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Chemical characterization and immunomodulatory potential of the moss *Hypnum cupressiforme* Hedw. extracts

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#These authors contributed equally to this study



Chemical characterization and immunomodulatory potential of the moss *Hypnum cupressiforme* Hedw. extracts

Graphical abstract

1. Moss collection site

- Vršačke Planine Mts., Serbia



Hypnum cupressiforme Hedw.



2. Moss extraction

- Soxhlet extractor
- Four extracts (ethanol, water/ethanol, ethyl-acetate, water)

3. Chemical characterization

- Spectrophotometric assays (total phenolic, total phenolic acid, total flavonoid, total flavonol, total triterpenoid, and total coumarin contents)
- Liquid Chromatography–Mass Spectrometry

4. Biological activities

- Antioxidant (DPPH, total reduction power, and β -carotene bleaching assays)
- Antidiabetic (α -glucosidase and α -amylase inhibition)
- Antineurodegenerative (acetylcholinesterase and tyrosinase inhibition)
- Anti-neuroinflammatory (BV2 cell line)
- Antitumor (HCT-116 and MDA-231 cell lines)



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Abstract:

This study aimed to examine the chemical composition and immunomodulatory potential of the moss *Hypnum cupressiforme* Hedw. extracts. The corresponding extracts were obtained utilizing Soxhlet extractor and further characterized by spectrophotometric assays and liquid chromatography coupled to mass spectrometry (LC-MS). The **antioxidant activity** was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH), total reduction power, and β -carotene bleaching assays. The inhibitory activities on α -glucosidase, α -amylase, acetylcholinesterase, and tyrosinase were tested for potential **antidiabetic and antineurodegenerative activity**. Additionally, **biocompatibility, antitumor, and anti-inflammatory potential** were tested on MRC-5, HCT-116, MDA-MB-231, and BV2 cells, respectively.

Major compounds identified by LC-MS in *H. cupressiforme* extracts were kaempferol and five phenolic acids: *p*-hydroxybenzoic, protocatechuic, *p*-coumaric, gallic, and caffeic acid. Biochemical assays revealed the significant immunomodulatory potential of examined extracts. Moreover, **significant antiproliferative potential** against human breast cancer cells – MDA-MB-231 (inhibitory rate up to 50%) and acceptable biocompatibility were observed. Also, a **significant decrease in NO production**, observed in lipopolysaccharide-stimulated BV2 cells, implies potential anti-neuroinflammatory application. Obtained results qualify the moss *H. cupressiforme* as a highly promising candidate for more detailed examination and also putative therapeutical application.

Keywords: *Hypnum cupressiforme*; antioxidant; antidiabetic; anti-neuroinflammatory/antineurodegenerative; antitumor activity



Introduction – *Hypnum cupressiforme*

- ❑ Mosses belong to the second largest group of higher plants – bryophytes
- ❑ These plants are recognized as promising sources of **novel biologically active compounds**
- ❑ *Hypnum cupressiforme* Hedw. is a common moss species found in a variety of habitats
- ❑ Studies have reported good **antimicrobial, antioxidant, and antiproliferative potential** of this moss

