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Original Research Article

Chemical Composition of Essential Oil from *Piper sarmentosum* Fruit and Neuroprotective Activity

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ABSTRACT

A number of complementary medicines have been investigated for their neuroprotection and treatment of neurodegenerative diseases. This study aimed to analyze the chemical compounds and the neuroprotective activity *Piper sarmentosum* Roxb. fruit essential oil. The essential oil from the fruits of *P. sarmentosum* (Piperaceae) was extracted by hydrodistillation. Chemical components of the oil were analyzed by Gas chromatography-Mass spectrometry (GC-MS). The effects of essential oil from *P. sarmentosum* fruit on P19-derived neuron cell viability, neuron length, and branching numbers were investigated. The results showed that the yield of essential oil obtained from *P. sarmentosum* fruit distillation was 1.47% w/w. The essential oil from *P. sarmentosum* fruit contained major compounds, namely; myristicin (88.9%), *E*-caryophyllene (3.7%), elemicin (1.4%), pentadecane (1.0%), apiole (1.0%), *α*-copaene (0.7%), and *β*-copaene (0.2%). The essential oil at 1 and 10 ng/mL significantly increased cell viability, elongated neuron length, and enhanced branching numbers. Therefore, it was suggested that the oil extracted from *P. sarmentosum* fruit may have promising neuroprotective and neuritogenic effects on neurons and prevent neurodegenerative diseases.

Keywords: *Piper sarmentosum*, Essential oil, Neuroprotective effect, Neuritogenic effect, P19 derived-neurons, Myristicin.

Introduction

Neurodegenerative disease is a common cause of mortality and morbidity in elderly people worldwide. The diseases are caused by irreversible damage of neurons resulting in impairments in memory, decision making, judgment, and orientation to physical surroundings.[1] Many of these diseases are caused by genetic mutations, alcoholism, toxins, chemical substance, and viruses. Neurodegenerative diseases can be life-threatening, depending on the type and severity of the diseases. Recently, the number of patients with neurodegenerative diseases has been increasing; however, there is no current cure for this group of diseases. Research and development in neurodegenerative diseases have provided an advanced understanding of these diseases, both in chemistry and biological aspects. This may help to generate important tools for the treatment of the disease.

Essential oils have been extensively used as a traditional medication due to their abilities to treat a variety of ailments and disorders.[2, 3] There are numerous reports on the neuroprotective and anti-aging properties of essential oils.[4,5] Essential oils possess neuroprotective and anti-aging potentials, which effectively prevent dementia, anxiety, and other neurological disorders. Elliot et al reported that essential oils from *Lavandula angustifolia* and *Melissa officinalis* significantly inhibited muscarinic M_1, 5-HT_{2A}, histamine H_3 receptors, and $GABA_A$ receptors, which could help manage agitation in patients with severe dementia.[6] *S*-Limonene from the essential oil of lemon was shown to increase GABA content in the brain and reduce glutamate concentration, resulting in the attenuation of stress responses.[7] *L. angustifolia* (lavender), *Santalum album* (sandalwood), *Salvia officinalis* (sage), *Citrus sinensis* (orange), *Melissa officinalis* leaf oil (lemon balm oil), *Anthemis nobilis* (roman chamomile), *Rosa damascena* (rose), and *Pelargonium* spp. essential oils are strong anxiolytic agents in clinical studies.[8-12] Kochaphum et al reported that essential oil from rhizomes of *Curcuma alismatifolia* at 1 ng/mL exhibited neuritogenic and neuroprotective activities.[5]

Piper sarmentosum Roxb. (Piperaceae) has been used in South East Asian countries for its culinary and medicinal properties to treat many diseases and disorders. The aerial parts of the plant are edible as vegetables and different parts of the plant are used for folk medicines to treat various symptoms.[13] However, the scientific reports of chemical compositions and the activities of *P. sarmentosum*, especially in the fruits are still limited. Qin *et al* demonstrated that *P. sarmentosum* leave oil had strong antifeedant activities on *Brontispa longissima* larvae.[14] Chieng et al also reported that the essential oil from leaves of *P. sarmentosum* had toxicity against the larvae of *Artemia salina*.[15] In this study, the chemical components of essential oil from *P. sarmentosum* fruits were investigated. The effects of the essential oil from the fruits of *P. sarmentosum* on cell viability, neuron length, and branching numbers were observed, suggesting the neuroprotective and neuritogenic activities of the oil.

