Original Article

Eurycomanone from *Eurycoma longifolia* Jack upregulates neurotrophin-3 gene expression in retinal Müller cells *in vitro*

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Photoreceptor degeneration decreases light sensitivity and leads to vision loss and various retinal diseases. Neurotrophin-3, originating from Müller glial cells in the retina, plays a key role in protecting photoreceptors from damage induced by light or hypoxia. This neuroprotective approach is important because there are no established methods to regenerate lost photoreceptors. Dietary supplements are one of the useful methods for improving eye health. *Eurycoma longifolia* (*E. longifolia*) Jack, which is native to the tropical forest of Malaysia and other Southeast Asian countries, exhibits several medicinal properties. In the present study, we demonstrated that the water extract of *E. longifolia* roots enhanced neurotrophin-3 gene expression in primary rat Müller cells. Using a stepwise bioassay-guided fractionation and purification of *E. longifolia* root extracts, we isolated the active compound underlying neurotrophin-3 gene-enhancing activities. Mass spectrometry and nuclear magnetic resonance spectral data identified the compound as eurycomanone. This study provides evidence for the efficacy of *E. longifolia* and eurycomanone in enhancing neurotrophin-3 expression in Müller cells *in vitro*. Although the biological significance of this effect and its underlying mechanism remain to be elucidated, this study suggests that *E. longifolia* may be promising for improving eye health and must be further investigated.

Key Words: neurotrophin-3, photoreceptor, retina, glia, functional food

The retina contains a specialized type of neuroepithelial cells called photoreceptors that convert light into electrical signals that the brain can interpret. Degeneration of photoreceptors significantly reduces the light-sensing ability of the retina, leading to vision loss and, consequently, a significantly reduced quality of life.[1] Müller glial cells in the retina provide structural stability and trophic support for photoreceptors.[2,3] They also play an important role in the maintenance of homeostasis in the retina and the maintenance of ions, water, pH levels, and the recycling of neurotransmitters.[2]

Neurotrophin-3 (NT3) belongs to the family of neurotrophic factors, which includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-4, and mainly binds to the tropomyosin receptor kinase C (TrkC). Müller cells are the main source of neurotrophin production, including NT3, which is supplied to adjacent photoreceptor cells. Notably, Müller cells and NT3 are key factors in the protection against and treatment of diseases involving photoreceptor degeneration.[4,5] LaVail *et al.*[4] reported that the administration of exogenous NT3 considerably protected photoreceptor cells from light-induced damage in rats. Moreover, NT3 produced by Müller cells protects against photoreceptor apoptosis caused by hypoxia-induced oxidative stress in mice.[5] Harada *et al.*[3] demonstrated that light-induced damage promotes the expression of TrkC but not TrkA, TrkB, or p75[NTR] in rat photoreceptors. These findings suggest that NT3 may contribute more directly to photoreceptor cell protection compared to NGF or BDNF. Overall, these findings suggest that increased NT3 expression in Müller cells may protect photoreceptors. Moreover, efficient radical treatments to fully restore photoreceptor loss are lacking,[6] warranting the identification of effective neuroprotective approaches against retinal pathologies.

Several studies[7] have demonstrated the efficacy of dietary supplement-based interventions in improving eye health. Carotenoids, such as lutein and zeaxanthin, which are found in plants, have been reported to slow the progression of certain retinal pathologies, such as age-related macular degeneration (AMD).[8] Therefore, it is worth exploring food materials that support eye health. *Eurycoma longifolia* (*E. longifolia*) Jack, popularly known as "Tongkat Ali", native to the tropical forest of Malaysia and other Southeast Asian countries, has several medicinal properties. The roots of *E. longifolia* have been traditionally used as herbal medicine against several diseases, including male infertility,[9–12] malaria,[13] cancer,[14,15] obesity,[16] mental stress,[17] and muscle disorders.[9] The safety of dietary supplementation of *E. longifolia* has also been reported.[18,19] Eurycomanone, a quassinoid, is the major active ingredient in *E. longifolia*. Similar to the crude extracts of *E. longifolia*, eurycomanone exhibits several physiological activities.[20] Therefore, eurycomanone is often utilized as an indicator of quality in many commercially available *E. longifolia* extracts.[21]

In our preliminary experiments, we conducted *in vitro* screening to assess the ability of several plant extracts with the potential to enhance *NT3* expression. Subsequently, we observed significant activity in *E. longifolia*, prompting us to further

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doi: 10.3164/jcbn.23-73
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J. Clin. Biochem. Nutr. | **May 2024** | vol. 74 | no. 3 | 199–206

investigate it. Therefore, in the present study, we aimed to explore the efficacy of the water extract of *E. longifolia* roots in enhancing *NT3* expression in Müller cells and identify its bioactive compound.

Materials and Methods

Materials and reagents. The standardized water extract of *E. longifolia* root was obtained from a commercial batch of Physta® from ASK Intercity Co., Ltd. (Ichikawa, Japan). The Physta® *E. longifolia* extract powder contained 0.8–1.5% eurycomanone, ≥35% glycosaponin, ≥30% polysaccharide, and >22% protein. Eurycomanone (CAS 84633-29-4, purity >94.8%) used in the *in vitro* assay was purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). Quassin (CAS 76-78-8; purity >95%) was purchased from Toronto Research Chemicals (Toronto, Canada).

Preparation of a standardized plant extract library for screening. The standardized plant extract library owned by FANCL Corp. (Yokohama, Japan) was used in this study. It was prepared as follows: plant extracts (10 mg) were dissolved in 10 ml dimethyl sulfoxide (FUJIFILM Wako Pure Chemical Corp.), the samples were then centrifuged at 10,000 rpm for 10 min to remove any residues, and the supernatants were collected in microplates and stored at −80°C until assay.

