (**4**)	H	H	OH	$OCOCH_3$
Acetylallosylglucosyl-isoscutellarein-4′-methylether (**5**)	H	CH_3	OH	$OCOCH_3$
Acetylallosyl-glucosyl-hypolaetin (**6**)	OH	H	OH	$OCOCH_3$
Acetylallosyl-acetylglucosyl-isoscutellarein-4′-methylether (**7**)	H	CH_3	$OCOCH_3$	$OCOCH_3$
7-*O*-(Acetylallosyl-glucosyl)-4`-*O*-methylhypolaetin (**9**)	OH	CH_3	OH	$OCOCH_3$
7-O-(Acetylallosyl-acetylglucosyl)-4′-*O*-methylhypolaetin (**10**)	OH	CH_3	$OCOCH_3$	$OCOCH_3$
7-*O*-(Allosyl-6-*O*-acetylglucosyl)-isoscutellarein (**11**)	H	H	$OCOCH_3$	OH

Fig. 1. Structure of compounds **1**–**11**.

2.9. Statistics

GraphPad Prism 9 software was used in order to conduct statistical analysis. If not stated otherwise, the values are given as means ± SD of at least three separate experiments. Each individual sample value was compared to the values from control samples. Comparison of multiple samples were analyzed by performing one-way ANOVA, followed by Dunnett's test.

3. Results and discussion

3.1. Isolation of compounds

In this work, eleven polyphenolic compounds were isolated from *S. scardica* and identified, see Fig. 1. These compounds have previously been reported from the plant, but knowledge of their biological activities is limited.

3.2. Inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE)

Cognitive impairment is associated with anticholinergic effects, and blockade of cholinergic transmission may lead to the development of cognitive decline (Campbell et al., 2009). Studies today are actively searching for compounds acting as new drug candidates that interact with brain biotargets, such as cholinesterase inhibitors acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are reported to catalyze the hydrolysis of brain acetylcholine, and reduced level of the neurotransmitter acetylcholine is observed in Alzheimer's disease (Giacobini, 2003). Also, AChE and BuChe have been linked to the formation of amyloid beta (Aβ) fibrils (Mushtaq et al., 2014). In an *in vivo* Alzheimer mice model, extracts of *Sideritis spp* have shown to reduce intracerebral levels of Aβ and improve cognitive functions (Hofrichter et al., 2016). Previously, *S. scardica* extracts has been shown to inhibit aggregation and toxicity of amyloid-β in the nematode *Caenorhabditis elegans* used as a model for Alzheimer's disease (Heiner et al., 2018). Hence, it is of interest to evaluate the nootropic properties of compounds isolated from *S. scardica* acting as inhibitors of AChE and BuChE. However, none of the isolated compounds, fractions or crude extract showed inhibitory activity against AChE or BuChE, as shown in Table 1. Compounds **1** and **2**, isolated from the BuOH fraction, demonstrated no ability to inhibit neither AChE nor BuChE even at the highest concentration tested (100 μg/mL). Previously, compound **1** has been described in AChE and BuChE assays as both inactive (Filho et al., 2012; Tang et al., 2016) and active (Bae et al., 2014). Likewise, results for compound **2** point in both directions in AChE and BuChE assays, expressing it as both active (Generalić Mekinić et al., 2016; Kwon et al., 2010) and inactive (Oboh et al., 2013) in previous literature. Thus, the literature values of compounds **1** and **2** in AChE and BuChE assays are conflicted. Compound **3**, isolated from the EtOAc-fraction, was inactive (>100 μg/mL) in both the AChE and BuChE assays (no inhibition at 100 μg/mL, 35 ± 5 % inhibition at 100 μg/mL (173 μM), respectively). One previous study has reported inhibitory activity of compound **3** against AChE and BuChE with IC_{50} value estimated at > 400 μM (53.5 ± 0.2 % inhibition at 500 μM) and 96.5 ± 0.2 μM, respectively. These literature values display compound **3** with low AChE inhibitory effect, but as a somewhat

Table 1
Inhibition of 15-lipoxygenase (15-LOX), xanthine oxidase (XO), acetylcholinesterase (AChe), buturylcholinesterase (BuChe) and scavenging of the DPPH radical. Activities are shown as IC_{50} values (μg/mL). For isolated compounds, molar activities are shown in parenthesis (μM). Quercetin and tacrine were used as positive controls. Results are presented as means ± SD (n = 4). n.t. = not tested, – = not applicable. Quercetin and tacrine were used as positive controls. Substances **9**–**11** were not available in sufficient amounts and purity for testing.