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Materials and Methods

Plant collection and identification

The fruits of *Piper sarmentosum* Roxb. were collected from a herbal garden in Prachinburi, Thailand in March 2017. The identification of the fruit of the plant was done by Dr. Sirivan Athikomkulchai. A voucher specimen number "LOYP_H_001" of this plant was deposited in a herbarium at the Faculty of Pharmacy, Srinakharinwirot University, Nakhonnayok, Thailand.

Chemicals

The P19 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Alpha minimal essential medium (α-MEM), and antibiotics-antimycotic solution were purchased from Invitrogen (Carlsbad, California, USA). Fetal bovine serum (FBS) and newborn calf serum (NCS) were purchased from Gibco (Waltham, MA USA.) Analytical grade dimethyl sulfoxide (DMSO), 1:250 porcine trypsin, all trans-retinoic acid, poly-L-lysine (MW=300,000), cytosine-1-β-D-arabinoside, and quercetin were purchased from Merck (Darmstadt, Germany).

Extraction of essential oil from P. sarmentosum fruits

The fresh fruits of *P. sarmentosum* were washed and dried at room temperature, and weighed. Then they were roughly pounded and extracted by hydro-distillation in a Clevenger-type apparatus. The oil was distilled at 100°C using 3 L of water for 4 h. The essential oil was separated and the excess water was absorbed by using anhydrous sodium sulfate. The oil was stored at 2 - 8°C until use.

Chemical analysis of essential oil constituents by gas chromatography and mass spectrometry

The chemical constituents of *P. sarmentosum* fruit essential oil was analyzed by gas chromatography-mass spectrometry (GC-MS) (GC Ultra, Thermo Electron Corporation, USA). A ZB-5 fused silica column linked to a methyl silicon capillary column (30 m x 0.22 mm i.d; 0.25 µm film thickness was used and equipped with DSQ Quadrupole detector.[16] The column temperature was maintained at 60 °C for 1 minute, then increased to 250 °C (at a rate of 3°C/min). The injector and transfer line temperature temperatures were set at 180°C and 275°C, respectively. Helium was used as a carrier gas at a constant flow rate of 1.0 mL/min with a split ratio of 1:100. The ion source was maintained at 200 °C. The MS operated in Electric Impact mode. The mass scan spectrum was acquired with a mass scan range of 40-650 amu at a rate of 500 amu/sec. The essential oil composition was identified by comparing the peaks obtained from GC with the Kovats gas chromatographic retention indices and comparing their MS with the MS database (NISTO5 library) and in the literature.[17,18]

Cell culture and differentiation

P19 cells were cultured in a culture medium composing of α-MEM supplemented with 7.5% new born calf serum, 2.5% FBS, and 1% antibiotics-antimycotic solution at 37°C, 5% CO_2. Cells were cultured until reaching a stable exponential growth and subcultured every 2 days.

Differentiation of P19 cells into P19-derived neurons

P19 cells (2 x10^6 cells/mL) were suspended in a mixture of α-MEM, 5% FBS, 1% antibiotics-antimycotic solution, and 0.5 µM all trans-retinoic acid (RA). The cell suspension (10 mL) was then seeded into a 100-mm culture dish. After treated with RA for 4 days, the cells formed large aggregates. The cell aggregates were centrifuged to remove retinoic acid. The embryoid bodies were dissociated and plated at 7 x10^4 cells in multi-well plates which were pre-coated with poly-L-lysine (50 µg/mL) in PBS, and allowed to expose UV light for 30 min. The cells were incubated with α-MEM containing 10% FBS, 1% antibiotics-antimycotic solution for 24 h. Then cytosine-1-β-D-arabinoside or Ara-C (10 µM) was added into the cell culture. After 14 day-differentiation, P19 derived-neurons were used.

