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Neuroprotective and Antioxidant Constituents from *Curcuma zedoaria* Rhizomes

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Abstract: This study investigates the effect of phytochemical constituents from medicinally important plant *Curcuma zedoaria* (Christm.) Rosc., on hydrogen peroxide induced oxidative stress in mouse neuroblastoma-rat glioma hybridoma cells NG108-15. Phytochemical investigation of *C. zedoaria* rhizomes resulted in the isolation of nine sesquiterpenes (germacrone **1**, dehydrocurdione **2**, curcumenol **3**, isoprocurcumenol **5**, curcumenone **6**, procurcumenol **7**, zerumbone epoxide **8**, zederone **9** and gweicurculactone **10**) and one labdane diterpene (zerumin A **4**). Curcumenol (**3**) and dehydrocurdione (**2**) showed 100% protection of the NG108-15 cells at the concentrations of 4 and 10 µM, respectively. Procurcumenol (**7**), isoprocurcumenol (**5**), zerumbone epoxide (**8**), zerumin A (**4**) and germacrone (**1**) showed moderate activity (80-90% protection). In the oxygen radical antioxidant capacity (ORAC) assay, all the test compounds showed strong antioxidant activity except curcumenol (**3**) which showed moderate antioxidant activity, as compared to the reference standard quercetin.

Keywords: Curcumenol; dehydrocurdion; zerumbone epoxide; ORAC assay; NG108-15 cells.

1. Introduction

Increased prevalence of neurodegenerative disorders in the aged population is affecting both economically and the quality of life of the patient and carer. The most prevalent neurodegenerative disorders include Alzheimer's disease (AD) and Parkinson's disease (PD). Scientists have predicted that by the year 2050, 1 in 85 people will be affected by AD, which is characterised by neurofibrillary tangles and senile plaques [1]. Parkinson's disease (PD) is another common neurological disease, in which the dopaminergic neurons in the substantia nigra of midbrain and their terminals in the striatum undergo cell death [2]. It leads to movement-related problems in the early stages, and thinking and behavioural symptoms in the later stages. Both AD and PD are multi-factorial which involve different etiopathogenic mechanisms implicating various genetic and environmental factors [3, 4]. Oxidative

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stress, which is the result of mitochondrial dysfunction, has long been considered to be one of the major causes of neurodegeneration. Being the most energy intensive organ (20% of whole body's need), the brain is highly dependent on mitochondrial aerobic oxidative phosphorylation (OXPHOS) to produce ATP. During OXPHOS, electrons leak from the electron transport chain (ETC) as superoxide ($O_2^{\bullet-}$) and give rise to various reactive oxygen species (ROS) including diatomic oxygen, hydrogen peroxide and hydroxyl radical [5]. In normal physiological condition, 1-2% of our consumed oxygen is converted into ROS, which is neutralised by the body's redox systems. As a second messenger system, the highly diffusible gaseous nitric oxide (NO) plays a vital role in the physiological functions in the CNS. The half-life of NO is very short and can convert into various reactive nitrogen species (RNS), including nitric oxide ($NO^{\bullet}$) by neuronal nitric oxide synthase (nNOS) and nitric oxide ($NO^{\bullet}$) derived free radicals, including peroxynitrile ($ONOO^{-}$), nitrate ($NO_3^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$). Large amount of various ROS and RNS are produced in pathological conditions where the redox homeostasis is insufficient for the neutralisation of excess oxidative radicals [6]. This will then lead to catastrophic events, which include modifications of biomolecules, *e.g.*, lipid peroxidation, DNA mutations, and structural, functional alteration or fragmentation of proteins, leading to protein denaturation and neurodegeneration [7].

Plant extracts, including those rich in antioxidants are of great interest for their therapeutic and preventive role in neurodegenerative disorders. Numerous studies have been done on plant extracts and plant derived natural products for their neuroprotective activity. Some of the most highly studied plants include curcuma, ginger, ginko biloba, cinnamon, saffron, coffee, green tea and berries [8]. Phytochemical constituents including monoterpenes, sesquiterpenes, triterpenes, alkaloids, flavonoids and lignans were found to be the active component for the observed biological activity [9].