Cell culture. Primary Müller cells were cultured as previously described,[22] Briefly, retinas were isolated from 10 seven-day-old Sprague–Dawley rats (either sex) (Japan SLC, Inc., Hamamatsu, Japan) and collected in 15 ml tubes containing HBSS (Gibco, Thermo Fisher Scientific, Waltham, MA) on ice. The protocol for retinal isolation was approved by the Ethics Committee of Yokohama University of Pharmacy (approval number: 2020-004). The isolated retinas were trypsinized with 0.25% trypsin-EDTA (Sigma-Aldrich, St. Louis, MO) in a 37°C water bath for 30 min, mechanically dissociated into small pieces, and centrifuged at 2,400 rpm for 5 min. The supernatant was aspirated, and the cell pellet was suspended with an appropriate volume of DMEM-F12 (Gibco) containing 10% fetal bovine serum (Corning Inc., Corning, NY) and 1% penicillin/streptomycin (Sigma-Aldrich) (hereafter referred to as complete medium). After filtering with a 100 μm cell strainer (Falcon, Corning), the number of viable cells was counted using trypan blue staining. The cells were then transferred equally into 3–5 cell culture flasks of 75 mm² (Sumitomo Bakelite, Tokyo, Japan) containing 10 ml complete medium and cultured in an incubator (37°C, 5% CO₂). On Day 3, 10 ml of the complete medium was added to each flask. On Day 7, Müller cells were purified according to the incomplete pancreatic enzyme digestion method described by Song *et al.*[23] On Day 8, purified Müller cells were trypsinized, seeded into cell culture plates, and incubated overnight. All assays using Müller cells were performed on Day 9.

Screening of plant extracts enhancing *NT3* expression in retinal Müller cells. Primary Müller cells seeded in 96-well cell culture plates (Falcon) were prepared for screening. The medium was aspirated, and cells were incubated for 90 min in DMEM-F12 containing 1% fetal bovine serum to induce serum-starved condition. Serum starvation was performed to decrease factors in the serum that could affect NT3 expression and to increase responsiveness to the test substance. Each screened material was then added to the medium at a final concentration of 10 μg/ml. After 90 min of treatment, the culture medium was aspirated, and the cells were lysed in RLT buffer (Qiagen, Hilden, Germany). Total RNA was isolated, and a real-time polymerase chain reaction (real-time PCR) was performed to analyze *NT3* expression. Materials that showed activity were confirmed for reproducibility and concentration dependence using cells seeded in 48-well cell culture plates (Sumitomo Bakelite).

RNA isolation and real-time PCR. Total RNA was isolated from cultured Müller cells using the Qiagen RNeasy 96 Kit (96-well plate assay) or the RNeasy mini Kit (48-well plate assay) according to the manufacturer's instructions. DNase I (Qiagen) was used to remove DNA contaminants during RNA purification. Total RNA was reverse transcribed using a Prime-Script™ RT reagent Kit (Takara Bio Inc., Shiga, Japan) in a 10 μl reaction. Real-time PCR was performed on the QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific) using TaqMan Fast Advanced Master Mix and Gene Expression Assays, specifically # Rn00579280_m1 (*NT3*) and # Rn01462662_g1 (*GAPDH*). The mRNA expression level of *NT3* was normalized to that of the reference housekeeping gene *GAPDH* in the same samples. Data were analyzed using the comparative Ct method.[24]

Fractionation and isolation of pure compounds from *E. longifolia* extract. The *E. longifolia* root extract was purified using a stepwise purification procedure (Fig. 1). The fractions in each step were evaluated for their bioactivity to enhance *NT3* expression, and those showing positive effects were subjected to the next step of fractionation. Briefly, the water extract of *E. longifolia* roots (55 g) was partitioned into ethyl acetate and water fractions using liquid–liquid extraction (LLE; step-I). The biological activity of the ethyl acetate and water fractions was evaluated by adding 10 and 50 μg/ml of the fractions, respectively, to primary Müller cells and assessing their effects on *NT3* expression. The active water fraction was further partitioned into n-butanol and water fractions using LLE (step-II), and the resultant fractions were tested for their bioactivity. The active fraction obtained in step-II was treated with 30% ethanol and separated into soluble and insoluble (residue) parts by filtration (step-III). After testing the bioactivity of the fractions at step-III, the soluble part was subjected to a solid-phase extraction using a Strata® C18-E column (55 μm, 70A 50 g/150 ml, Phenomenex Co., Torrance, CA). The column was initially equilibrated with water, followed by elution with a stepwise gradient of 0, 5, 10, 20, and 50% of methanol in water (step-IV). The resulting five fractions were designated fractions A through E (Fr. A–E) and subjected to bioactivity assay. The bioactive fractions were further purified by HPLC using a Capcellpack C18 UG-120 column (5 μm, 20 mm I.D. × 250 mm, Osaka Soda Co., Ltd., Osaka, Japan) (step-V). The separation was carried out using 10% acetonitrile aqueous solution and 0.1% formic acid as the mobile phase and a flow rate of 20 ml/min. Detection was performed at 240 nm using UV spectroscopy. The resulting five fractions were designated fractions BC-1 to BC-5 and subjected to bioactivity assay.