Neuronal viability assay

The cell viability of P19-derived neurons was tested by using an XTT assay.[19] At day 14 after the differentiation process, the α-MEM containing 10% FBS, 10 µM Ara-C, and 1% antibiotics-antimycotic solution (P19SM) was removed, and the essential oil of the *P. sarmentosum* fruit at concentrations of 0.001, 0.01, 0.1, 1, and 10 µg/mL in 0.5% DMSO, diluted with P19SM was added. For control, P19SM was added to P19-derived neurons. The cells were then incubated at 37°C for 18 h. After removal the samples, the cells were then incubated with 50 µL of XTT solution (1 mg/mL XTT in α-MEM) and phenazine methosulfate (25 µM). The cells were further incubated at 37°C for 4 h, and then 100 µL of PBS were added. The absorbance value was measured using a microplate reader spectrophotometer at a maximum wavelength of 450 nm. The data were presented as the mean ± S.D. Cells in the medium was used as a control representing 100% cell viability. The IC_{50} value of essential oil was calculated from dose-effect curves by non-linear regression using GraphPad Prism 7.0.

Neuroprotective assay by serum deprivation method

The neuroprotective assay was carried out with P19-derived neurons plated in a 96-well plate.[20] The cell culture medium was removed. The 1 and 10 ng/mL essential oil in 0.5% DMSO, diluted with α-MEM plus 10 µM Ara-C (samples), P19SM (control), α-MEM plus 10 µM Ara-C (serum-deprived), 0.5% DMSO in α-MEM plus 10 µM Ara-C (solvent control of serum-deprived), and 1 nM quercetin in α -MEM plus 10 µM Ara-C (positive control) were added into the cells (150 µL/well). The cells were incubated for 18 h at 37°C, 5% CO_2. After incubation, samples were removed and XTT solution containing 25 µM PMS in α-MEM was added to the cells (50 µL/well). The cells were then incubated at 37°C, 5% CO_2 for 4 h. Then PBS was added (100 µL/well). The absorbance was determined by using a microplate reader spectrophotometer at a maximum wavelength of 450 nm. Cells in the medium as a control represented 100% cell viability.

Neuritogenicity assay

The P19-derived neurons were cultured in a 6-well plate.[19] The cell culture medium was removed. Then 1 and 10 ng/mL essential oil in 0.5% DMSO, diluted with P19SM (samples), 0.5% DMSO in P19SM, 1 nM quercetin in P19SM (positive control), and P19SM (control) were added into the cells (2,000 µL/well). The cells were incubated for at 37°C, 5% CO_2 for 18 h. The morphology of P19-derived neurons was observed under a microscope (ECLIPSE E200; Nikon, Japan). The length and number of neurites were measured under the microscope.

Statistical analysis

The software Graphpad Prism 7.0 (GraphPad Software, San Diego, CA, USA) was used for the statistical evaluation of the results. Data are presented as mean ± SD. One-way analysis of variance (ANOVA) was used to analyze the difference between the means of more than two groups. The Newman–Keuls post hoc test was used to determine the significance of the differences. In all cases, the probability level of $p < 0.05$ was used as the criterion of significance.

Results and Discussion

Yield of P. sarmentosum fruit oil distillation

The essential oil obtained from the water distillation of *P. sarmentosum* fruit was a clear yellow liquid. The distillation of the *P. sarmentosum* fruit yielded 1.47% w/w of essential oil.

The Essential Oil Composition Analyzed by GC-MS

Seven compounds from the fruits of *P. sarmentosum* were analyzed by GC-MS (Table 1). The major compounds in *P. sarmentosum* oil grown in Thailand were Myristicin (88.90%), *E*-Caryophyllene (3.70%), Elemicin (1.38%), Pentadecane (0.98%), Apiole (0.98%), *α*-Copaene (0.66%), and *β*-Copaene (0.20%).

