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Inhibitory effects of four naturally occurring compounds from *Epicoccum nigrum* on acetylcholinesterase activity and nitric oxide production in LPS-activated RAW 264.7 cells

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Abstract

Background: The role of nitric oxide in the pathogenesis and progression of neurodegenerative illnesses is becoming prominent over the years and the inhibition of acetylcholinesterase (AChE) is considered as a promising strategy in the management of such diseases. This study aims to assess the inhibitory potential of **(1)** beauvericin, **(2)** para-hydroxybenzaldehyde, **(3)** indole-3-carboxylic acid and **(4)** quinizarin isolated from endophytic fungi *Epicoccum nigrum* on acetylcholinesterase activity and on nitric oxide production in LPS-stimulated macrophages.

Methods: The inhibition of acetylcholinesterase activity was determined using Ellman's colorimetric method. Nitric oxide released from macrophages was determined by measuring the nitrite concentration in culture supernatant using the Griess reagent and the cell viability was determined by MTT assay.

Results: The tested compounds exerted concentration-dependent activity against both AChE and NO production. At the highest dose assessed (20 µg/mL) compounds **1** and **4** produced the smallest amount of NO (0.25 µM and 0.5 µM respectively) and their cell viability varied from 88.49% to 93.04%. Compound **1**exhibited the most potent anti-AChE activity (IC_{50} = 13.49 µg/mL) which however was lower compare to the reference compound galantamine (IC_{50} = 8.22 µg/mL), while compound **2** and **3** showed moderate activity.

Conclusions: This study revealed that beauvericin is endowed with a potential against AChE activity and NO production. Taking into account the respective role of NO and AChE in the occurrence and the prevention of neurodegenerative diseases, our findings indicate thatbeauvericincan be considered as a good drug candidate that could be further developed for the prevention of neurodegenerative disorders such as Alzheimer's disease.

Keywords: Acetylcholinesterase, Nitric oxide, Natural compounds, Endophytic fungi, *Epicoccum nigrum*

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Background

Nitric oxide is a small signalling, highly diffusible, and reactive molecule with a short lifetime that is generated by nitric oxide synthase (NOS) through enzymatic conversion of L-arginine to L-citrulline [1].The physiological roles of NO depend on its local concentrations, its availability as well as the nature of downstream target molecules. Under normal conditions, NO is produced by NOS and can induce physiological responses such as vasodilation. However, excessive NO produced by inducible nitric oxide synthase (iNOS) has been linked to a number of human pathological conditions including inhibition of cardiac contractility, the formation of reactive nitrogen species, impairment of mitochondrial respiration, apoptosis, and especially neuronal cell death [2].The role of nitric oxide in the pathogenesis and progression of neurodegenerative illnesses such as Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease (HD), has become prominent over the years [3]. Alzheimer's disease (AD) is a progressive neuro-degenerative disorder associated with memory impairment and cognitive deficit. It's one of the most common forms of dementia, characterized by low levels of acetylcholine in the brain of AD patients [4]. Acetylcholinesterase is an acetylcholine hydrolase enzyme with esterase activity which plays a key role in neural functioning via the cholinergic pathways. It is mainly localized in the synaptic gaps of the central and peripheral nervous system where it terminates nerve impulses by catalyzing the hydrolysis of the cholinergic system neurotransmitter acetylcholine (ACh) [5].Inhibition of acetylcholinesterase (AChE) which breakdown acetylcholine has been considered as a promising strategy in the management of Alzheimer's disease (AD). According to the cholinergic hypothesis, the inhibition of acetylcholinesterase (AChE) increases the levels of acetylcholine in the brain, thus improving cholinergic functions in AD patients [4].There are no available treatments that can stop or reverse AD progression, several compounds have been designed and approved in order to inhibit AChE breakdown in the brain that can increase the activity of ACh, and moderate AD symptoms [6]. Galantamine, a powerful AChE inhibitor (AChEI), was the first compound isolated from a plant source. Nowadays, the most commonly used AChEIs are physostigmine, tacrine,and donepezil. However, these inhibitors are associated with a number of adverse effects such as hepatotoxicity and gastrointestinal complaints [7].There is an urgent need to search for new, effective and safe AChE inhibitors, especially those which act through multiple receptor sites, but do not elicit undesirable effects. In this regard, the evaluation of compounds from natural sources can be a rational approach. Medicinal plants had been used to isolate and characterize directly the bioactive metabolites. However, the discovery of fungal endophytes inside these plants with capacity to produce the same compounds shifted the focus of new drug sources from plants to fungi. Bioactive natural products from endophytic fungi, isolated from different plant species, are attracting considerable attention from natural product researchers during the recent years [8]. *Epicoccum nigrum* Link is a widespread mitosporic ascomycete that colonizes different types of substrates and is associated with plant primary decomposition. Similar to other ubiquitous fungi, *E. nigrum* can display an endophytic lifestyle in a variety of plants including *Entada abyssinica* [9].In our previous study, we isolated and chemically characterized compounds from *E. nigrum* associated with *Entada abyssinica* and we evaluated their cytotoxicity, antioxidant and antibacterial activity [10]. Therefore, we hypothesized that, these compounds could be endowed with health benefits towards neurodegenerative illnesses especially Alzheimer's disease. In this regard, this study was undertaken to assess the inhibitory potential of compounds isolated from endophytic fungi *Epicoccum nigrum* on acetylcholinesterase activity as well as on nitric oxide production in LPS-stimulated macrophages.

