Neuroprotective activity of galloylated cyanogenic glucosides and hydrolysable tannins isolated from leaves of *Phyllagathis rotundifolia*

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Abstract

The galloylated cyanogenic glucosides based on prunasin (1–7), gallotannins (8–14), ellagitannins (15–17), ellagic acid derivatives (18, 19) and gallic acid (20) isolated from the leaves of *Phyllagathis rotundifolia* (Melastomataceae) were investigated for their neuroprotective activity against hydrogen peroxide (H₂O₂)-induced oxidative damage in NG108-15 hybridoma cell line. Among these compounds, the gallotannins and ellagitannins exhibited remarkable neuroprotective activities against oxidative damage in vitro as compared to galloylated cyanogenic glucosides and ellagic acid derivatives in a dose-dependent manner. They could be explored further as potential natural neuroprotectors in various remedies of neurodegenerative diseases.

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1. Introduction

Neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, stroke, and dementia account for 8% of total death worldwide [1]. These severe neuronal diseases have been found to be caused by oxidative stress-induced cell damage mediated by reactive oxygen species (ROS), mainly superoxide anion and hydrogen peroxide. Antioxidants scavenge the free radicals directly or activate the protective mechanisms inside the cells that prevent the damage reaction on deoxyribonucleic acid, proteins and lipids. Almost all organisms have natural antioxidant defenses such as glutathione peroxidase, catalase and superoxide dismutase [2,3] that prevent oxidative stress. However, these systems are insufficient to prevent the damage entirely [4,5]. The imbalance between antioxidant and free radicals in the body can cause degenerative diseases and neurodegenerative diseases [6].

Hydrogen peroxide (H₂O₂) induces the oxidative stress or apoptosis of various neuron cells such as SK-N-MC neuroblastoma cell line, SH-SY5Y human neuroblastoma cells and PC12 cells which is associated with the implications of ischemic brain, Alzheimer’s disease and other neurological diseases [7–9]. Therefore, plant polyphenols such as tannins play an important role as antioxidants [10] and have demonstrated a variety of biological activities including marked anti-viral, anti-HIV, inhibition of lipid peroxidation, anti-tumor [11], anti-bacterial against *Helicobacter pylori*, anti-methicillin-resistant *Staphylococcus aureus* (MRSA) and anti-leishmanial activity [10,12,13].

*Phyllagathis rotundifolia* (Jack) Bl. is a creeping herb from the family of Melastomataceae. This plant occupies the shady or partly shady places in the lowland and montane forest of Peninsular Malaysia and Sumatra. It has heart-shaped leaves with dark green upper surface and reddish lower surface, short stem and pink or magenta flower [14,15]. Traditionally, a decoction of the leaves of *P. rotundifolia* is used in the treatment of malaria, fever and stomachache, in parturition and as tonic [16–18]. Locally, they are known as “Tapak
Gajah" or “Tapak Sulaiman”. Previous phytochemical analysis on the leaves of *P. rotundifolia* showed the presence of prunasin based cyanogenic glucosides with galloyl esterification together with prunasin, gallo-tannins, ellagitannins and ellagic acid derivatives [19,20]. In this study, we report for the first time the neuroprotective activity of these compounds against H₂O₂-induced oxidative damage in NG108-15 hybridoma cells.

2. Experimental

2.1. Plant material

The leaves of *P. rotundifolia* were collected from Pasoh Forest Reserve, Negeri Sembilan and the reference specimens (A624–A633) were identified and deposited at the specimen collection of Genetic Laboratory in Forest Research Institute Malaysia (FRIM).

2.2. Solvents and chemicals

All reagents and solvents used were of analytical and HPLC grades. All solvents were purchased from Merck, Germany. Catechin as a positive control was purchased from Sigma, USA. The chemicals used in the analysis were Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), penicillin, streptomycin, amphotericin B, Hypoxantine-Aminopterin-Thymidine (HAT) medium, Phosphate Buffered Saline (PBS), accutase enzyme, hydrogen peroxide (H₂O₂), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and analytical grade dimethyl sulfoxide (DMSO).