The GC-MS analysis exhibited that myristicin was the major chemical component found in the fresh fruit oil of *P. sarmentosum.* The proportion of myristicin shown in this study had the highest percentage peak area (88.90%) among the previous reports. Chanprapai and Chavasiri reported that among 63 compounds in the essential oil from fresh leaves of *P. sarmentosum*, myristicin was the major component (27.27%), followed by *E*-caryophyllene (18.30%).

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The results agreed with our GC-MS analyses showing that myristicin was the major compound found in the essential oil of *P. sarmentosum* fruit, followed by *E*-caryophyllene.[21] However, compared to the fresh leaves, the essential oil distilled from the fresh fruits contained a significantly larger proportion of myristicin. The result was supported by Rameshkumar *et al*, showing that myristicin was identified as the major compound (84.2%) of *P. sarmentosum* fruit oil.[22] The major component of *P. sarmentosum* fruit oil varied depending on the origin of the plant. *P. sarmentosum* fruit oil in Thailand contained myristicin as the major constituent which was agreed consistent with the essential oil of fruit of *P. sarmentosum* fruit grown in India and China. However, the major compounds of *P. sarmentosum* fruit oil were found to be different from those from Malaysia and Vietnam.[22]

Neuronal cell viability

The toxicity of the essential oil from *P. sarmentosum* fruits against P19-derived neurons determined by the XTT reduction method showed that at 1 ng/mL and 10 ng/mL of the essential oil, % cell viability was more than 100%, indicating non-toxic concentrations of the oil (Figure 2). The IC_{50} of the oil was 1,104 ng/mL. The non-toxic concentrations, 1 and 10 ng/mL, of essential oil, were selected for further investigation of the neuroprotective and neuritogenic effects of the essential oil.

Neurodegenerative diseases are caused by excessive neuronal cell damage and death. Examples include Alzheimer's disease which is characterized by dementia and compromised cognitive and memory functions of patients. Parkinson's disease is another example of neurodegenerative disorder, characterized by progressive movement disorders such as tremors and slow movement. Severe neuronal degeneration is a common pathological characteristic of neurodegenerative disorders. Although the etiology of the most neurodegenerative diseases is still unknown and there is no treatment that can cure the diseases, several researchers have aimed to discover the new compound to retard the development of the disease. In this study, essential oil from *P. sarmentosum* fruit was analyzed for its chemical constituents and demonstrated its neuroprotective and neuritogenic activities *in vitro*.

The XTT assay was used to measure cellular metabolic activity as an indicator of differentiated neuronal cell viability. The colorimetric assay is sensitive and reliable, based on the reducing reaction of a tetrazolium salt to water-soluble formazan product by metabolically active cells. In this study, XTT was used to determine the neuronal toxicity of essential oil from *P. sarmentosum* fruits. The results showed that an increase in the oil concentration resulted in a decrease in the number of living P19-derived neurons suggesting a decrease in the mitochondrial dehydrogenase activity of cells after exposure with the oil. However, at low concentrations i.e. 1 and 10 ng/mL, the oil was shown to be non-toxic to P19-derived neurons. Therefore, the oil at these two concentrations was further used to investigate their neuroprotective and neuritogenic activities.

Neuroprotective activity assay

The % viability of P19-derived neurons after exposure to 1 and 10 ng/mL of essential oil were 59.87 and 49.63%, respectively, whereas the percentage cell viability of neurons under serum-deprived conditions was 15.94%. The results suggested that essential oil at 1 and 10 ng/mL had neuroprotective activities by significantly increasing the number of viable neurons. The % cell viability of P-19 derived neurons treated with essential oil was significantly different from that of cells treated with the positive control (1nM of quercetin) as shown in Figure 3.