Curcuma zedoaria (Christm.) Rosc. (Zingiberaceae), locally known as "temu putih" in Malaysia, is a perennial herb largely found in tropical countries including Malaysia, Indonesia, India, Japan and Thailand. It is one of the medicinally important species from the genus *Curcuma*. Also known as 'Ezhu' in Chinese and 'Krachura' in Sanskrit, *C. zedoaria* is used alone or in combination in the herbal remedies of Malaysia, Indian Ayurveda and Chinese medicine [10]. In traditional medicine, the rhizomes of *C. zedoaria* is used for the treatment of menstrual disorders, dyspepsia, stomachic, vomiting and cancer [11]. The plant is reported to have antimicrobial [12], antiulcer [13], analgesic [14], anti-inflammatory [15], hepatoprotective [16], and cytotoxic [17] activity. The isolated bioactive compounds from *C. zedoaria* include curcumenol, dihydrocurdione, curcumin, dihydrocurcumin, tetrahydromethoxycurcumin, tetrahydrobismethoxycurcumin as the analgesic principles [14], curcumin, demethoxycurcumin, bisdemethoxycurcumin, curcumenol (**3**) as cytotoxic against various cancer cell lines [18, 19], furanodiene, germacrone (**1**), curdione, neocurdione, curcumenol (**3**), isocurcumenol, aerugidiol, zedoarondiol, curcumenone (**6**), curcumin as hepatoprotective [16], curzenone and dehydrocurdione (**2**) as anti-inflammatory agents [20].

Despite numerous studies on *C. zedoaria*, it has never been investigated for neuroprotective activity. In our continuous effort to study the bioactive compounds in medicinal plants used in Malaysia and South East Asian region [21-23], we have attempted the isolation of natural compounds from *C. zedoaria* and evaluated them for neuroprotective activity against H_2O_2-induced oxidative stress in NG108-15 cells. Antioxidant activity of the compounds under investigation was further carried out to correlate their antioxidant ability towards the neuroprotective activity.

2. Materials and Methods

2.1. Plant material

The rhizomes of *Curcuma zedoaria* (Christm.) Rosc. were collected from Tawamangu, Indonesia and a voucher specimen (KL 5764) was deposited at the Herbarium, Department of Chemistry, University of Malaya.

2.2. Extraction and isolation

The air dried, powdered rhizomes (1.0 kg) of *C. zedoaria* was successively extracted by maceration with *n*-hexane (Hex) and dichloromethane (DCM). The *n*-hexane extract (20.2 g, yield