Identification of bioactive compounds of *E. longifolia*. To identify the active compounds in the bioactive fraction of the *E. longifolia* root extract, the *NT3* expression-enhancing fraction obtained by preparative HPLC of the fractions obtained at step-V was subjected to liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance spectroscopy (NMR) analyses. The retention time, UV spectrum, and molecular weight of the compounds were determined using LC-MS (Waters Co., Ltd., Milford, MA) analysis using a CapcellCORE C18 column (2.7 μm, 2.1 mm I.D. × 100 mm; Osaka Soda) with a mobile phase containing 7% acetonitrile aqueous solution and 0.1% formic acid and a flow rate of 0.35 ml/min. MS was performed under the following conditions: Ion mode electrospray ionization (ESI) (+/−); Capillary voltage, 3.0 kV; Cone voltage, 25 V; Desolvation Temp, 400°C; Desolvation gas, 800 L/h; Cone gas, 20 L/h; Acquisition mode, MSSCAM. NMR spectral data were acquired at 298 K and recorded on a JEOL JNM-ECS400 or JNM-ECX-400 spectrometer for ¹H (400 MHz) and ¹³C (100 MHz) spectra. ¹H NMR chemical shifts (referenced to residual solvent signals CD₂HOD: δH 3.31, CD₂HOH: δH 3.31, CHCl₃: δH 7.26) and ¹³C NMR chemical shifts (referenced to the

Eurycoma Longifolia root extract
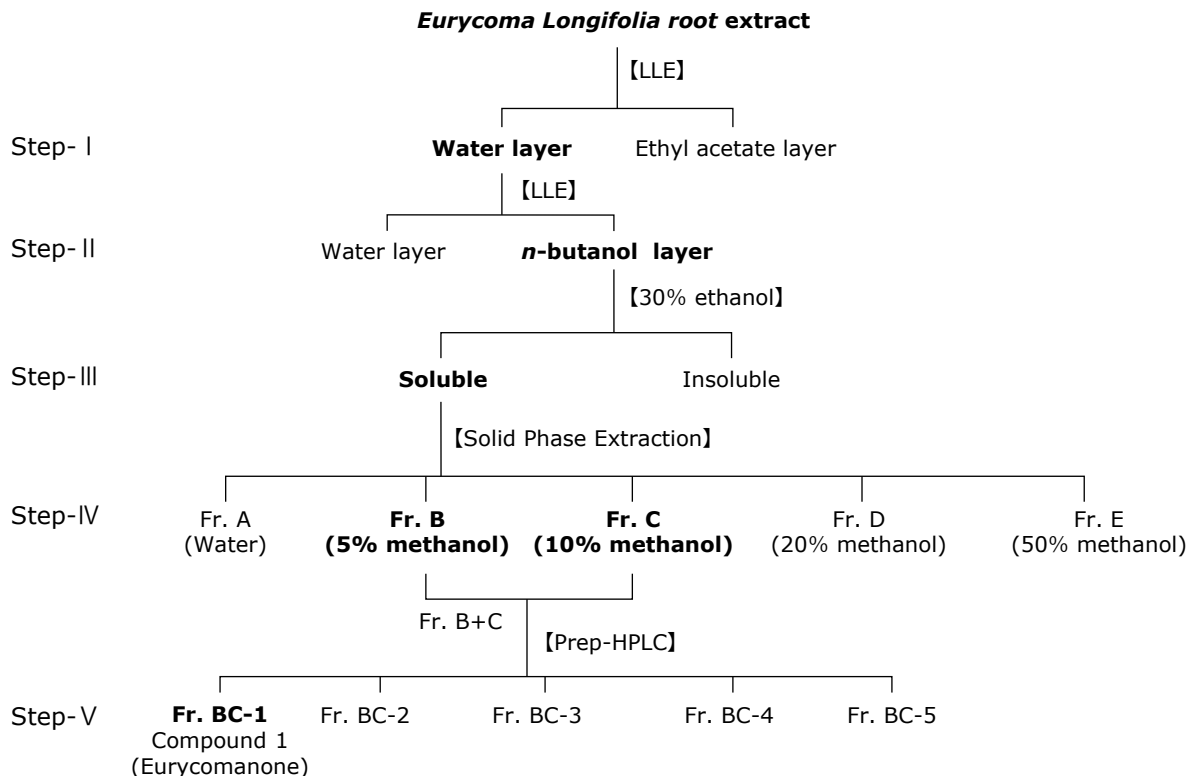
Fig. 1. Stepwise procedure for the purification of *Eurycoma longifolia* extract. Active fractions are indicated in bold. LLE, liquid–liquid extraction; Fr, Fraction; Prep-HPLC, preparative HPLC.

residual solvent signals. CD$_3$OD: δC 49.0, CD$_3$OH: δC 49.0, and CDCl$_3$: δC 77.16) were assigned using a combination. To determine the concentration of the calibration compound, each substance was dissolved in a CD$_3$OD solution. Eurycomanone purchased from Cayman Chemical (Ann Arbor, MI) was used as the standard.

Experimental instrumentation. The analytical equipment used in this study was as follows: NMR spectra were recorded using JEOL JNM-ECS400 and JNM-ECX-400 FT NMR spectrometers. Mass spectra were obtained using a Waters LC-MS system equipped with a photodiode array detector (UPLC-H-Class) and mass analyzer (Xevo-TQD). Preparative LC was performed using a multiple preparative HPLC LC-FORTE/R. LC was performed using a JASCO extrema HPLC system.

Statistical analysis. All data with error bars are expressed as means ± SD of three independent experiments. For comparisons between more than two groups, statistical analyses were performed using a one-way analysis of variance (ANOVA), followed by Dunnett's test. Differences were considered statistically significant at $p<0.05$. The data without error bars were expressed as the mean of two independent experiments.

Results

Identification of plant extracts that enhance *NT3* expression in Müller cells. Screening of potential *NT3* expression enhancers revealed that the water extract of *E. longifolia* roots enhanced *NT3* expression in Müller cells. The addition of 10 and 50 μg/ml *E. longifolia* extract to the culture medium resulted in a 1.24- and 1.75-fold increase in gene expression, respectively, compared to that in the control (Fig. 2).