	Fraction or compound	**15-LO inhibition**		**XO inhibition**		**DPPH scavenging**		**AChE inhibition**		**BuChE inhibition**	
	IC_{50}-values in μg/mL and μM	IC_{50}(μg/mL)	IC_{50} (μM)	IC_{50} (μg/mL)	IC_{50} (μM)	IC_{50} (μg/mL)	IC_{50} (μM)	IC_{50} (μg/mL)	IC_{50} (μM)	IC_{50} (μg/mL)	IC_{50} (μM)
	EtOH (Crude extract)	232 ± 9	–	238 ± 74	–	41 ± 3	–	>200	–	>200	–
	DCM fraction	249 ± 7	–	>333	–	>333	–	>200	–	>200	–
	EtOAc fraction	65 ± 6	–	31.2 ± 8.7	–	47 ± 4	–	>200	–	>200 (45 ± 2 % inhibition at 200 μg/mL)	–
	EtOAc precipitate	120 ± 5	–	>167	–	24 ± 1	–	>200	–	>200	–
	BuOH fraction	98 ± 6	–	>167	–	26 ± 3	–	>200 (49 ± 4 % inh. at 200 μg/mL)		>200	–
	Water fraction	>333	–	>333	–	137 ± 13	–	>200	–	>200	–
1	Verbascoside	>167	> 268	>167	>268	30 ± 2	47 ± 3	>100	–	>100	–
2	Chlorogenic acid	71 ± 2	201 ± 7	>167	>472	92 ± 14	240 ± 40	>100	–	>100	–
3	Echinaticin	137 ± 12	237 ± 21	27 ± 6	47 ± 10	>167	>288	>100	–	>100 (35 ± 5 % inh. at 100 μg/mL)	173
4	Acetylallosyl-glucosyl-isoscutellarein	>83	>127	>167	>256	65 ± 15	100 ± 23	>100	>153	>100	>153
5	Acetylallosyl-glucosyl-isoscutellarein 4′- methylether	>167	>235	>83	117	33 ± 4	46 ± 6	>100	>141	>100	>141
6	Acetylallosyl-glucosyl-hypolaetin	n.t.	n.t.	n.t.	n.t.	93 ± 7	139 ± 10	n.t.	n.t.	n.t.	n.t.
7	Aacetylallosyl-acetylglucosyl-isoscutellarein 4′-methylether	n.t.	n.t.	n.t.	n.t.	>150	>212	n.t.	n.t.	n.t.	n.t.
8	Leucoseptoside A	n.t.	n.t.	n.t.	n.t.	27 ± 1	11 ± 2	n.t.	n.t.	n.t.	n.t.
–	Quercetin	27 ± 1	89 ± 3	5.5 ± 1.1	18.1 ± 4	6.0 ± 0.6	11.8 ± 0.5	n.t.	n.t.	n.t.	n.t.
–	Tacrine	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	15.0 ± 0.4 ng/mL	75 ± 2 nM	14 ± 1 ng/mL	69 ± 5 nM

more active BuChE inhibitor in comparison with our data (Malik et al., 2012). Compounds **4** and **5** were inactive against AChE and BuChE (>100 μg/mL). To our knowledge, this is the first time the inhibitory activity of *S. scardica* extracts and compounds **4** and **5** against AChE and BuChE has been investigated. The obtained results demonstrate that the polyphenols from *S. scardica* are not promising inhibitors of either AChE or BuChE, and that other modes of mechanisms are involved in the putative improvement of cognitive effects for this herb.