2.4%) was chromatographed on a silica gel column (0.063-0.200 mm) with a gradient elution system using *n*-hexane and ethyl acetate (EtOAc) (100:0-0:100). Based on the TLC pattern, fractions were pooled together to get a total of 21 fractions. Fraction 6 was chromatographed on a silica gel column (0.043-0.063 mm) using a gradient elution system (Hex:EtOAc 100:0-0:100), followed by purification through PTLC (Hex:EtOAc 90:10, 3 times run) to get germacrone (**1**, 21.6 mg). Fraction 7 was subjected to size exclusion chromatography using methanol (MeOH) and DCM in a 1:1 ratio, followed by PTLC (Hex:EtOAc 90:10) and HPTLC (Hex:EtOAc:MeOH 96:3:1) to afford dehydrocurdione (**2**, 34.5 mg). Fraction 8 was separated by PTLC using petroleum ether (40-60°C) (PE) and EtOAc in a ratio of 85:15 for the first run and 82:18 for the second run to get curcumenol (**3**, 15.5 mg), and zerumin A (**4**, 9.8 mg). Isoprocurcumenol (**5**, 10.2 mg) was isolated from fraction 9 by successive development on PTLC using PE:EtOAc in the ratio of 90:10 and 85:15, respectively. Curcumenone (**6,** 16.4 mg) was purified from fraction 12 by PTLC using three times run with PE:EtOAc:MeOH in a ratio of 85:14:1. Procurcumenol (**7**, 8.9 mg) was isolated from fraction 15 as a colourless oil by PTLC (PE:EtOAc:formic acid 85:14.5:0.5). Zerumbone epoxide (**8**, 11.9 mg) was isolated from silica gel (0.043-0.063 mm) column of fraction 16 using a gradient elution system of Hex and EtOAc (100:0-0:100), followed by three times run on HPTLC (Hex:EtOAc:1,4-dioxane (85:14:1). The DCM extract (10 g) was fractionated on a silica gel column (Hex:EtOAc 95:5-0:100) to give 23 fractions. Fraction 2 was subjected to silica gel column (0.043-0.063 mm) with a gradient elution system of Hex and EtOAc (100:0-0:100) followed by RP-HPLC (H_2O:MeOH 40:60, run time 80 min, flow rate 2.5 mL/min) which afforded zederone (**9**, 24.4 mg) and gweicurculactone (**10**, 3.6 mg) at the retention time of 15.26 and 20.16 min, respectively. The spectral data of the isolated compounds (**Fig. 1**) were in agreement with the literature [24-27].

2.3. Cell culture

NG108-15 was obtained from the American Type Culture Collection (ATCC) and cultured in DMEM (Sigma Aldrich) composed of 10% (v/v) heat inactivated FBS, 2% penicillin/streptomycin, 1% amphotericin B (all from PAA, Austria) and HAT (Sigma Aldrich). NG108-15 cells were cultured and conditioned at 5% CO_2 moist atmosphere at 37°C and checked routinely under inverted microscope (Motic) for any contamination. Cells with confluency of 70-80% were selected for the neuroprotective experiment. Cells exposed to vehicle alone (10% FBS DMEM, DMSO ≤ 0.5% v/v) was used as the control group.

2.4. Assessment of neuroprotective activity

The neuroprotective effect of the isolated compounds against H_2O_2-induced apoptosis in NG108-15 cells was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [28]. Cells were plated at a total density of 5×10^3 cells/well in a 96-well plate. The cells were left to adhere for 48 h and treated with test compounds (2 h) prior to H_2O_2 (400 µM) exposure for 24 h. Aliquots of MTT solution (20 µL) was added into each well and incubated at 37°C for another 4 h. The absorbance was measured on a microplate reader (ASYS UVM340) at 570 nm (reference wavelength: 650 nm).

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100\%$$

2.5. ORAC assay

Oxygen radical antioxidant capacity (ORAC) assay was done as described by Cao et al. with slight modification [29]. The assay was performed on a 96-well black microtitre plate, with 25 µL of the samples, standard (Trolox), blank (solvent/PBS) or positive control (quercetin) in each well. Subsequently, 150 µL of working fluorescein solution was added to each well of the assay plate. The plate was incubated at 37°C for 5 min. Aliquot of (25 µL) AAPH working solution was added to each well, making up a total volume of 200 µL. Fluorescence was recorded at an excitation wavelength of 485 nm and emission wavelength of 538 nm. Data were collected every 2 min during an observation period of 2 h, and were analysed by calculating the differences of area under fluorescence decay curve

(AUC) of samples, standard or positive control against blank. The antioxidant capacity was expressed as Trolox equivalent (TE)/100 µg sample.