Fractionation and isolation of bioactive compounds from *E. longifolia*. *E. longifolia* water extract was partitioned into ethyl acetate and water fractions using LLE. Treatment of
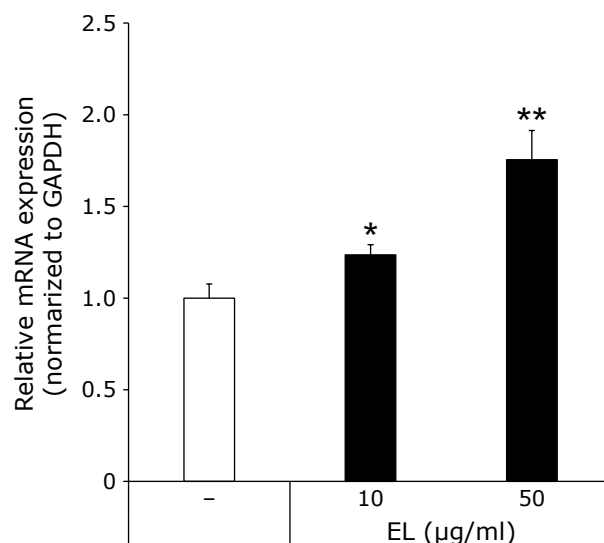
Fig. 2. Effects of *Eurycoma longifolia* on *NT3* expression in Müller cells. Rat primary Müller cells were treated with water extract of *E. longifolia* root. After incubation for 90 min, cells were lysed, and *NT3* mRNA levels were analyzed using real-time PCR. Values are represented as the mean ± SD of three independent experiments, normalized to *GAPDH* mRNA levels. *$p<0.05$, **$p<0.01$ vs control. EL, the water extract of *E. longifolia* root.

primary Müller cells with the resulting water fraction enhanced *NT3* expression (Fig. 3A). The water fraction was further partitioned into *n*-butanol and water using LLE, and the resulting *n*-butanol fraction was active (Fig. 3B). The soluble portion (4.61 g) of concentrated residue from the *n*-butanol fraction,
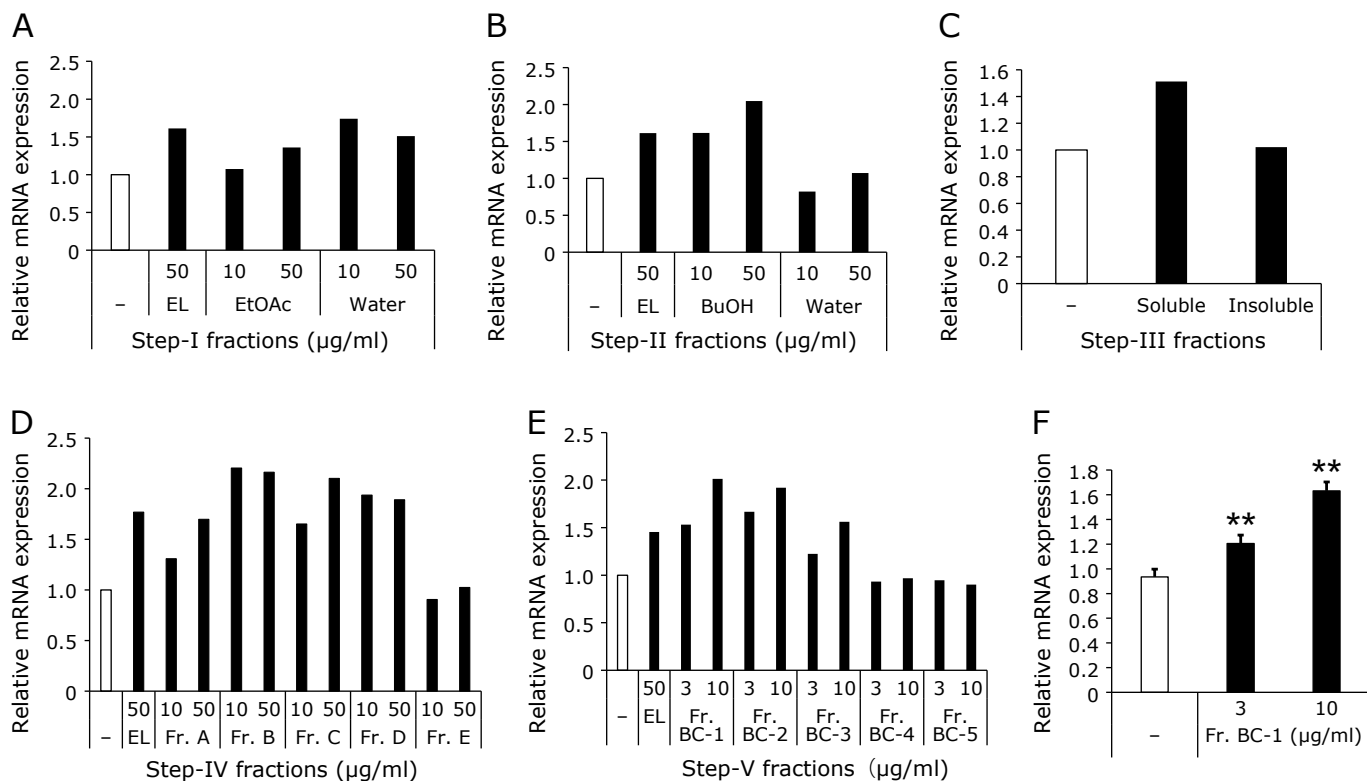
Fig. 3. Effects of fractions obtained from *Eurycoma* extract on *NT-3* expression in Müller cells. Rat primary Müller cells were treated with each fraction obtained from the *E. longifolia* purification process. Each graph shows the effect of (A) fractions obtained from LLE to ethyl acetate and water (step-I), (B) fractions obtained from LLE to n-butanol and water (step-II), (C) the soluble and insoluble parts separated from the n-butanol layer treated with 30% ethanol (step-III), added at 10 µg/ml respectively, (D) fractions obtained from solid-phase extraction of the soluble part and elution using a stepwise gradient of 0, 5, 10, 20, and 50% methanol (step-IV), and (E) the fractions obtained from preparative HPLC of Fr. BC (step-V). (F) Confirmed result of the activity of Fr. BC-1. After incubation for 90 min, the cells were lysed, and *NT-3* expression levels were analyzed via real-time PCR. Data values without error bars are expressed as the mean of two independent experiments, normalized to *GAPDH* mRNA levels (A–E). Data values with error bars are represented as the mean ± SD of three independent experiments, normalized to *GAPDH* mRNA levels (F). *$p < 0.05$, **$p < 0.01$ vs control. EL, the water extract of *E. longifolia* root; LLE, liquid–liquid extraction; EtOAc, ethyl acetate; BuOH, n-butanol; EtOH, ethanol; MeOH, methanol.