Methods

Chemicals

Galantamine, acetylthiocholine iodide (ATCI), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), acetylcholinesterase (AChE) enzyme from *Electrophorus electricus* (electric eel) lyophilized (type VI-S lyophilized powder), Bovine serum albumin (BSA), Sodium dodecyl sulphate, sodium chloride (NaCl), $MgCl_2 \cdot 6H_2O$, ferrous sulfate, indomethacin, 15-lipoxygenase from *Glycine max,* and sodium nitrite were obtained from Sigma (Germany) while Tris (hydroxymethyl)aminomethane were purchased from Sigma, (Switzerland). Penicillin/streptomycin/fungizone (PSF), Dulbecco's modified Eagle's medium (DMEM) and foetal calf serum (FCS) were purchased from Highveld Biological Products (South Africa). Trypsin and phosphate buffered saline (PBS) were provided by Whitehead Scientific (South Africa). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and quercetin were provided by Sigma-Aldrich St. Louis, MO, USA.

Natural compounds

The four compounds (beauvericin, parahydroxybenzaldehyde, indole-3-carboxylic acid, and quinizarin) studied in this work were isolated from

an endophytic fungus *Epicoccum nigrum* associated with *Entada abyssinica.* Their isolation procedure and structure elucidation were previously described [10].

Nitric oxide inhibitory activity and viability of LPS-activated RAW 264.7 macrophages

The RAW 264.7 macrophages cells were seeded in 96 well-microtitre plates and were activated by incubation in medium containing 1 µg/mL LPS alone (control) or lipopolysaccharide with different concentrations of the samples dissolved in DMSO. Quercetin served as a positive control NO inhibitor for the reduction of NO production. The amount of nitric oxide released from macrophages as well as the cell viability were determined as previously described [11].

Acetylcholinesterase inhibition activity

Inhibition of acetylcholinesterase activity was determined using Ellman's colorimetric method as previously described [11].Then, for the samples with at least 50% inhibition, the IC_{50} value was calculated by plotting the percentage of inhibition against the concentrations.

Statistical analysis

The results were presented as means of three experiments. Values were expressed as mean ± standard deviation. Statistical analysis was performed with GraphPadInStat Software and results were compared using the Fisher's least significant difference (LSD) at a 5% significance level.