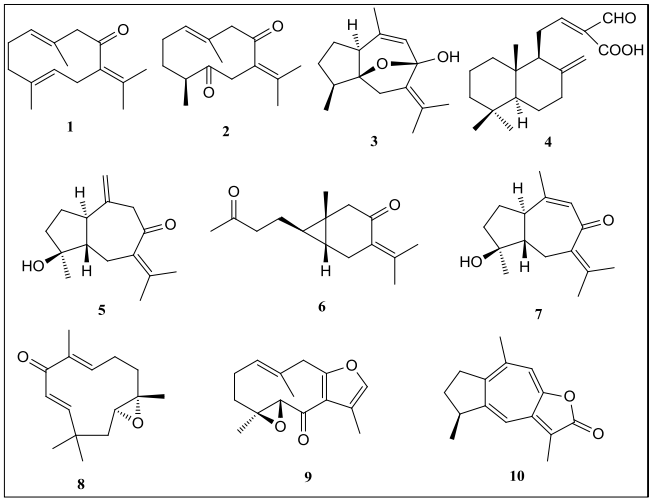

Figure 1. Structures of the isolated compounds from *C. zedoaria* rhizomes.

3. Results and Discussion

As summarized in **Table 1**, all the ten compounds tested, protected the NG108-15 cells from H_2O_2 induced oxidative stress at various degrees. Exposure to H_2O_2 (400 µM) reduced the viability of NG108-15 cells to 67.6% after a period of 24 h. Pretreatment of the cells with curcumenol (**3**) showed the maximum protection (100%) of the cells at the concentration of 4 µM but the effect reduced to 97.7% as the concentration increased to 30 µM. Dehydrocurdione (**2**) also showed strong activity (100% protection) at the concentration of 10 µM, and the activity reduced by either an increase of decrease in the concentration. Germacrone (**1**), zerumin A (**4**), isoprocurcumenol (**5**), procurcumenol (**7**), and zerumbone epoxide (**8**) showed moderate activity (80-90% viability) as compared to the control.

In the ORAC assay, zerumbone epoxide (**8**) showed the highest antioxidant activity with a TE of 35.41 µM/100 µg of sample. Isoprocurcumenol (**5**), zederone (**9**), dehydrocurdione (**2**), germacrone (**1**) showed higher antioxidant capacity than quercetin, used as standard in this assay. While the antioxidant activity of zerumin A (**4**), gweicurculactone (**10**), curcumenone (**6**), procurcumenol (**7**) were close to that of quercetin, curcumenol (**3**) showed a lesser extent of activity (Table 2).

Table 1. Neuroprotective evaluation of compounds against H_2O_2-induced cell death in NG108-15 cells.

Compound	Cell viability (%)					
	1 µM	4 µM	8 µM	10 µM	15 µM	30 µM
Control	100					
H_2O_2	67.63±0.86					
Germacrone (**1**)	79.29±1.05*	77.80±1.15*	81.25±1.72*	79.61±2.38*	89.99±2.01*	78.68±1.39*
Dehydrocurdione (**2**)	82.90±1.77*	90.76±1.45*	98.05±2.33*	100.60±1.72*	99.98±2.60*	88.59±1.75*
Curcumenol (**3**)	78.03±1.23*	103.04±2.17*	100.73±2.63*	100.60±1.72*	99.98±2.60*	97.79±2.41*
Zerumin A (**4**)	77.89±1.95*	77.98±1.09*	82.69±1.12*	86.84±1.76*	91.14±1.42*	74.41±1.45
Isoprocurcumenol (**5**)	76.72±0.88*	79.51±0.91*	76.42±1.61*	77.08±1.07*	80.96±0.91*	75.94±1.01*
Curcumenone (**6**)	66.25±1.50	59.74±1.86	66.93±1.66	73.86±1.18	70.58±1.13	79.18±1.43*
Procurcumenol (**7**)	77.75±0.95*	79.08±1.289*	77.00±1.25*	75.73±2.22*	80.00±0.71*	72.87±0.71
Zerumbone epoxide (**8**)	75.66±0.68*	71.29±1.24	78.15±0.98*	73.66±1.70	74.63±1.27	84.32±1.09*
Zederone (**9**)	62.50±1.41	61.47±1.53	72.41±0.97	76.33±1.19*	70.79±2.88	74.51±0.85
Gweicurculactone (**10**)	59.84±2.19	64.24±1.32	59.44±0.78	67.54±0.89	75.45±1.04	75.21±1.47

All experiments were done in triplicate and results expressed as mean ± S.E.
*P<0.05 vs H_2O_2 treated cells, One way ANOVA followed by Dunnett's test

Table 2. Antioxidant capacity of the compounds in ORAC assay.