treated with 30% ethanol, was also active (Fig. 3C). The soluble part (3.15 g) of the *n*-butanol fraction was then subjected to solid-phase extraction, and five fractions were obtained. Of these, Fr. B (5% methanol) and Fr. C (10% methanol) were active (Fig. 3D). As their chromatographic profiles showed a similar pattern (Supplemental Fig. 1*), Fr. B and Fr. C were mixed into a single fraction, termed Fr. BC (839.36 mg), and subjected to further purification. Although Fr. A (water) and Fr. D (20% methanol) also showed activity (Fig. 3D), their chromatogram showed multiple small peaks in addition to those common to Fr. B and Fr. C. Thus, Fr. BC was further purified via preparative HPLC and five fractions were obtained—Fr. BC-1 to Fr. BC-5. Of these five fractions, Fr. BC-1, Fr. BC-2, and Fr. BC-3 exhibited activity (Fig. 3E). The chromatogram of these active fractions is shown in Supplemental Fig. 2*. The yields of these three active fractions were 64.12 mg (Fr. BC-1), 8.62 mg (Fr. BC-2), and 16.52 mg (Fr. BC-3). The active compound in Fr. BC-1 with the highest yield was designated compound 1; it exhibited significant activity (Fig. 3F) and was selected for further investigation as a potential bioactive component in *E. longifolia*.

Identification of the bioactive principle. Fr. BC-1 was concentrated, and the residue was collected to obtain compound 1, a colorless powder. From 55 g of *E. longifolia* root, 64.12 mg of compound 1 was obtained. It was characterized based on LC-MS and NMR data and compared with the literature. Compound 1 exhibited ESI-MS spectra, with peaks at *m/z* = 409 (M+H) and 407 (M−H). UV spectroscopy showed that compound 1 showed

maximum absorbance at 244 nm (Waters LC-MS). The molecular weight of compound 1 was determined to be 408.4, which is the same as that of the quassinoid eurycomanone (MW: 408.4),[25] a known component of *E. longifolia*. Furthermore, the LC-MS retention time and UV spectrum of compound 1 showed the same profile as those of commercial eurycomanone used as a standard. Compound 1 was further characterized using NMR to confirm that it was eurycomanone. ¹H and ¹³C NMR spectral data for compound 1 and eurycomanone are shown in Table 1. The ¹H-NMR spectrum of compound 1 indicated signals of 4 oxymethine protons δ 3.96 (s, ¹H, H-12), 4.31 (s, ¹H, H-1), 4.80 (dd, ¹H, H-7), and 4.73 (s, ¹H, H-15); 3 olefin protons δ 5.44 (d, ¹H), 5.56 (d, ¹H), and 6.06 (q, ¹H, H-3); and 2 methyl protons δ 2.03 (d, ³H, H-18) and 1.20 (s, ³H, H-19). Two protons of the oxymethylene group (-CH₂O-) were determined by resonance signals at δ 3.84 (¹H, d, H-20a) and 3.68 (¹H, d, H-20b), respectively. The ¹³C-NMR spectrum of compound 1 displayed a structure with 20 carbons and indicated signals of 4 olefinic carbons δ 121.5 (C-21), 126.0 (C-3), 146.1 (C-13), and 165.2 (C-4); 2 methyl carbons δ 10.0 (C-19) and 22. 9 (C-18); 7 oxygenated carbons δ 71.7 (C-7), 84.3 (C-1), 80.8 (C-12), 79.3 (C-14), and 77.1 (C-15); 2 carbonyl groups δ 174.5 (C-16) and 198.9 (C-2); and acetal carbon δ 109.2 (C-11). Based on these results, compound 1, which showed similar profiles to eurycomanone, was identified as eurycomanone (Table 1 and Fig. 4). The chemical structure of eurycomanone (compound 1) is shown in Fig. 5.