2.3. Extraction, fractionation and purification

Dried and ground leaves of *P. rotundifolia* (330 g dry weight) were extracted five times using methanol at room temperature which yielded 60.8 g methanol extract after removal of solvent by rotary evaporator at 40 °C (Fig. 1). Then, the methanol extract was suspended in water and extracted consecutively with hexane and ethyl acetate. Each extract was evaporated under reduced pressure at 40 °C and yielded a hexane extract (10.5 g), ethyl acetate extract (11.1 g) and water extract (36.0 g). The ethyl acetate and water extracts were fractionated and purified by a combination of column chromatography utilizing MCI gel CHP 20P column chromatography with a solvent system of methanol (50-100%) in water. These two compounds were purified by preparative HPLC, using a Waters 600 HPLC system (Milford Massachusetts, USA) equipped with a photodiode array (PDA) detector. A Symmetry C-18 reversed-phase semi-preparative column (7.8 i.d.×300 mm, 7 μm) was used. The mobile phase consisted of a linear gradient of acetonitrile in water from 20 to 30% over 25 min at a flow rate of 2.88 ml/min. All the compounds were identified using Bruker DRX 300 NMR spectrometer (300 MHz for ¹H-NMR and 75 MHz for ¹³C-NMR) and the mass of the compounds were determined using LTQ Orbitrap high resolution mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).

2.4. Cell culture

NG108-15 cells were cultured using Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated Fetal Bovine Serum (FBS), 50 IU/ml penicillin, 50 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 100 μM Hypoxanine-Aminopterin-Thymidine (HAT) medium consisting of 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine as complete growth medium. The cells were cultured in 5% CO₂ atmosphere with 95% humidity at 37 °C (CO₂ incubator chamber, RSBiotech).

2.5. Neuroprotective experiment design

NG108-15 cells were raised to confluence, rinsed with Phosphate Buffered Saline (PBS) and harvested by using accutase enzyme. The cells were seeded at concentration of 5×10⁴ cells/well in 96-well plate and incubated in 5% CO₂ at 37 °C for 48 h. After 48 h incubation, the cells were pretreated with compounds 1–20 at various concentrations of 6.25–100 μM for 2 h and followed by 2 mM H₂O₂-induced oxidative damage for 10 h.

2.6. MTT cell viability assay

The NG108-15 cells viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Mitochondrial dehydrogenases converted the MTT to insoluble purple formazan crystals whereas the formation of purple formazan crystals was proportional to the cell viability. 20 μL of 5 mg/ml MTT solution was added and incubated for 4 h. The medium was discarded and 150 μL of DMSO was
added to dissolve the formazan crystal. Each plate was shaken to
dissolve the formazan crystals and the absorbance values were
measured at 570 nm (650 nm as reference wavelength) by
using a microplate reader (ASYS UVM340). The percentage of
cell viability was calculated according to the equation below:

\[
\text{Percentage of cell viability} = \left( \frac{A_0}{A_c} \right) \times 100 \%
\]

where, \( A_0 \) is the absorbance of treated cells and \( A_c \) is the
absorbance of control cells.

2.7. Statistical analysis

Results were given as means ± SE values. Three indepen-
dent experiments were conducted for each sample in tripli-
cate. One way analysis of variance (ANOVA) follow by
Dunnett’s multiple comparisons were carried out and the sta-
tistical significant limit was set at \( p < 0.01 \).

3. Results and discussion

NG108-15 cells are formed by Sendai virus-induced fu-
sion of the mouse neuroblastoma clone N18TG-2 and rat gli-
oma clone C6 BV-1. This neuroblastoma-glioma hybrid cell
line has been commonly applied for neuronal model in elec-
trophysiology and pharmacology research [21-23]. Nearly
all sources of oxidative stress generated \( \text{H}_2\text{O}_2 \). \( \text{H}_2\text{O}_2 \)-induced
DNA damage via fenton reaction in the presence of oxygen
and transition metal ion [24] and the toxicity were attributed
to its high membrane permeability [23,25]. It acted not only
as precursor for oxidizing radicals such as hydroxyl radicals
[26] but also affected various mechanisms such as perturbing
intracellular calcium homeostasis [27] and intracellular ATP
[28], inducing DNA damage [29] as well as apoptosis of the cells [30].