Compound	TE*	Compound	TE*
Germacrone (**1**)	24.86 ± 2.33	Procurcumenol (**7**)	20.46 ± 1.88
Dehydrocurdione (**2**)	26.18 ± 2.59	Zerumbone epoxide (**8**)	35.41 ± 2.25
Curcumenol (**3**)	12.62 ± 2.67	Zederone (**9**)	27.78 ± 2.53
Zerumin A (**4**)	19.86 ± 3.92	Gweicurculactone (**10**)	18.26 ± 1.66
Isoprocurcumenol (**5**)	26.43 ± 1.88	Quercetin	21.73 ± 2.87
Curcumenone (**6**)	21.16 ± 2.12		

*TE: Troloxequivalent in μM per 100 μg of sample; Results expressed as mean±SE

In normal physiologic condition, small amount of H_2O_2 is produced during aerobic metabolism in the cell and is neutralised by redox systems of our body. This does not pose significant threat at young age due to the presence of strong antioxidant defence system. However, in aged person, an increased production of ROS and functional decline of neutralising systems creates an imbalance, leading to detectable level of oxidative damages. As a result of cumulative deposition of the pathogenesis, the effects get more noticeable which includes lack of coordination, imbalance, cognitive dysfunction and reduced muscle tone [30].

Use of external H_2O_2 to create oxidative stress in NG108-15 cell is a popular method of studying neuroprotective effect of natural products [31]. Due to the neuronal glial properties, NG108-15, a mouse neuroblastoma-rat glioma hybridoma cell line is used extensively as a neuronal model in electrophysiological and pharmacological research [32]. An excess application of H_2O_2 to NG108-15 cells mimic a similar condition as it takes place during the oxidative stress in the body. Superoxide dismutase (SD), an important enzyme of antioxidant system of the body converts superoxide into H_2O_2 which is further scavenged by catalase or glutathione redox pathway [33]. When there is an increased level of H_2O_2, the neutralisation by catalase or tissue thiols fail, giving rise to various reactive oxygen species. Application of excess exogenous H_2O_2 creates oxidative stress that is beyond the manageable level of the endogenous antioxidant system [32]. Cell death may happen through two major mechanisms, necrosis and apoptosis. Apoptosis is the most noticeable programmed cell death mechanism and is associated with distinct morphological changes, such as membrane blebbing, cell shrinkage and DNA fragmentation. On the other hand, necrosis is the premature death of cells associated with the loss in cell membrane integrity followed by uncontrolled release of cell death products into the intracellular space [34].

In the present investigation, nine sesquiterpenes and one labdane diterpene isolated from the rhizomes of *C. zedoaria* were tested for their possible neuroprotective role in H_2O_2-induced oxidative stress in NG108-15 cells. Among these, the guaiane type sesquiterpene, curcumenol (**3**) showed 100% protection of the cells at 4 μM concentration. The compound showed very little change in its activity even at the concentration of 30 μM, indicating that the compound might not be toxic to the cell within a wide concentration range. Dehydrocurdione (**2**), a germacrane type sesquiterpene also showed maximum protection of the cells at the concentrations of 10 and 15 μM, which reduced by 10% at 30 μM concentration. Curcumenol (**3**) and dehydrocurdione (**2**) are the two major phytoconstituents of *C. zedoaria* and their content in the rhizomes is more than that of any other part of the plant. However, seasonal variation can change the ratio of these two constituents in the plant [14]. Biogenetically, these two compounds are also closely related which is evident from a biomimetic transformation study where dehydrocurdione (**2**) was converted into curcumenol (**3**) in a highly selective manner [35].