Table 1. ^{1}H and ^{13}C NMR spectral data for compound 1 and commercially available eurycomanone (400 and 100 MHz in CD$_3$CD)

No.	Compound 1 ^{1}H δ ppm	Compound 1 ^{13}C δ ppm	Eurycomanone ^{1}H δ ppm	Eurycomanone ^{13}C δ ppm
1	4.31 (s, ^{1}H)	84.3	4.31 (s, ^{1}H)	84.3
2	—	198.9	—	198.9
3	6.06 (q, J = 1.4 Hz, ^{1}H)	126.0	6.06 (q, J = 1.4 Hz, ^{1}H)	126.0
4	—	165.2	—	165.2
5	3.05 (dd, J = 2.7, 12.3 Hz, ^{1}H)	42.8	3.05 (dd, J = 2.7, 12.3 Hz, ^{1}H)	42.8
6	2.09 (ddd, J = 2.9, 12.3, 15.0 Hz, ^{1}H) 2.33 (ddd, J = 2.7, 2.9, 15.0 Hz, ^{1}H)	26.1	2.09 (ddd, J = 2.9, 12.3, 15.0 Hz, ^{1}H) 2.33 (ddd, J = 2.7, 2.9, 15.0 Hz, ^{1}H)	26.1
7	4.80 (dd, J = 2.9, 2.9 Hz, ^{1}H)	77.1	4.80 (dd, J = 2.9, 2.9 Hz, ^{1}H)	77.1
8	—	53.1	—	53.1
9	3.07 (s, ^{1}H)	48.0	3.07 (s, ^{1}H)	48.0
10	—	46.5	—	46.5
11	—	109.2	—	109.2
12	3.96 (s, ^{1}H)	80.9	3.96 (s, ^{1}H)	80.9
13	—	146.1	—	146.1
14	—	79.3	—	79.3
15	4.73 (s, ^{1}H)	71.7	4.73 (s, ^{1}H)	71.7
16	—	174.5	—	174.5
17	—	—	—	—
18	2.03 (d, J = 1.4 Hz, ^{3}H)	22.9	2.03 (d, J = 1.4 Hz, ^{3}H)	22.9
19	1.20 (s, ^{3}H)	10.0	1.20 (s, ^{3}H)	10.0
20	3.74 (d, J = 9.1 Hz, ^{1}H) 3.89 (d, J = 9.1 Hz, ^{1}H)	68.0	3.74 (d, J = 9.1 Hz, ^{1}H) 3.89 (d, J = 9.1 Hz, ^{1}H)	68.0
21	5.44 (d, J = 0.9 Hz, ^{1}H) 5.56 (d, J = 0.9 Hz, ^{1}H)	121.5	5.44 (d, J = 0.9 Hz, ^{1}H) 5.56 (d, J = 0.9 Hz, ^{1}H)	121.5

Confirmation of *NT3* expression-enhancing activity of eurycomanone *in vitro*. Evaluation of the *NT3* expression-enhancing activity in primary Müller cells treated with 3, 10, and 30 μM pure eurycomanone revealed enhanced *NT3* expression in a dose-dependent manner (Fig. 6A). Furthermore, evaluation of the bioactivity of quassin, the basic ring of quassinoids, showed no activity (Fig. 6B).

Discussion

In the present study, we demonstrated that the *E. longifolia* root water extract can enhance *NT3* expression in retinal Müller cells. We focused on *E. longifolia* in this study based on the positive outcomes observed in our preliminary screening experiments, wherein we assessed various plant extracts, including *E. longifolia*, for their *NT3* expression-enhancing properties.

In this study, guided by bioassays using *NT3* expression levels as an indicator, *E. longifolia* water extract was fractionated and purified to isolate the bioactive component. One bioactive compound (Compound 1) from the fraction (Fr. BC-1) was identified with the highest activity and yield. Compound 1 was identified to be eurycomanone using NMR spectroscopy. Eurycomanone enhanced *NT3* expression in Müller cells in a dose-dependent manner. However, as shown in Fig. 3, during the purification process, in addition to Fr. BC-1, four fractions—Fr. A, Fr. D, Fr. BC-2, and Fr. BC-3—were also active. Since each peak in Fr. BC-2 and Fr. BC-3 was different from those in Fr. BC-1 (Supplemental Fig. 2*), it was inferred that the activity of Fr. BC-2 and Fr. BC-3 could be due to compounds other than eurycomanone. Both Fr. A and Fr. D of step-IV seemed to contain the peak indicating eurycomanone (Supplemental Fig. 1*, retention time 10 min). However, because of their crude nature and the presence of multiple peaks, the possibility of the presence of other active compounds cannot be ruled out. In addition to eurycomanone, *E. longifolia* contains various types of quassinoids.[26] In the present study, we demonstrated that quassin, the basic ring of quassinoids, does not contribute to the bioactivity of eurycomanone, indicating that not all chemicals classified as quassinoids induce *NT3* expression in retinal Müller cells. However, we could not identify the active structure of eurycomanone conferring the *NT3*-upregulating activities. Therefore, further studies are required to elucidate the molecular mechanism underlying the *NT3* expression-enhancing effects of *E. longifolia*.