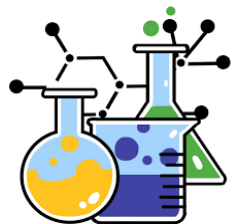


Chemical composition of mosses

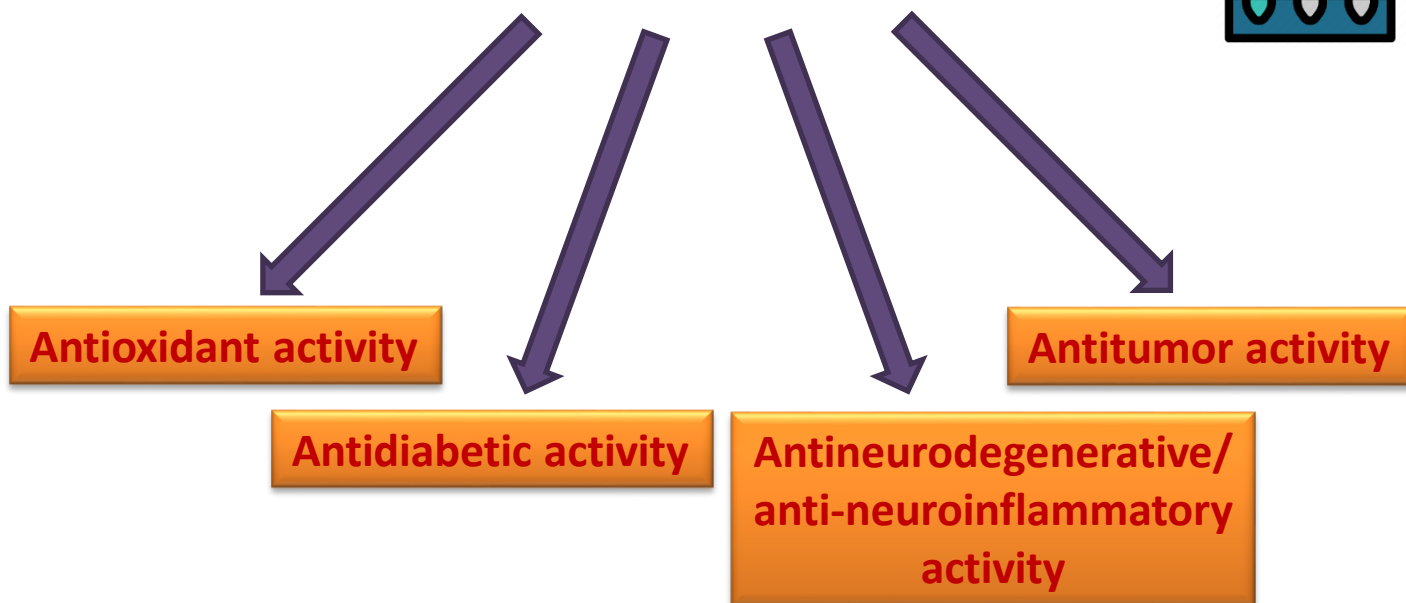
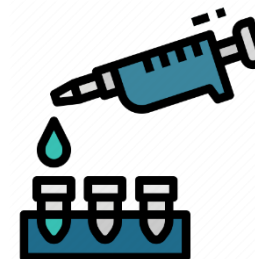
- ❑ Moss phytochemistry has been overlooked in the past
- ❑ Secondary metabolites found in bryophytes can be divided into two main groups – **polyphenols and lipids**
- ❑ The majority of secondary metabolites from mosses belong to **flavonoids, terpenoids, and bibenzyls**
- ❑ **Diverse biological activities** of these metabolites: cytotoxicity, antimicrobial, antifungal, antitumor, antioxidant, anti-inflammatory, antidiabetic, and many other



The aim of this study



1. **Extraction** of biologically active compounds from *H. cupressiforme*
2. **Chemical characterization** of the obtained moss extracts
3. Evaluation of potential **biological activities** of the extracts



Extracts chemical characterization

Extraction yield for *Hypnum cupressiforme* moss extracts

	Solvent	Moss weight (g)	Extract weight (g)	Yield (%)
E1	Ethanol (96%)	10	0.42	4.2
E2	Water-ethanol (1:1, vol%)	10	0.80	8.0
E3	Ethyl-acetate	10	0.06	0.6
E4	Water	7.6	2.00	26.3

Chemical characterization of moss *Hypnum cupressiforme* extracts

	TPC (mg GAE/g extract)	TPAC (mg CAE/g extract)	TFC (mg QE/g extract)	TFIC (mg QE/g extract)	TTC (mg UAE/g extract)
E1 (Ethanol)	6.25 ± 0.48	67.41 ± 6.97	35.00 ± 1.34	ND ¹	88.37 ± 1.55
E2 (Water-ethanol)	7.38 ± 0.34	7.08 ± 2.36	12.43 ± 0.49	ND	75.93 ± 2.97
E3 (Ethyl-acetate)	15.33 ± 0.95	339.93 ± 14.03	58.86 ± 2.82	14.11 ± 1.33	235.95 ± 4.09
E4 (Water)	18.21 ± 0.73	8.31 ± 3.48	2.04 ± 0.29	ND	43.33 ± 0.86

CAE – caffeic acid equivalents; GAE – gallic acid equivalents; ND – not detected; QE – quercetin equivalents; TPAC – total phenolic acid content; TFC – total flavonoid content; TFIC – total flavonol content; TPC – total phenolic content; TTC – total triterpenoid content; UAE – ursolic acid equivalents