Serum deprivation has been shown to induce substantial oxidative stress leading to ATP reduction, potassium loss, and apoptosis in neurons. Since oxidative stress, apoptosis, and target deprivation were shown to play important roles in progressive neurodegenerative diseases, the serum deprivation was used as an *in vitro* model to test the activity of essential oil in neuroprotection.[23-25] Quercetin was used as a positive control. The neuroprotective effects of quercetin have been extensively investigated. At low concentrations, quercetin reduced oxidative stress which caused toxicity in neurons and suppressed neuro-inflammatory processes by downregulating pro-inflammatory cytokines and stimulating neuronal regeneration.[26]

Neuritogenic activity assay

The results of neuritogenic activity demonstrated that the essential oil from *P. sarmentosum* fruit at 1 and 10 ng/mL significantly increased the branching numbers and elongated the length of the cultured neurons, compared with the control group (Figure 4 and 5). The results suggested that the oil from *P. sarmentosum* fruit could enhance the neurite outgrowth of P19 derived-neurons.

In this study, the essential oil of *P. sarmentosum* fruits exhibited neuroprotective and neuritogenic effects against P19-derived neurons. These effects might be a result of myristicin which was identified as a major active component of the oil. Myristicin was shown to protect hypoxia-induced apoptosis in rat dorsal root ganglion neurons via inhibition of the endoplasmic reticulum stress pathway.[27] Ghorbanian *et al* also reported that pre-treatment of nutmeg extract containing myristicin as a major component could reduce seizure, decreased hippocampal neuronal death, and ameliorated glial activation in pentylenetetrazol-induced mice.[28] The protective effect of myristicin against ulcerative colitis in acetic acid-induced ulcerative colitis mice through regulating anti-oxidative responses, NF-κB, and subsequent pro-inflammatory cytokines production.[29] The neuronal loss attenuation might be a result of the anti-inflammatory effect of myristin.[30] The low dose of essential oil (i.e. less than 10 ng/mL (50 nM)) was suggested to be used to protect neurons since they were non-toxic to the cells. The result was supported by another study showing that myristicin at a concentration as high as 1 mM induced apoptosis in human neuroblastoma cells.[31]

Table 1: Chemical composition of *P. sarmentosum* fruit essential oil evaluated by the GC-MS

Compounds	Retention time	Kovats retention index[a]	% Peak area
α-Copaene	23.95	1376	0.66
E-Caryophyllene	25.81	1419	3.70
β-Copaene	28.36	1432	0.20
Pentadecane	29.09	1500	0.98
Myristicin	30.16	1518	88.90
Elemicin	31.16	1557	1.38
Apiole	35.96	1678	0.98

[a]Kovats retention index was identified by Adams RP (2004) and Davies NW (1990)

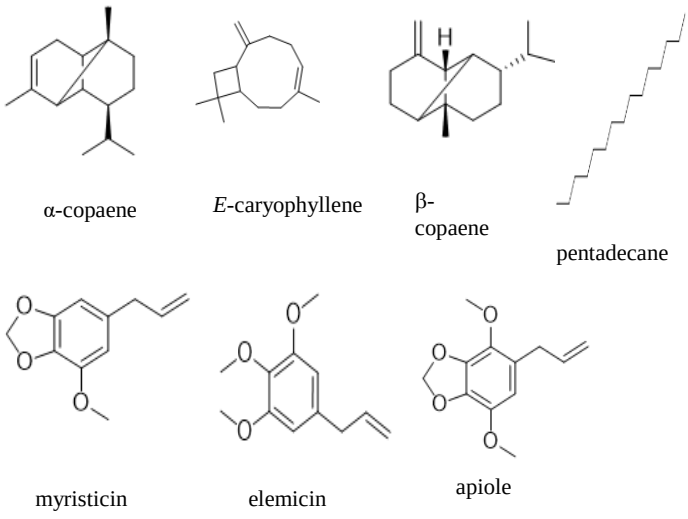

Figure 1: Chemical structures of compounds in the essential oil of *P. sarmentosum* fruit.