E. longifolia and its main component, eurycomanone, have been reported to exhibit several biological activities. However, our study is the first to demonstrate the effects of *E. longifolia* on retina-derived cells and the expression of neurotrophic factors. Neurotrophins, such as NT3 and BDNF, have been extensively studied for their central and peripheral neuroprotective effects.[27,28] However, their clinical application presents challenges owing to their short plasma half-life (1.28 and 0.92 min for NT3 and BDNF, respectively) after intravenous administration.[29] To overcome these limitations, the potency of adeno-associated virus (AAV) gene therapy to increase intracellular NT3 levels has been explored for several diseases, including sarcopenia,[30] Charcot-Marie-Tooth disease,[31] and cochlear synaptopathy.[32,33] Furthermore, intravitreal injection of AAV-encoding BDNF or ciliary neurotrophic factor effectively promotes the viability and regeneration of injured adult retinal ganglion cells in rats.[34] While intraocular injections represent an important technique for treating many retinal conditions that can lead to vision loss, they are not less invasive. Therefore, increasing endogenous NT3 expression through orally administered methods, such as dietary sources, could serve as an effective and less invasive neuroprotective strategy. Additionally,
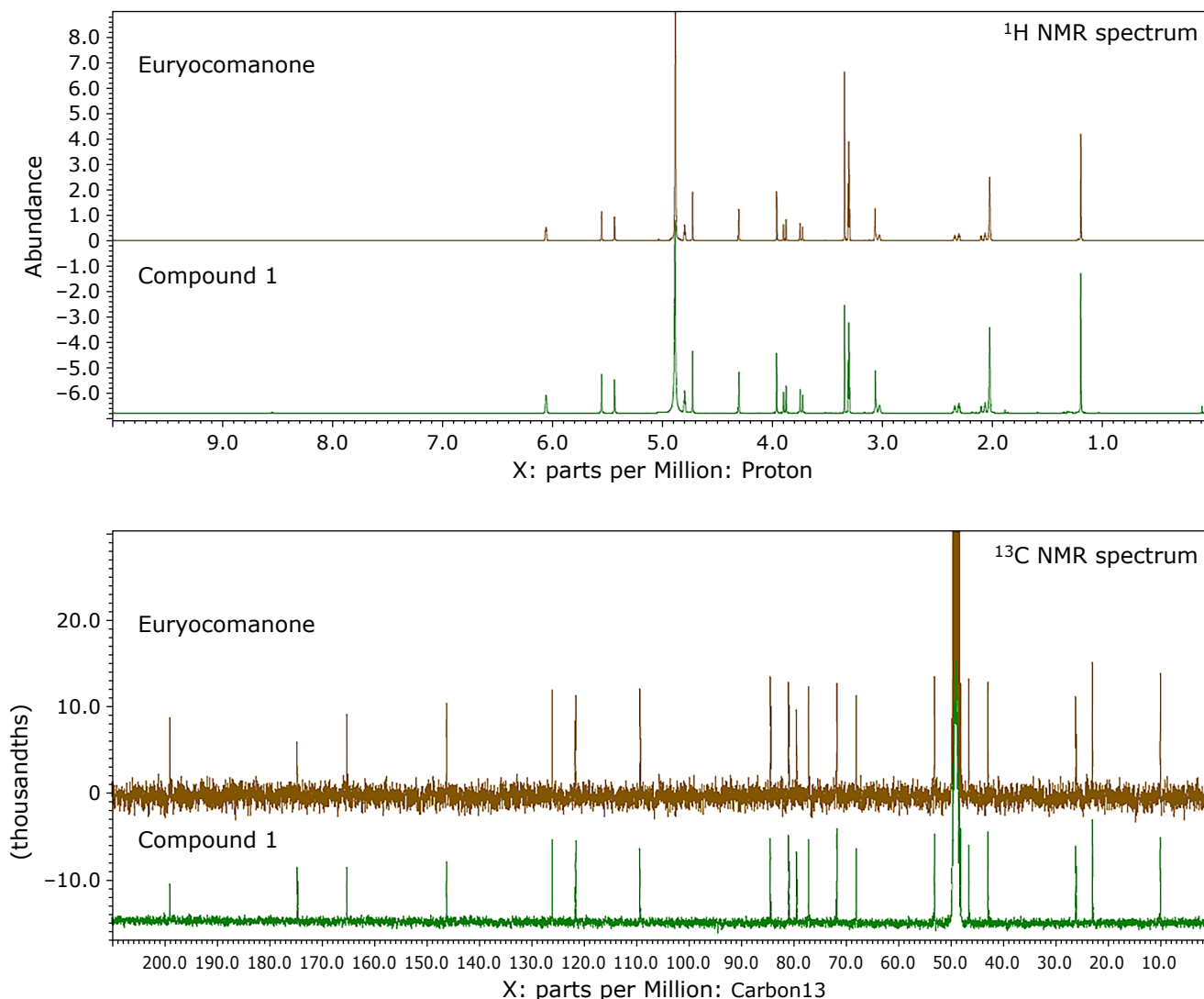
Fig. 4. Overlay display of ¹H NMR and ¹³C NMR spectra of eurycomanone (brown) and compound 1 (green). See color figure in the on-line version.
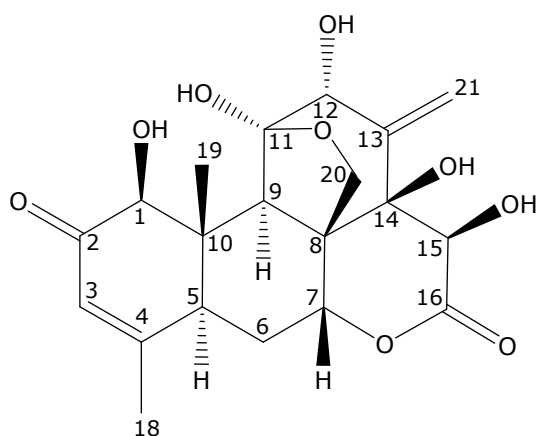

Fig. 5. Chemical structure of eurycomanone.

Suzumura *et al.*[35] reported that oral administration of eicosapentaenoic acid ethyl ester and one of its metabolites induces BDNF upregulation in retinal Müller cells and restores retinal function in diabetic retinopathy. These findings suggest that oral administration is an effective drug delivery method for retinal pathologies.