In a previous study, curcumenol (**3**) protected hepatic cells from D-GalN-induced cytotoxicity and inhibited LPS (lipopolysaccharide)-induced nitric oxide (NO) production in mouse macrophage [16]. Although inducible NO synthase (iNOS) is not expressed in normal brain, presence of cytokines and LPS can result in its expression in microglia and astocytes. Neuroinflammations trigger immunoresponses resulting in the infiltration of T cells and macrophage at the inflammatory site. These cells release immunomodulatory molecules including NO which leads to a more widespread CNS-injury [36, 37]. A low level of NO is present in brain for cellular signalling, but a high level of NO can be observed in neurodegenerative disorders causing neuronal cell death by inhibiting mitochondrial cytochrome oxidase [38]. Thus, the inhibition of NO production by curcumenol (**3**) can also contribute towards the neuroprotective activity of this compound.

In another study, curcumenol (**3**) was isolated as the major anti-inflammatory agent from *Curcuma phaeocaulis* using COX-2 inhibitory assay [39]. There is strong evidence that COX-2 level increases significantly in neurodegenerative disease conditions. In AD, a direct correlation has been found with the COX-2 level with that of amyloid plaque and neuronal atrophy [36]. While in PD, injection of LPS in brain resulted in the increased level of inflammatory factors including COX-2 and iNOS prior to the death of dopaminergic neurons [40]. Therefore, the neuroprotective effect of the curcumenol (**3**) may be partly due to its anti-inflammatory action stemming through the inhibition of COX-2 enzyme [41].

To correlate the antioxidant activity of the compounds under investigation toward their neuroprotective activity, ORAC assay was performed. ORAC assay is considered as a complementary antioxidant test for neuroprotective assay [30]. It is a reliable method of testing antioxidant activity of biological samples including natural products due to its sensitivity towards broader class of compounds [42]. Moreover, it is the only assay which involves the use of peroxyl radical and quantifies the antioxidant capacity via area under curve (AUC) technique [43]. The result obtained from this assay is from the direct quenching of free radicals, which is related to the antioxidant capacity of the molecule itself [43]. All the compounds (**1**-**10**) tested, showed strong to moderate antioxidant activity in the order of zerumbone epoxide (**8**)> zederone (**9**)> isoprocurcumenol (**5**)> dehydrocurdione (**2**)> germacrone (**1**)> curcumenone (**6**)> procurcumenol (**7**)> zerumin A (**4**)> gweicurculactone (**10**)>curcumenol (**3**). While zerumbone epoxide (**8**), a humulane type sesquiterpene, showed the highest antioxidant capacity (35.41 TE/100 µg sample), and the activity was stronger than that of quercetin (21.16 TE/100 µg sample); in the neuroprotective assay, this compound showed a maximum of 84.32% protection of the cells at the highest concentration tested (30 µM). Interestingly, curcumenol (**3**), which showed the strongest neuroprotective activity (100%) among all the compounds tested, exhibited a moderate antioxidant activity (12.62 TE/100 µg sample). This suggests that the neuroprotective activity of this compound is not the sole contributor from its antioxidant activity, rather a combined effect of its anti-inflammatory, antioxidant and NO-production inhibitory activity. In case of zerumbone epoxide (**8**), the effect might be chiefly due to its antioxidant property. Dehydrocurdione (**2**), the other compound with strong neuroprotective activity, showed an antioxidant capacity of 26.18 TE/100 µg sample, higher than the standard, quercetin.

This is the first report of neuroprotective activity of the constituents from *C. zedoaria* rhizomes. Curcumenol (**3**) and dehydrocurdione (**2**), two major constituent of 'Zedoary turmeric oil' were the most active compounds providing protection of NG108-15 cells. These two compounds have been the attention of researchers for their various pharmacological actions [14]. Present investigation dictates further investigation of curcumenol (**3**), dehydrocurdione (**2**) and other active constituents of *C. zedoaria* rhizomes for *in vitro* and *in vivo* studies to explore their usefulness in the development of potential drug or drug lead for the treatment of neurodegenerative disorders.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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