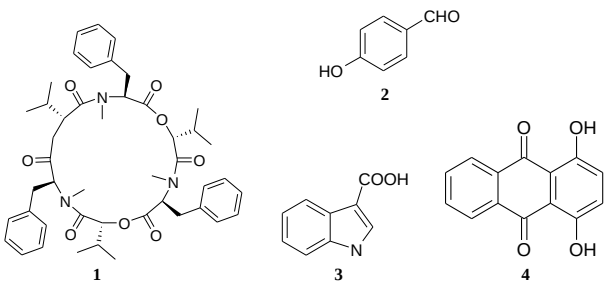

Figure 1. Chemical structures of the tested compounds.
1: Beauvericin, **2**: para-hydroxybenzaldehyde, **3**: indole-3-carboxylic acid, **4**: quinizarin.

Results and discussion

Neurodegenerative disorders especially Alzheimer's disease (AD) are associated with memory loss, cognitive dysfunction, behavioural turbulence and abnormalities in activities of daily life. Excessive NO production has been identified as one of the major causative reasons for the pathogenesis of several neurodegenerative diseases. Moreover, excessive NO synthesis under neuroinflammation leads to the formation of reactive nitrogen species and neuronal cell death [12].As for now, the use of cholinesterase inhibitors to reverse the cholinergic deficit is the symptomatic treatment. However NO inhibitors should also be considered in the therapy of such diseases. Therefore, the search for compounds with dual activity that could both inhibit excessive NO production and AChE activity will be more useful in focused neurodegenerative diseases therapies. In this study, the NO production and the AChE inhibitory activity of four natural compounds were evaluated.

Nitric oxide inhibitory activity

To evaluate the ability of compounds to inhibit NO production in activated RAW 264.7 macrophage cell lines, the amount of nitrite released in the culture supernatant was evaluated 24hours after the treatment of cells with compounds. Results are shown in Figure 2. The treatment of RAW264.7 cells with LPS resulted in a significant increment of nitrite concentration in the medium (control). All the four compounds showed various extent of inhibition of NO production activity. In general, a decline in the amount of nitrite produced was observed when the concentration of the tested compounds increased. At the highest dose assessed (20 µg/mL) compound **1**and **4** produced the smallest amount of NO (0.25 µM and 0.5 µM respectively) and the cell viability varied from 88.49% to 93.04%. A the lowest concentration tested (0.5 µg/mL), compound **1** showed the highest NO inhibitory activity with 3.92 of NO released. However, unlike the highest concentration, at the lowest concentration, the activity of compound 1 was found to be more potent than the positive control quercetin which showed 5.01 µM of NO released. The overall cell viability was more than 80%, therefore consistent with the fact that the observed NO inhibition was not related to the cell toxicity. Compound **1** (beauvericin) is a cyclic hexadepsipeptide, which was previously shown to possess biological properties including antimicrobial, antiviral and cytotoxic activities [13, 10].Plant secondary metabolites generally produced for their defense mechanisms have been implicated in the therapeutic properties of most medicinal plants. Plants, therefore, provide an invaluable useful resource as leads in the development of therapeutic compounds [14].Many compounds from medicinal plants have been demonstrated as inhibitors of NO production in LPS-activated macrophages. Their structures can be categorized as flavonoids, alkaloids, sesquiterpene, polyacetylenes, and lignans [15].