It is important to note that the biological significance of the effect of *E. longifolia* on enhancing *NT3* expression in Müller cells, as observed in this study, needs to be carefully examined. While Müller cells are responsible for maintaining photoreceptors by providing trophic and anti-oxidant support in healthy retinas,[36,37] they can also be activated in response to various pathological conditions.[38] Activated Müller cells proliferate and undergo physiological changes, including alterations in ion transport properties and signaling molecules;[39,40] this process is believed to protect the retina from further damage and facilitate repair after pathological damage.[41] However, under certain conditions, such as diabetic retinopathy, activated Müller cells can exacerbate pathological conditions.[39,40] In diabetic retinopathy, Müller cells have been reported to produce vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor glycoprotein (PEDF), which are involved in diabetes-induced retinal angiogenesis.[42] Thus, Müller cells play crucial roles in the retina under both physiological and pathological conditions. Therefore, the mechanism underlying the ability of *E. longifolia* to enhance *NT3* expression and its potential
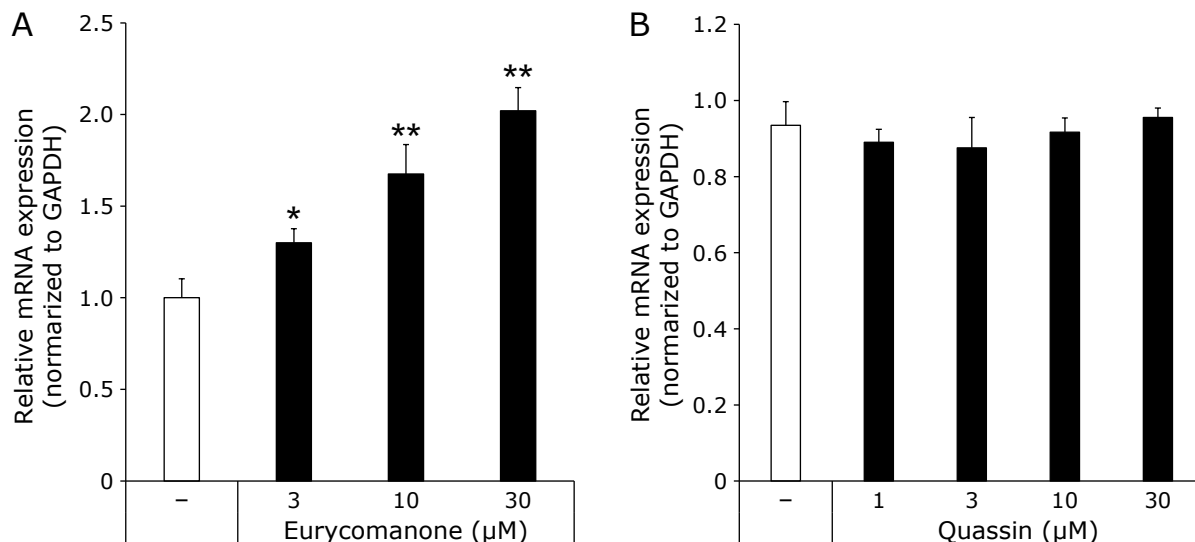
Fig. 6. Effects of eurycomanone and quassin on *NT3* expression in Müller cells. Rat primary Müller cells were treated with eurycomanone (A) or quassin (B). After incubation for 90 min, cells were lysed, and *NT3* mRNA levels were analyzed using real-time PCR. Values are represented as the mean ± SD of three independent experiments, normalized to *GAPDH* mRNA levels. *$p < 0.05$, **$p < 0.01$ vs control.

effects on trophic factors, cytokine, and glutamate metabolism must be investigated in detail. The effects on photoreceptor cell damage should also be verified via co-culture systems.

In this study, we used a standardized water extract of *E. longifolia* root (Physta®). Although the water extract of *E. longifolia* root has been used as a food product, its toxicity should be considered for its applications. Rehman *et al.*[26] reported in their review that the acute oral lethal dose 50 (LD50) of the alcoholic extract of *E. longifolia* in mice is between 1,500 and 2,000 mg/kg, whereas the acute oral LD50 of the water extract is over 3,000 mg/kg, suggesting that the alcohol extract is more toxic than the water extract. Choudhary *et al.*[19] reported that the oral NOAEL of *E. longifolia* water extract in rats is more than 1,000 mg/kg and nontoxic for 90 days. The European Food Safety Authority (EFSA) has recommended that the water extract of *E. longifolia* can be safely used as a dietary supplement, with no nutritional disadvantages, with a daily intake of up to 200 mg.[43] However, the genotoxicity of *E. longifolia* is controversial, as it has been shown to induce DNA damage in *in vitro* mammalian chromosome aberration tests and *in vivo* comet assays.[43] Therefore, when applying *E. longifolia* clinically, it is necessary to consider the extraction method and intake amount, as safety is not guaranteed under all conditions.

In conclusion, in this study, we demonstrated that *E. longifolia* extract can enhance endogenous *NT3* expression in Müller cells and that its major compound, eurycomanone, contributes to this activity. We postulate that oral consumption of *E. longifolia* may contribute to eye health. This study has a few limitations. First, we only focused on the effect of one compound on one neurotrophin at the transcription level *in vitro*. Furthermore, we were unable to provide experimental evidence for NT3 expression in *E. longifolia* at the protein level due to the very low expression of NT3. Further research is necessary to ascertain the role of eurycomanone in eye health and elucidate its mechanism of action in enhancing *NT3* expression.

Author Contributions

YS: conceptualization; data acquisition; data analysis and interpretation; and writing – original draft, review & editing. MY: data acquisition; data analysis and interpretation (for extraction, purification, and identification of active ingredients); and writing – original draft, review & editing. TW: technical support and study supervision. AY: data acquisition (extraction, purification, and identification of active ingredients). MF: technical and material support. NI: technical and material support. HM: critical revision of the manuscript.

Abbreviations

BDNF	brain-derived neurotrophic factor
Fr	fraction
LC-MS	liquid chromatography-mass spectrometry
LLE	liquid–liquid-extraction
NGF	nerve growth factor
NMR	nuclear magnetic resonance spectroscopy
NT3	neurotrophin-3
real-time PCR	real-time polymerase chain reaction
TrkC	tropomyosin receptor kinase C

Conflict of Interest

YS, MY, TW, and AY are employees of FANCL Corporation (FANCL). This study was funded by FANCL.

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