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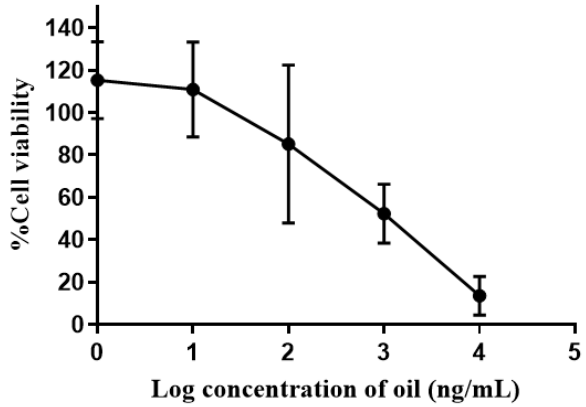

Figure 2: P19-derived neuronal viability after treatment with essential oil from *P. sarmentosum* fruits for 18 h. Data are presented as mean ± SD (n = 3).

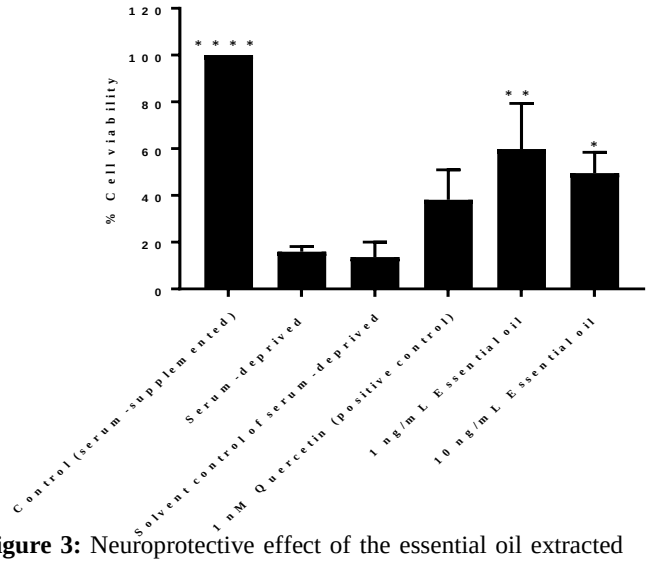

Figure 3: Neuroprotective effect of the essential oil extracted from *P. sarmentosum* fruit on P19 derived-neurons under the serum deprivation. Data are presented as mean ± S.D. (n = 3) **p* < 0.05, ***p* < 0.01, and *****p* < 0.0001 compared with the serum-deprived group.