3.3. Activation and inhibition of the human complement system

The complement system, consisting of more than 30 proteins circulating in plasma, is a cascade system integrated in the human immune system. The system is rooted in the innate immune system, but as the name implies, it is functioning as a complement to the adaptive immune system. Thus, the complement system facilitates the interplay between the innate and adaptive immune system in defense against pathogens and in the homeostatic processes. Dysregulation of the complement system has been associated with infectious diseases, as well as neurological and immunological disorders (Ricklin et al., 2016). Hence, compounds that are able to modulate the activity of complement could be candidates for novel therapeutics or health beneficial substances. In order to determine whether the extracts and compounds of *S. scardica* cause stimulation or inhibition of human complement, extracts or compounds were incubated with serum for either 0 min or 30 min prior to addition of erythrocytes. Inhibition of complement components comes into force nearly immediately, while on the contrary full activation of complement require some time before hemolysis. Correspondingly, compounds functioning as inhibitors of complement components should give similar IC_{50} values at different incubation times, while activators of complement components should reflect higher activity over time. Both stimulation and inhibition of the complement system result in reduction of hemolysis (Alban et al., 2002; Inngjerdingen et al., 2006). As shown in Table 2, among the extracts, the DCM extract showed the strongest interaction with human complement, leading to a reduction in complement-induced hemolysis of sheep erythrocytes with 50 % inhibitory concentration of hemolysis (ICH_{50} values of < 12.5 μg/mL). The activity of the DCM fraction was at least 10-fold higher after 30 min of incubation time compared to 0 min of incubation time (ICH_{50} value of 125 ± 12 μg/mL versus < 12,5 μg/mL). The EtOAc and BuOH fraction showed some degree of activity after 30 min incubation time (ICH_{50} value of > 200 μg/mL versus 121 ± 34 μg/mL, >400 μg/mL versus > 349 μg/mL, respectively). These results suggest that these three fractions are able to interact with and modulate human complement, more specifically acting as activators of complement components. The strong activity of the DCM fraction demonstrates the immunomodulatory potential of this plant. This may provide evidence that lipophilic compounds from *S. scardica* are the ones responsible for the complement fixating effects. Even though the low molecular weight flavonoids, compounds **3**, **4** and **7**, did not show any activity in this assay, flavonoids have previously been reported to exhibit complement fixating activity (Shahat et al., 1996). Our report is the first describing complement-modulating activities of *S. scardica*.

Table 2
Inhibition of serum-induced hemolysis of sheep erythrocytes. BP-II (a polysaccharide isolated from *Biophytum petersanium*) was used as positive control. Results are presented as means ± SD (n = 3).

Compound	ICH_{50} values (μg/mL) 0 min incubation	ICH_{50} values (μg/mL) 30 min incubation
DCM fraction	125 ± 12	<12,5
EtOAc fraction	>200	121 ± 34
BuOH fraction	>400	>349
1	>200	>200
2	>200	>200
3	>100	>100
4	>200	>200
7	>200	>200
8	>200	>200
BP-II	>250	19 ± 5