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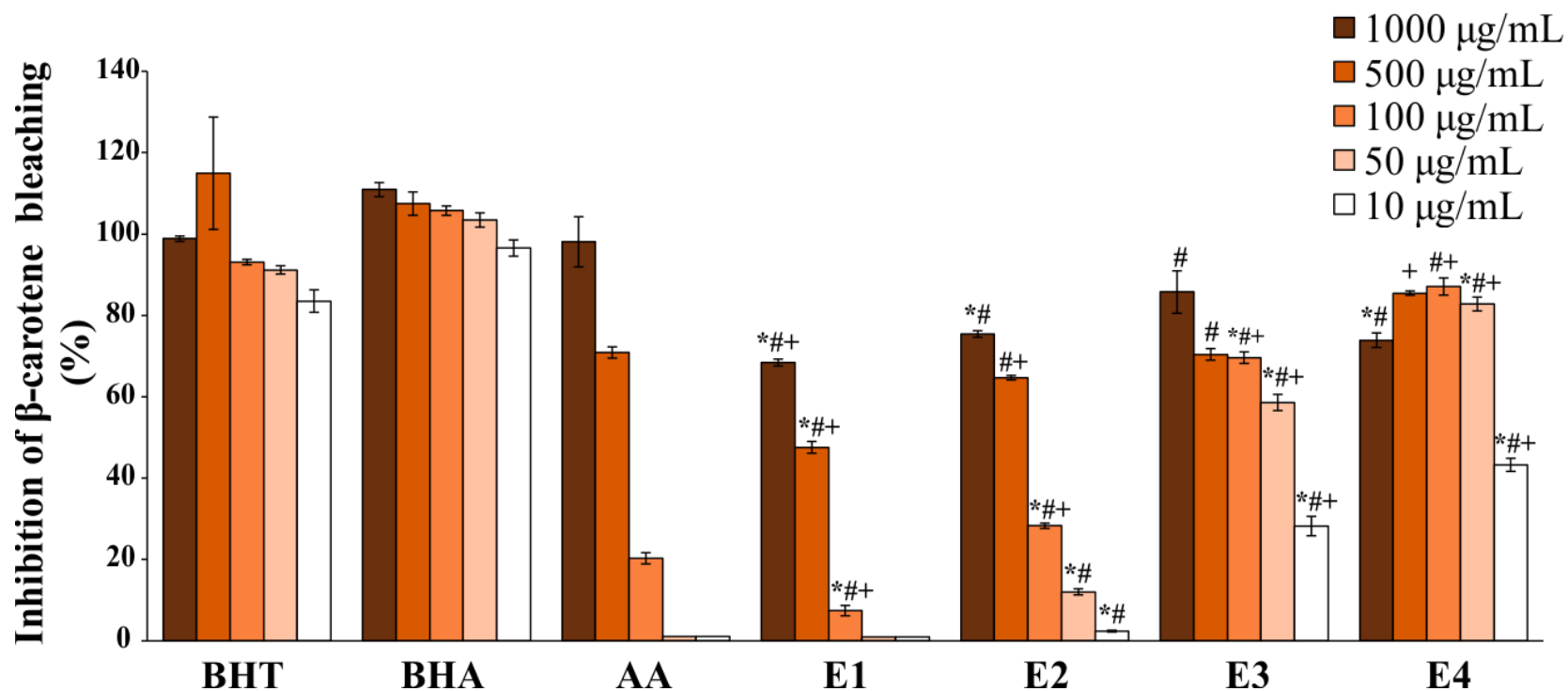
Extracts chemical characterization

LC-MS analysis of the investigated *Hypnum cupressiforme* extracts

mg/100 g extract	E1 (Ethanol)	E2 (Water-ethanol)	E3 (Ethyl-acetate)	E4 (Water)
Gallic acid	0.62	0.70	0.50	1.21
Protocatechuic acid	3.75	2.89	2.39	3.91
5-O-Caffeoylquinic acid	0.14	0.07	0.02	0.04
p-Hydroxybenzoic acid	4.56	3.17	5.78	4.62
Caffeic acid	0.65	0.42	0.13	1.10
Quercetin 3-O-rutinoside	0.09	0.06	0.01	0.03
p-Coumaric acid	2.60	2.33	0.46	4.40
Quercetin 3-O-glucoside	0.27	0.21	0.02	0.04
Isorhamnetin 3-O-glucoside	0.12	0.06	0.02	0.04
Eriodictyol	0.13	0.11	0.05	0.07
Apigenin	0.51	0.47	0.11	0.11
Naringenin	0.57	0.62	0.12	0.08
Kaempferol	7.35	6.60	0.21	0.47
Acacetin	0.21	0.15	0.09	0.02



Antioxidant activity



#,+,*p<0.05 different moss extracts vs. different standard substances.