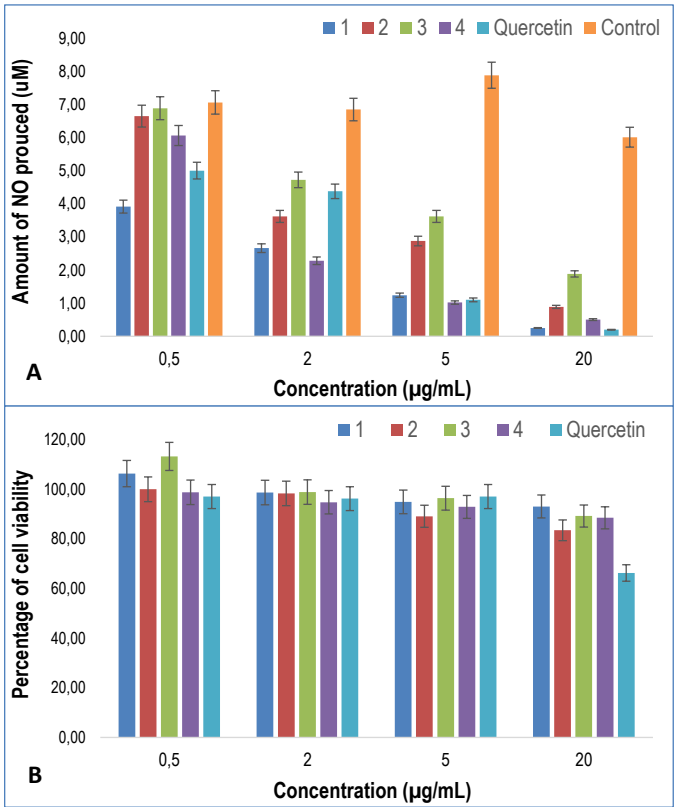

Figure 2. Inhibitory effects of compounds **1**, **2**, **3**, **4** and quercetin on NO production in LPS-activated RAW 264.7 macrophages.

AChE inhibitory activity

The compounds were tested for AChE inhibitory activity by Ellman's colorimetric method in 96-welled microplate. The results are shown in Table 1 representing the % inhibition at different concentration tested and IC_{50} value for those who had more than 50% inhibition. Galantamine was used as the standard AChE inhibitor in this study which showed IC_{50} of 8.22 µg/mL.The AChE inhibitory activity was highest with compound **1** followed by *compo*unds **2** and **3** with IC_{50} values of 13.49 µg/mL, 17.50 µg/mL, and 23.25 µg/mL respectively. Compound **4** showed weak activity and gave less than 50% of AChE inhibition. Many plant-derived products are either in the clinical trial phase or currently used in treatment of several ailments including Alzheimer's disease[16].Previous studies reported that cholinesterase inhibitors could act on multiple therapeutic targets such as prevention of the antioxidant activity and the formation of β-amyloid plaques. However, there is still a need for new AChE inhibitor lead compounds with lower toxicity and higher central nervous system (CNS) penetration. To this end, many plants have been studied by bioassay-guided approaches for the identification of new AChE inhibitors and different classes of plant-derived natural products have been considered as new AChE inhibitors potentially useful for AD treatment [17].

Table 1: Acetylcholinesterase inhibitory effects of compounds **1**, **2**, **3**, **4** and galantamine.

Compounds	Concentration (µg/mL)	% AChE inhibition	IC_{50} (µg/mL)
1	50	56.33±1.74	13.49±1.74[a]
	25	30.00±6.56	
	12.5	29.33±9.24	
	6.25	25.33±2.52	
2	50	75.00±9.64	17.50±1.74[b]
	25	64.33±6.35	
	12.5	38.00±8.72	
	6.25	25.33±10.41	
3	50	66.67±8.14	23.25±1.74[c]
	25	50.33±5.51	
	12.5	36.67±5.77	
	6.25	25.67±4.62	
4	50	65.67±12.34	>50
	25	41.67±3.06	
	12.5	33.33±7.51	
	6.25	34.00±6.08	
Galantamine	50	83.00±8.89	8.22±2.73[d]
	25	69.67±4.16	
	12.5	57.67±4.04	
	6.25	38.00±1.00	

Values with different letters are significantly different at $p < 0.05$

Conclusions

In this study, natural compounds isolated from endophytic fungi *Epicoccum nigrum* inhibited the activity of AChE and the NO production in LPS-stimulated RAW264.7macrophage cells. To our knowledge, the activities of compounds investigated herein are reported for the first time. From this study, new insights on the beneficial therapeutic potential of beauvericin especially towards neurodegenerative diseases are provided.

Abbreviations

AChE: acetylcholinesterase
AChEI: acetylcholinesterase inhibitor
AD: Alzheimer's disease
LPS: lipopolysaccharide
NO: nitric oxide

Authors' Contribution

JPD carried out this study and designed the experiments. RM and ATT isolated the compounds and performed the chemical analysis. JPD and RM wrote the manuscript. DGWFK and BTG contributed to chemical structural elucidation. LJM and JNE supervised the work. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no competing interests.

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