3.4. NO-reduction of LPS + IFNγ-activated dendritic cells

Dendritic cells (DCs) are a part of the innate immune system, serving as messengers between the innate and the adaptive immune system. Under inflammation, exposure of pathogen-associated structures, e.g. lipopolysaccharide (LPS), activates the DCs which then further initiates pro-inflammatory responses. The co-stimulator, interferon-γ (IFNγ), promotes the activation of DCs by enhancing antigen presentation for T cell activation, which again regulates the immune response. Upon activation of DCs, inflammatory mediators such as nitric oxide (NO) and cytokines are released (Bogdan, 2001). NO is a signaling and effector molecule in the immune system and is synthesized in various physiological and pathological processes. However, in case of overproduction, NO can contribute to neurodegenerative and autoimmune diseases (Bogdan, 2001; Doherty, 2011). Hence, compounds that are able to reduce NO production may be considered as inflammatory modulators. This might in turn contribute to the prevention and treatment of inflammatory diseases. Extracts and isolated compounds from *S. scardica* were tested for their ability to reduce LPS + IFNγ-induced NO production in murine D2SC/1 dendritic cells. An MTT assay was conducted thereafter to evaluate the viability on the same cells. As shown in Fig. 2, compounds **1** and **2** reduced NO levels without affecting cell viability. The BuOH extract and other isolated compounds (**3**, **4**, **7** and **8**) were inactive (data not shown). Treatment with the DCM extract led to a potent reduction in NO in a dose-dependent manner. However, the results corresponded inversely with cell viability, indicating a cytostatic or cytotoxic effect on the cells. Previously, one study revealed no toxicity or mutagenicity in rats after oral treatment of *S. scardica* 20 % (v/v) ethanol extract in acute and repeated doses (Feistel et al., 2018). Compounds **1** and **2**, the only ones available in sufficient amounts for testing, showed NO inhibitory activity, but less than the positive control quercetin, and less than the DCM and EtOAc extracts. This is the first report describing the NO inhibitory effects of *S. scardica* extract on dendritic cells. However, one study performed on endothelial cells, provided evidence that the methanolic extract (70:30 v/v) of aerial parts of *S. scardica* did not alter the NO levels in culture media (Woodcock et al., 2013). To our knowledge, this is the first report of NO inhibitory effects of compound **1** on dendritic cells. Compound **1** has previously demonstrated reduction of NO production of LPS-treated macrophages (RAW 264.7 cell line) with IC_{50} value of 17.8 ± 0.4 μM (Tran et al., 2021). However, in that work, viability assays were not investigated while our study showed no cytotoxic effects of compound **1**. Previous reports on compound **2** have revealed NO inhibitory effects in both dendritic cells and RAW264.7 cells in a setup similar to our assay (Ho et al., 2017), in accordance with our results. Macrophages and dendritic cells exhibit many similar properties, but they are considered to belong to different lineages due to unlike sets of surface phenotypes and functionalities (Ferenbach & Hughes, 2008). This implies that NO inhibitory results of macrophages and dendritic cells might not be comparable, because the isolated phenolic compounds might potentially act on different cellular targets in/on dendritic cells and RAW 264.7 cells. Both compounds **1** and **2** are major constituents in *S. scardica* extracts, and may therefore be important contributors of inflammatory modulation.

3.5. Inhibition of 15-lipoxygenase (15-LOX)

The inhibitory potency of the extracts and compounds from *S. scardica* against peroxidation of linoleic acid catalyzed by soybean 15-LOX was investigated (Table 1). The 15-lipoxygenase (15-LOX) is a peroxidative enzyme in the arachidonic acid pathway and considered as a target for AD therapy as it seems to modulate the metabolism of