Symbols *, #, + were used for standards BHT, BHA, and AA (ascorbic acid), respectively

✓ **Significant antioxidant activity** obtained for ethyl-acetate and aqueous extracts (E3 and E4) in the β -carotene bleaching test



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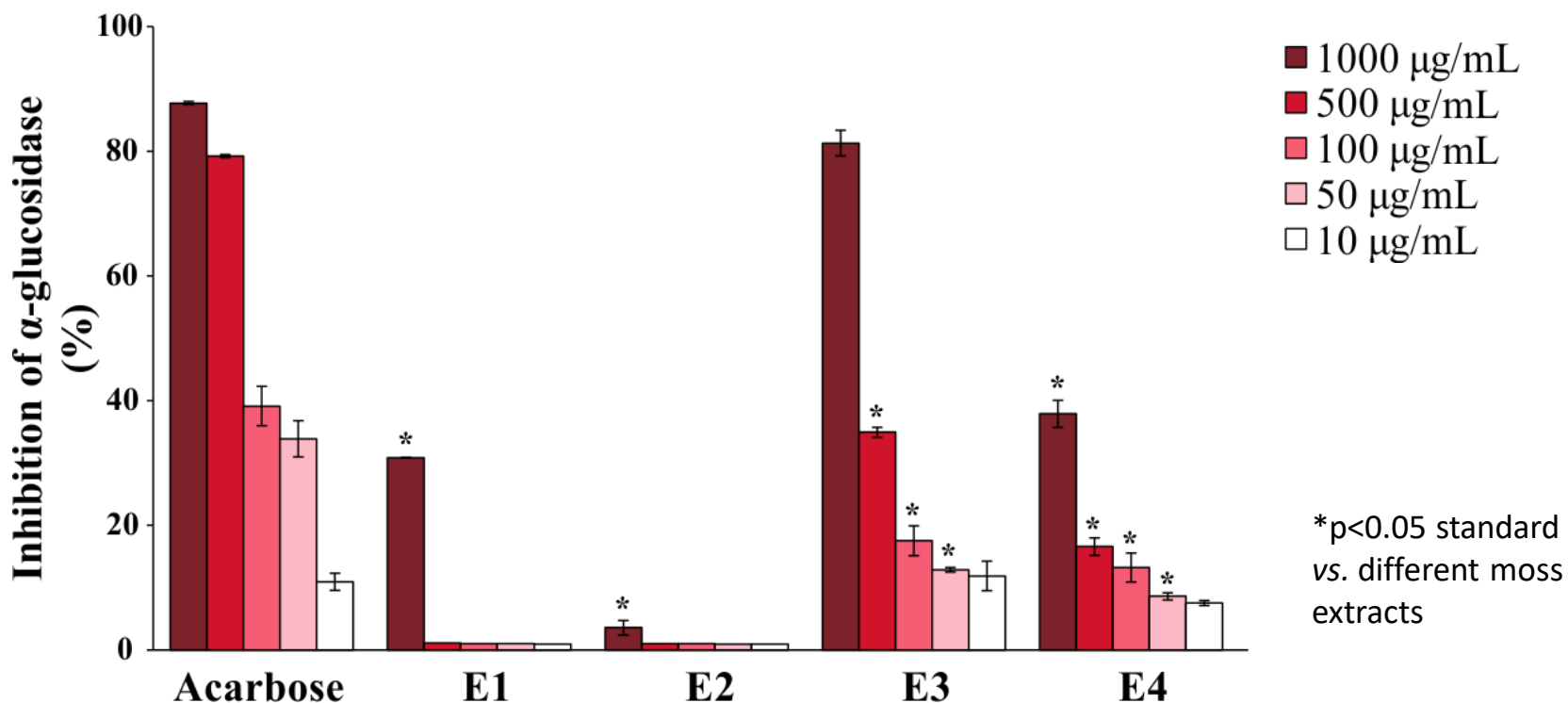
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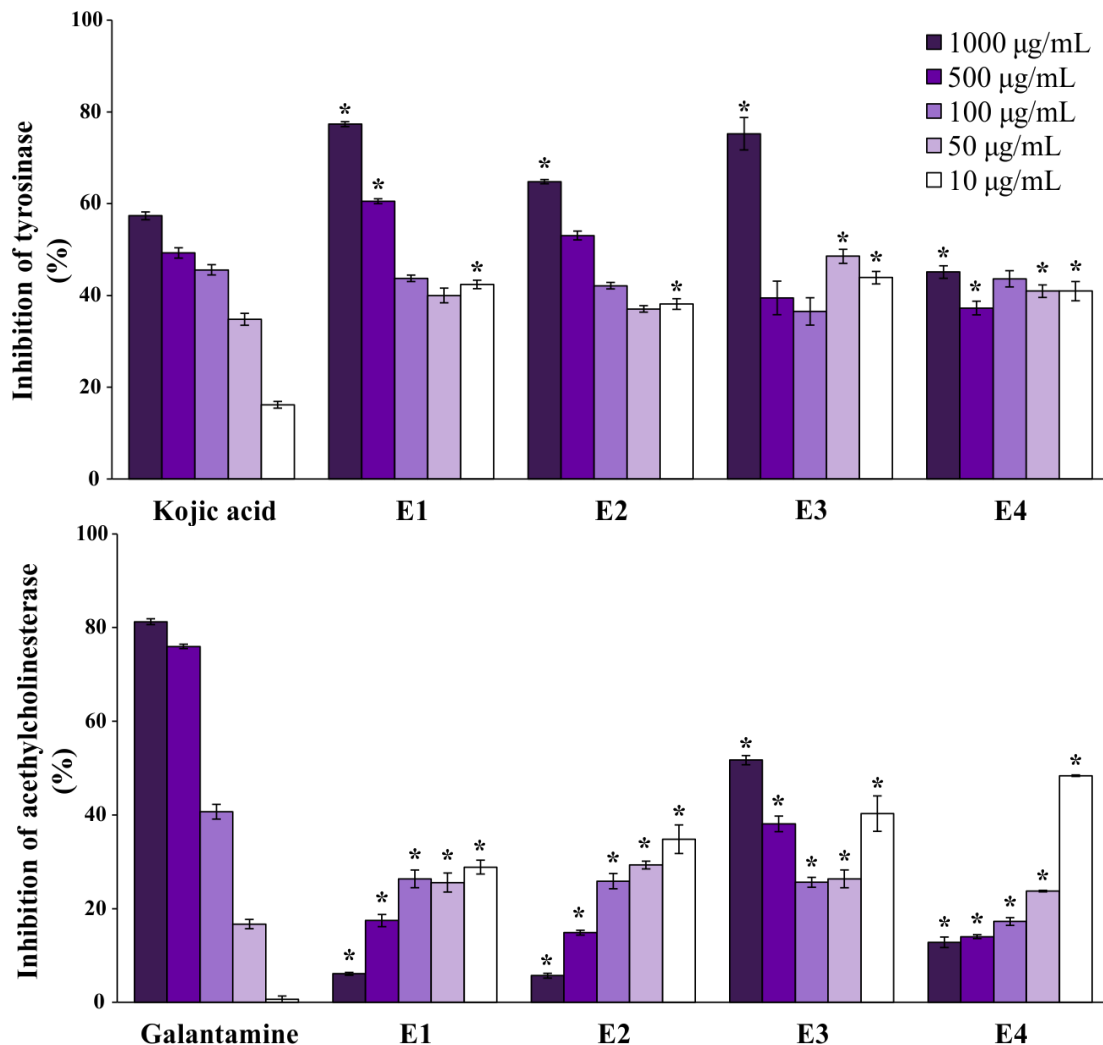
Antidiabetic activity