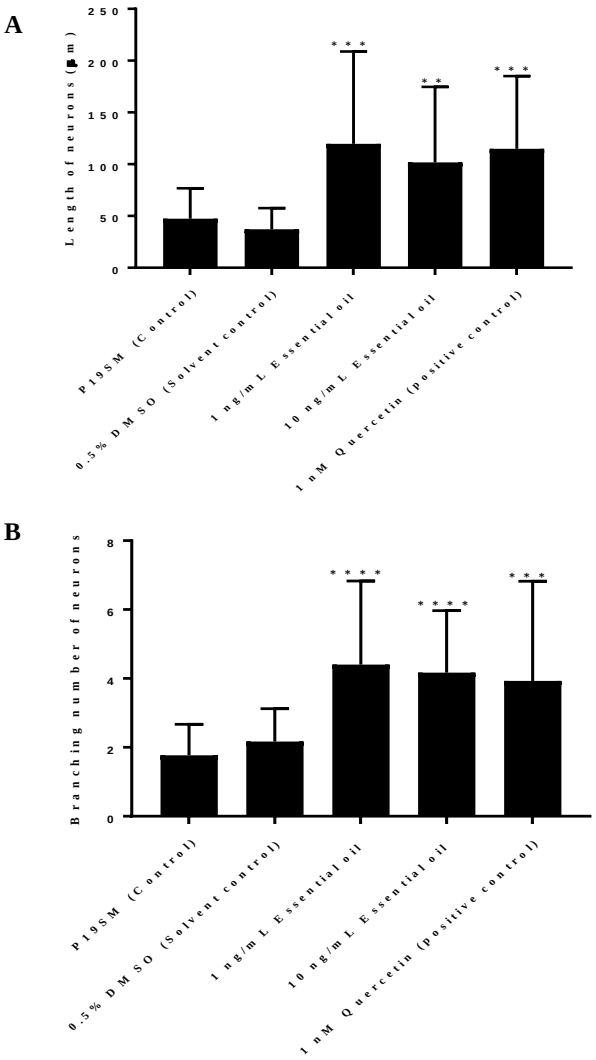

Figure 4: (A) Length of neurites and **(B)** branching numbers of neurites after treatment with essential oil or quercetin. Data are expressed as mean ± SD (n = 30). **, ***, **** indicated *p* < 0.01, *p* < 0.001and *p* < 0.001, respectively compared with the control group.

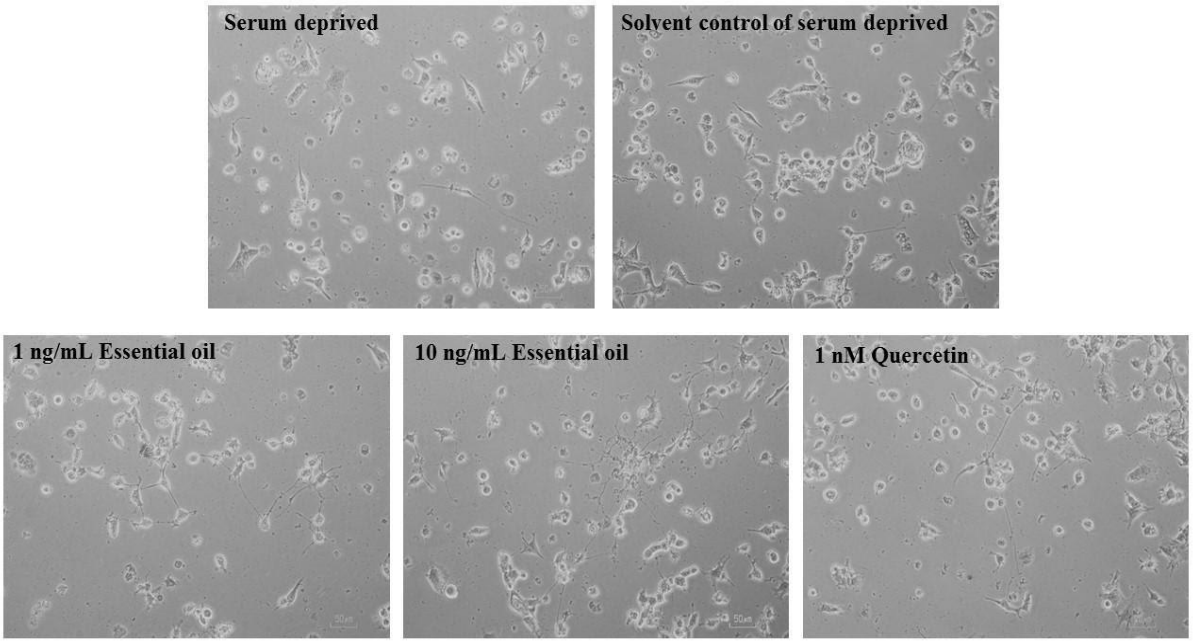

Figure 5: Number of neurites after serum deprivation and treatment of P19-derived neurons with essential oil and quercetin. ***, **** indicated *p* < 0.001 and *p* < 0.0001, respectively compared with the serum-deprived group

Conclusion

In summary, we found that the essential oil from *P. sarmentosum* fruit was able to protect neuronal cells against serum deprivation-induced neurodegeneration. Myristicin was the major active chemical constituent found in the essential oil. Therefore, the low dose of essential oil distilled from *P. sarmentosum* fruit may have a therapeutic potential in ameliorating neurodegenerative diseases.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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