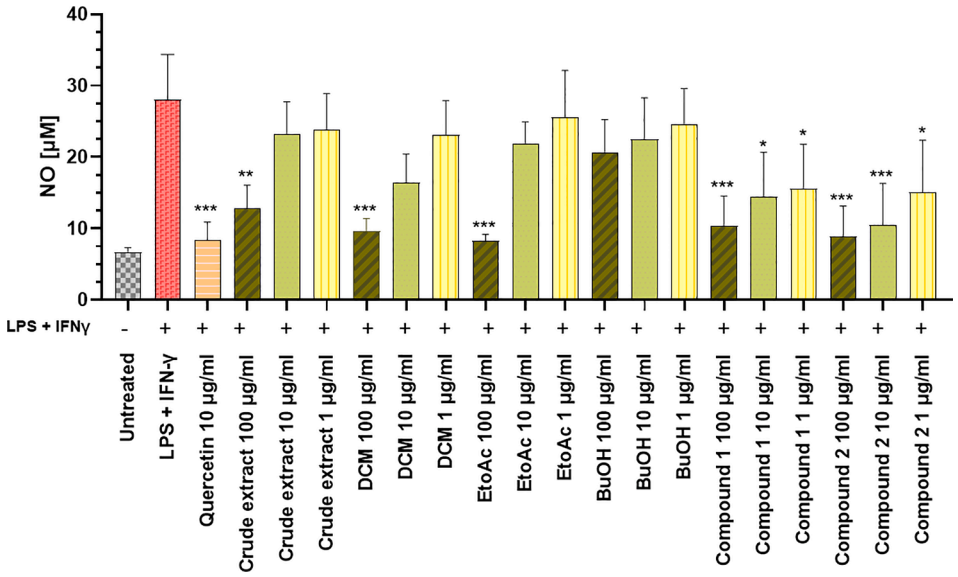


Fig. 2. Effects of *S. scardica* extracts and compound **1** and **2** on NO-release in LPS + IFNγ-induced D2SC/1 dendritic cells. Quercetin was used as a positive control. The concentrations of LPS and IFNγ were 500 ng/ml and 20 ng/ml, respectively. Results are expressed as means ± SD (n = 3). *** = $p < 0.001$, ** = $p < 0.01$, * = < 0.05, compared to control cells; LPS + IFNγ treated cells. Cell medium was used as a negative control (untreated).

amyloid-beta; Aβ- and the protein tau, which are both implied in AD (Di Meco et al., 2017). This enzyme generates reactive oxygen species (ROS) in vascular cells, and is widely expressed in the central nervous system. Vascular and ischemic damage, as well as neuronal death, have been ascribed to 15-LOX (van Leyen, 2013). In patients with AD or clinical diagnosis of mild cognitive impairment, the levels of 15-LOX are elevated. Also, 15-LOX has been suggested to be involved in the development of a number of other diseases such as atherosclerosis, cancer, psoriasis and diabetes (both type I and II) (Sadeghian & Jabbari, 2016). An *in vivo* study showed that feeding transgenic AD mice with a 15-LOX inhibitor for three months resulted in improved cognitive skills and lower Aβ levels compared to the control mice (Di Meco et al., 2017). Different types of LOX (5-LOX, 8-LOX, 12-LOX, 15-LOX) catalyze the lipid peroxidation reaction, and these enzymes respond differently to flavonoids as inhibitors. The presumed mechanism of action may be that flavonoids inhibit LOX by chelating or reducing Fe^{3+}. Other ways flavonoids can act is through scavenging of LOX-activating hydroperoxides or other ROS oxidants (Mladenka et al., 2010). Among the fractions obtained in this study, the BuOH and EtOAc fraction demonstrated moderate inhibitory activity against soybean 15-LOX (IC_{50} 98 ± 6 µg/mL and IC_{50} 65 ± 6 µg/mL, respectively), whereas the other fractions showed low inhibitory activity. The five isolated *S. scardica* compounds **1**–**5** exhibited low inhibitory enzymatic activity. Compound **3** expressed low inhibitory activity against 15-LOX with an IC_{50} value at 137 ± 1 µg/mL (237 ± 21 µM). Compared to our findings, a previous study found that compound **3** was more active (IC_{50} 48.2 ± 0.3 µM) (Malik et al., 2012). It appears that the inhibitory effects of compound **3** is associated with the presence of the 2,3-double bond and a 4-oxo group in the C-ring, which have shown in previous studies to be important features of flavonoids for the inhibition of 15-LOX (Sadik et al., 2003). This specific structure-activity relationship, in addition to electron delocalization from the B ring and hydroxyl groups at positions 3 and 5 providing hydrogen bonding to the oxo group, are central structural features for the antioxidant activity (Procházková et al., 2011). None of the flavonoids tested have a free 3-OH group, but compound **3** lacks the allose part of the molecule and has a 4-hydroxycinnamoyl group attached to its glucose moiety. This might be involved in the 15-LOX inhibitory activity of compound **3**. At the highest concentration tested, compounds **1, 4** and **5** did not show significant 15-LOX inhibition. Compound **2** has been described as a weak inhibitor of 15-LOX, which is in fair accordance with our results (IC_{50} 201 µM) (Shingai et al., 2011). IC_{50} values in the nanomolar range for compounds **1**, **4**, **6**, **7** and **8** have been reported, with a value for quercetin (positive control) of 215 nM (Charami et al., 2008). This value is, however, more than 100 times lower than what has been reported in other studies. In comparison, our positive control, quercetin, displayed an IC_{50} value at 27 ± 1 µg/mL (89 ± 3 µM). The 15-LOX inhibition of *S. scardica* crude extract and of compound **5** has previously not been reported.