- ✓ **Significant inhibitory effect** of ethyl-acetate and aqueous extracts (E3 and E4) on carbohydrate hydrolyzing enzyme **α -glucosidase**
- ✓ **Potential adjuvant therapy** to delay postprandial hyperglycemia



Antineurodegenerative activity



- ✓ **Significant inhibition** of tyrosinase and acetylcholinesterase
- ✓ Enzymes associated with the development of **Alzheimer's and Parkinson's disease**
- ✓ Potential therapeutic application in the **prevention/treatment of neurodegenerative diseases**

*p<0.05 standard vs. different moss extracts



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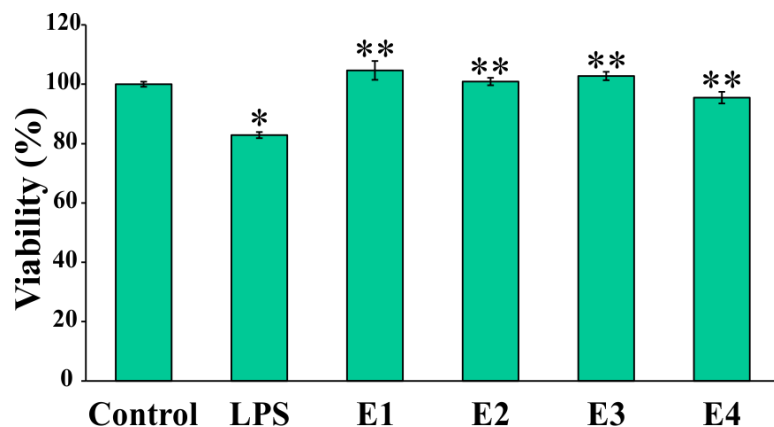
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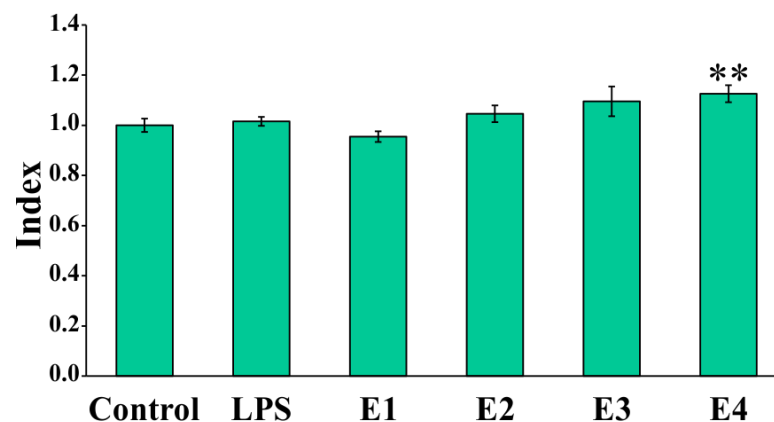
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Anti-neuroinflammatory activity – BV2 cells

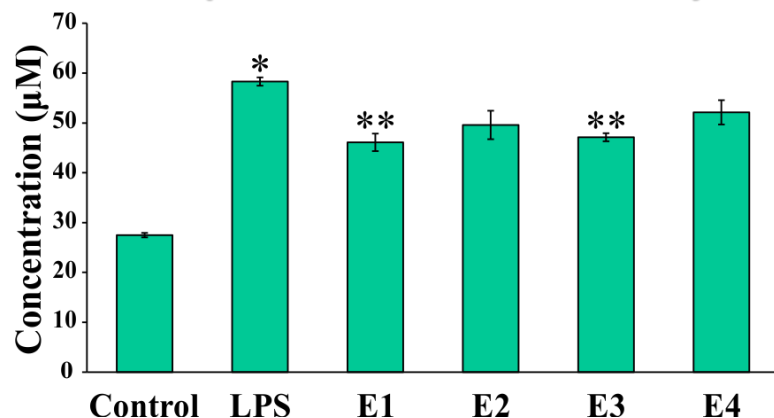
Cell viability – MTT assay



ROS production – NBT assay



NO production – Griess assay



*p<0.05 LPS-stimulated control cells vs. non-stimulated control cells;

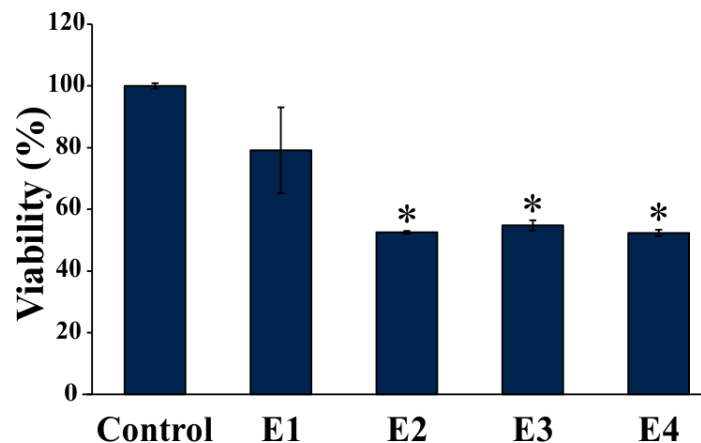
**p<0.05 extracts vs. only LPS-stimulated control cells