In the present study, sample pretreatment at various concentrations were performed to evaluate the neuroprotective effect of compounds against H2O2-induced oxidative damage. Exposure of NG108-15 cells to H2O2 leads to the reduction in cell viability significantly by 45.94%±1.24 as compared to control. Results showed that all the treated samples displayed a dose dependent manner in the neuroprotection model. The chemical structures of compounds (1–20) isolated from the leaves of *P. rotundifolia* are presented in Fig. 2. Neuroprotective activities of galloylated cyanogenic glucosides (1–7), gallotannins (8–14), ellagitannins, ellagic acid derivatives and aromatic compounds (15–20) are shown in Figs. 3, 4 and 5 respectively. As shown by the MTT cell viability assay, these compounds demonstrated neuroprotective activities against oxidative damage in NG108-15 cells challenged by H2O2 induction. Catechin was selected as a positive control in the present study since it has been reported as a neuroprotective agent [31].

Galloylated cyanogenic glucosides (1–7) in *P. rotundifolia* were first discovered by Ling et al. in 2002 [19], but there is yet no report on the study of neuroprotective activity for these compounds. This study demonstrated that they protected NG108-15 cells in a concentration-dependent manner when the cells were exposed to H2O2. Nevertheless, as compared to catechin, these compounds showed less neuroprotection on the cell damage except for prunasin 2′,3′,4′,6′-tetra-O-gallate (7). As shown in Fig. 3, the percentage of cell viability decreases from prunasin-trigallate (5–6), prunasin-digallate (2–4) to prunasin-monogallate (1). Among the two prunasin-trigallate isomers, prunasin 2′,3′,6′-tri-O-gallate (5) possessed higher neuroprotective activity as compared to prunasin 3′,4′,6′-tri-O-gallate (6). On the other hand, prunasin 2′,6′-di-O-gallate (2) has lower neuroprotective activity as compared to the other two isomers of prunasin-digallate (3–4). The mono-galloylated cyanogenic glucosides, prunasin 6′-O-gallate (1) showed lower neuroprotective effects against oxidative damage in the NG108-15 cells. However, it significantly protected the cells from oxidative damage at the concentration of 50 μM and 100 μM.

Similarly, the gallotannins (8–14) also increased the neuroblastoma-glioma hybrid cell viability in a dose-dependent manner (Fig. 4) which is in accordance to several reported neuroprotection studies [32,33]. The compound 1,2,3,4,6-penta-O-galloyl-β-D-glucose (14) and 1,2,3,6-tetra-O-galloyl-β-D-glucose (13) significantly inhibited H2O2-induced neuron cells damage in a dose-dependent manner at concentrations of 6.25–100 μM. The inhibitory activity of 1,2,3,6-tetra-O-galloyl-β-D-glucose (13) was comparable to that of catechin. However, the neuroprotective activity of 1,2,3,4,6-penta-O-galloyl-β-D-glucose (14) was more potent than that of catechin. This compound has also been reported to not only increase the cellular resistance to H2O2 but also highly protected neuronal cells from H2O2-induction damage via induction of HO-1 gene expression [32].

![Fig. 2. Compounds (1–20) isolated from the Phyllagathis rotundifolia.](image-url)
As shown in Fig. 4, the neuroprotective activity decreased when the number of galloyl esterification with glucose moieties was reduced, from trigalloyl-glucose (10–12) to 3,6-di-

O-galloyl-D-glucose (9) and 6-O-galloyl-D-glucose (8). Compounds 3,4,6-tri-O-galloyl-D-glucose (12), 1,2,3-tri-O-galloyl-β-D-glucose (10) and 1,4,6-tri-O-galloyl-β-D-glucose (11) presented noticeable differences in neuroprotective activity although they are the isomers of trigalloyl-glucose.