3.6. DPPH radical scavenging activity

Oxidative stress, the imbalance of prooxidants and antioxidants, is suggested to be deleterious and an important factor for the development of AD (Salehi et al., 2020). The antioxidant activity of *S. scardica* fractions and isolated compounds **1**–**8** was measured by the ability to scavenge the DPPH radical. The EtOH crude extract and four liquid-liquid extracted fractions (DCM, EtOAc, BuOH and water) showed concentration-dependent DPPH scavenging activities. As shown in Table 1, the BuOH fraction (IC_{50} 26 ± 3 µg/mL) demonstrated the strongest DPPH radical scavenging activity. This, indicates that compounds with strongest radical-scavenging capacity are of medium polarity. Also, the BuOH fraction was more potent than the EtOH crude extract (IC_{50} 41 ± 3 µg/mL). The DCM fraction (IC_{50} > 333 µg/mL) and the water fraction (IC_{50} 137 ± 13 µg/mL) exhibited little or no radical scavenging activity. Among the eight isolated compounds, compound **1**, **5** and **8** showed the most potent radical scavenging activity with IC_{50} values of 30 ± 2 µg/mL (47 ± 3 µM), 33 ± 4 µg/mL (46 ± 6 µM) and 27 ± 1 µg/mL (11 ± 2 µM), respectively. Compound **1** is reported to have an IC_{50} value comparable to what was found in our study (Malheiro et al., 2014). Previously reported results for compound **8** vary from 14.2 µg/mL (Delazar et al., 2004) to nearly inactive (IC_{50} 127 ± 1 µg/mL) (Harput et al., 2004). DPPH scavenging activity of compound **5** has not been previously reported. Structure-activity relationship studies indicate that polyphenols possessing hydroxyl groups in *ortho* or *para* positions show high DPPH radical scavenging activity (Ali et al., 2013). The phenomenon was observed with the most active compounds **1** and **8**, having OH groups in *ortho* positions (i.e., catechols). The glycosylation of flavonoids apparently reduced the DPPH radical scavenging activity, referring to glycosylated flavones of isoscutellarein, compound **4**, **6** and **7**. Our results are consistent with previous studies on antioxidant

activity of flavonoids being dependent on the position of substituted OH groups and with the presence of glycosides (De Martino et al., 2012).

3.7. Inhibition of xanthin oxidase (XO)

Intracellular metabolic processes, such as oxidation of hypoxanthine or xanthine to uric acid catalyzed by xanthine oxidase (XO) normally generate unstable reactive oxygen species (ROS). Oxidative stress, referred to as excess production of ROS relative to antioxidants, leads to inflammation and apoptosis resulting in neurodegeneration and memory loss (Czupryna & Tsourkas, 2012). Inhibition of XO is therefore believed to reduce the production of ROS and might accordingly prevent the development of different diseases with inflammatory components such as AD, Parkinson's disease, diabetes and rheumatism (Ramzan et al., 2020). The EtOAc fraction (IC_{50} 39 ± 4 μg/mL) was significantly more active than the crude extract (IC_{50} 238 ± 74 μg/mL), and less active than the positive control, quercetin (IC_{50} 5.5 ± 1.1 μg/mL). The other fractions were inactive (shown in Table 1). Among the compounds tested for inhibition of xanthine oxidase (XO), compound **3** showed moderate inhibitory activity (IC_{50} 27 ± 6 ug/mL, corresponding to 47 ± 10 μM). Compounds **1**, **2, 4** and **5** were inactive up to the highest concentration tested (167 ug/mL or 83 ug/mL). No previous *in vitro* studies have been carried out on XO inhibition on aerial parts of *S. scardica*, but one *in vivo* study reported no effects on XO activity by consumption of *S. scardica* decoction (Begas et al., 2018). This is the first time compound **3** has been investigated as potential inhibitor of XO. Structure-activity relations for flavonoids as XO inhibitors has been discussed previously (Cos et al., 1998). They found that the presence of hydroxyl groups in position 5 and 7, as well as a double bond between *C*2 and C3 were needed for high activity. None of the flavonoids tested have a free 7-OH group, but compound **3** lacks the allose part of the other molecules and has a 4-hydroxycinnamoyl group attached to its glucose moiety. This might be involved in the XO inhibitory activity of compound **3**. However, compound **2**, with a 3,4-dihydroxycinnamoyl group was inactive. There is considerable discrepancy in the results from previous studies on compound **1**, showing it as both inactive and active against XO (Flemmig et al., 2011; Huang et al., 2008). The XO inhibitory activity of compound **2** has been subject to many studies, and a variety of results exists displaying it as both inactive (Honda et al., 2014) and active with IC_{50} values estimated at 2.14 μM (Ozyürek et al., 2009) and 26.4 ± 2.8 μM (Wang et al., 2009) among others. The present study support previous studies indicating that compounds **1** and **2** do not inhibit XO. This is the first time the XO inhibitory activity of *S. scardica* DCM extract has been investigated, whilst one study has shown no XO inhibitory effect of aqueous *S. scardica* extract (Begas et al., 2018). This is the first time the XO inhibitory activity of compounds **4** and **5** has been investigated.