- ✓ Extracts **increased the viability** of LPS-stimulated **BV2 cells**
- ✓ The production of **NO** by activated microglia is **diminished**
- ✓ Another evidence for extracts **anti-neuroinflammatory potential**

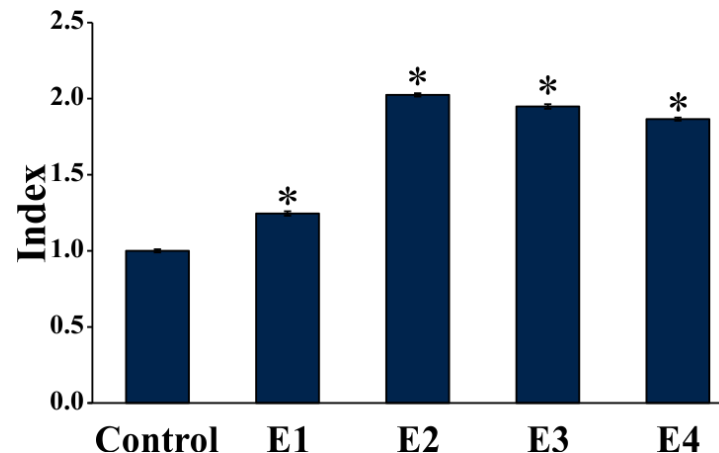


Antitumor activity – MDA-MB-231 cells

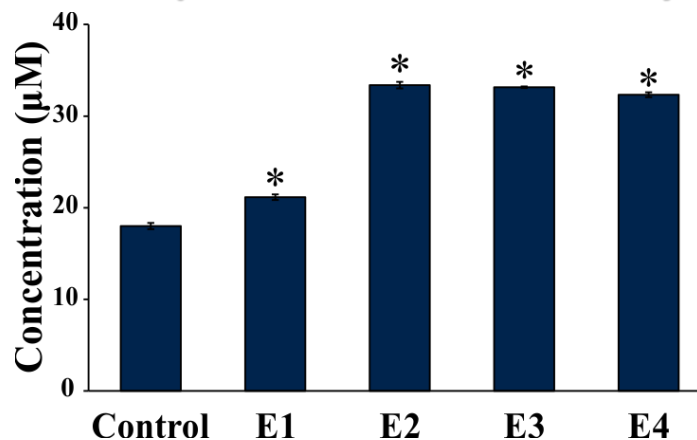
Cell proliferation – MTT assay



ROS production – NBT assay



NO production – Griess assay



*p<0.05 control cells vs. different moss extracts

- ✓ Significant **antiproliferative** activity against **MDA-MB-231 cells**
- ✓ All extracts **increased ROS and NO** production
- ✓ Potential **antitumor agents** in the prevention/adjuvant treatment of **breast cancer**



Conclusions

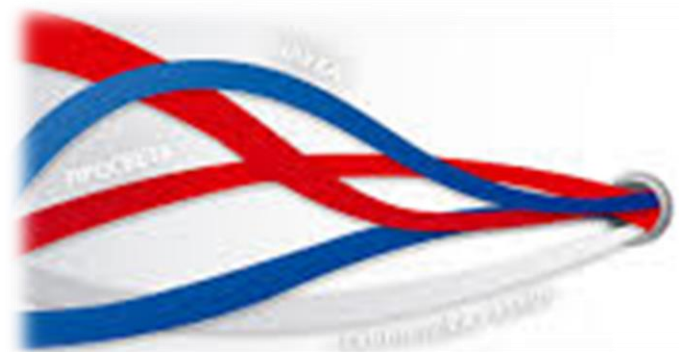
- ✓ **Flavonoids, phenolic acids, and triterpenoids** – important classes of secondary metabolites discovered in *H. Cupressiforme* extracts
- ✓ Extracts exhibited **good antioxidant activity** regarding the prevention of β -carotene bleaching
- ✓ High **tyrosinase and acetylcholinesterase inhibition** potential
- ✓ High **α -glucosidase inhibition** activity
- ✓ Promising **anti-inflammatory potential** (reducing the production of NO by LPS-stimulated BV2 cells)
- ✓ Significant **antiproliferative effects** against MDA-MB-231 cancer cells

Altogether, *H. cupressiforme* is a highly promising source of novel biologically active compounds



Acknowledgments

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