Ellagitannins are hydrolysable tannins that contained the hexahydroxydiphenoyl (HHDP) group. These compounds (15–17) also showed remarkable inhibition on the H$_2$O$_2$-induced oxidative damage in NG108-15 cells (Fig. 5) as compared to catechin. They highly protected the neuron cells at concentrations ranging from 6.25 to 100 μM in dose-dependent manner. The sequence of high to low neuroprotective activity of ellagitannins was in order of pterocarinin C (17), praecoxin B (16) and 6-O-galloyl-2,3-O-(S)-hexahydroxydiphenoyl-D-glucose (15). This result indicated that the number of galloyl esterification might influence the neuroprotective activity. The gallic acid (20) possessed similar level with 3’-O-methyl-3,4-methylenedioxyellagic acid 4’-O-β-D-glucopyranoside (18) and 3,3’,4-tri-O-methylellagic acid 4’-O-β-D-glucopyranoside (19) against H$_2$O$_2$-induced oxidative damage in NG108-15 cells. Among the ellagic acid derivatives (18–19), 3,3’,4-tri-O-methyl-ellagic acid 4’-O-β-D-glucopyranoside (19) showed considerably higher neuroprotective activity (Fig. 5). At the concentration of 100 μM, the compounds showing comparable neuroprotective activity with the positive control were in the sequence of 14 > 7 > 17 > 16 > 13 > 10 > 5 > 15 > 11 > 6 > catechin. Among them, three belonged to the galloylated cyanogenic glucosides, four belonged to the gallotannins and the remaining three compounds were ellagitannins.

Catechin is also related to the family of flavonoids and the sub-group flavan-3-ol. The antioxidant potential of catechin was mainly attributed to the OH substitution at 3’ and 4’ which provided the peroxyl radical absorbing activity [34].
while the hydroxyl group in position 3 has little influence in antioxidant due to its spatial position [35]. Other features in flavonoids such as 3’,4’,5’-triOH in ring B and 5,6-diOH 5,8-diOH, 6,7-diOH, 7,8-diOH, 5,6,7-triOH, 5,6,8-triOH, 5,7,8-triOH, 6,7,8-triOH and 5,6,7,8-tetraOH in ring A were also expected to impart the scavenging activity [36]. The oxidation potential was influenced by the position and numbers of hydroxyl group which played a role as hydrogen donating hydroxyl in radical neutralization [35,36]. The catechol B-ring particularly involved in electron transfer from the phenolate was also important and is most significant in scavenging ROS [37,38]. The study of bioavailability in rats showed that the main catechin metabolites were the glucuronidated derivatives [39], whereas methylated catechin metabolites were found mainly in the liver but much lesser in plasma and urine [40]. The hydrolysis of β-glycosidic bonds in the flavonoids glycosides only occurred in the colon by microorganisms and the aglycones were believed to be able to pass through the gut wall [41]. However, the mechanism for transporting the aglycones across the gut wall was still lacking [41]. The O-methylation of hydroxyl in the metabolism of catechin by enzyme catechol O-transf erase in catechol B-ring resulted in a decrease of the antioxidant activity [34,37]. Nevertheless, these metabolites retained significant radical scavenging activity which acted as better electron and hydrogen donors at pH 7.4 [37]. Their antioxidant activity was also strongly dependent on the pH of the medium which increased at greater pH values [37]. Thus, a potent neuroprotective compound is not only dependent on antioxidant activity but also capable to provide proper hydrophobicity that allows the permeability of membrane cell. Apart from the hydrophobicity, the number, pattern and position of hydroxyl, presence of unsaturated 2–3 bond in conjunction with 4-oxo, the number, position and structure of sugar and steric effect of the compounds [36,38,42], other factors such as phenolic O–H bond dissociation enthalpy (OH–BDE), energy-eigenvalue of the highest occupied molecular orbital (E_HOMO) and ionization potential (IP) were also important in determining the scavenging activity of antioxidant [43,44].

Our results indicated that as the number of galloyl esterification is increased in a compound, the neuroprotective activity is also increased accordingly [11]. The o-dihydroxy group is capable of donating hydrogen or electron and reducing iron [45,46]. However, the configuration and position of galloyl esterification would also affect the neuroprotective activity although these compounds have the same number of galloyl unit, possibly due to the spatial hindrance of the molecules which prevented from the free radicals or iron reaction [45]. It was suggested that the scavenging effects of tannins were generally dependent on the molecular size and the number of hydroxyl groups with orthodihydroxyl (catechol) and ortho-trihydroxyl (pyrogallol) structure in the molecule [11,12,36]. The antioxidant activities of hydrolysable tannins were also affected by their oxidation level [47]. The twenty compounds isolated from the leaves of *P. rotundifolia* showed potential as neuroprotective agents. However, the absorption, pharmacokinetics, bio-transformation and bioavailability of these type of compounds have not been well studied. Further investigation is required to ascertain the neuroprotective ability of these compounds.

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