4. Conclusion

Eleven phenolic compounds from the herb *S. scardica* were isolated and chemically characterized, and examined for their immunological and cytotoxic effects in a variety of bioassays. BuOH, DCM and EtOAc fractions were isolated from crude EtOH extract of aerial parts of *S. scardica* and further purified by chromatographic methods. To our knowledge, this is the first report addressing the NO inhibitory effects on dendritic cells (D2SC/1), complement-modulating activities and cholinesterase inhibitory effects of *S. scardica.* Structural elucidation of the eleven phenolic compounds revealed flavone-glycosides and phenylethanoids as predominant substances in the extracts. Compound **1** is abundant in *S. scardica* and is suggested to be an important contributor to the modulation of inflammatory effects. Compounds **1** and **2** from the BuOH extract were able to reduce NO levels in the murine cell line D2SC/1 dendritic cells moderately. The DCM extract reduced NO levels of dendritic cells, but affected cell viability. Nevertheless, compounds with favorable cell viability effects may exist in the DCM extract. This should be further investigated in future studies. The DCM extract strongly activated the complement cascade, indicating that lipophilic compounds are responsible for the immunomodulatory potential of *S. scardica*. In the DPPH assay, the BuOH fraction demonstrated the strongest activity, indicating that compounds with the strongest radical scavenging capacity are of medium polarity. DPPH radical scavenging activity was mainly attributed to compounds **1**, **5** and **8**. SAR analysis of compounds **1** and **8** correlate with high DPPH radical scavenging activity with –OH groups in *ortho* positions (i.e., catechols). Interestingly, other modes of mechanisms than AChE and BuChE inhibition appear to be involved in the putative neuroprotective activities of *S. scardica* as the extracts and isolated compounds were inactive in the cholinesterase assays. The assays for inhibition of XO and 15-LOX showed moderate to no effect of extracts and isolated compounds. The BuOH and EtOAc extract showed moderate activity in both of these antioxidant assays, as shown in Table 1. Of all isolated compounds tested, only compound **3** exhibited XO inhibitory activity. Synergistic effects between compound **1** and other substances, such as flavonoids, might conceivably play a role in the health beneficial and nootropic profile of *S. scardica.* In sum, *S. scardica* contains a pool of compounds indicating multifaceted activities with respect to the obtained results in various *in vitro* bioassays, and thus may serve as a potential candidate for supplementary treatment of Alzheimer's disease. The activity across a wide range of bioassays may explain the beneficial effects of *S. scardica*. The results obtained from this study indicate that further research is needed on the isolated and identified compounds of *S. scardica*, involving the characterization of mechanisms against Alzheimer's disease and other neurodegenerative diseases.

CRediT authorship contribution statement

Nastaran Moussavi: Conceptualization, Investigation, Methodology, Writing original draft, Writing review & editing. **Hasina Azizullah:** Investigation. **Karl E. Malterud:** Conceptualization, Methodology, Supervision, Writing review & editing. **Kari T. Inngjerdingen:** Methodology, Supervision, Writing review & editing. **Helle Wangensteen:** Conceptualization, Methodology, Project administration, Supervision, Writing review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Ethics statements file

No human or animal experiments were involved in the conducted research for this manuscript.

Appendix A. Supplementary material

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