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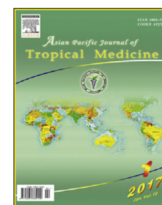

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Neuroprotective effect of *Spilanthes acmella* Murr. on pesticide-induced neuronal cells death

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ABSTRACT

Objective: To investigate protective effects of *Spilanthes acmella* (*S. acmella*) Murr. extracts against pesticide-induced neuronal cells death and to elucidate the underlying molecular mechanism in dopaminergic (SH-SY5Y) cells lines.

Methods: Cell viability of SH-SY5Y cells was studied by treating the cells with various concentration of pirimicarb for 24 h. Neuroprotective effect of *S. acmella* Murr. extracts was investigated by adding the plant extracts to the medium for 24 h prior to the incubation with 100 μM H_2O_2 or with pirimicarb for 24 h. Control-untreated cells were incubated with the culture medium. Cell viability was measured by MTT assay, calpain and calpastatin expressions were analyzed by Western blotting and immunocytochemistry.

Results: Pretreatment of SH-SY5Y cells with *S. acmella* Murr. extracts (1 μg/mL) for 24 h significantly increased the dopaminergic neurons in pirimicarb-induced neurotoxicity. In addition, pretreatment with the *S. acmella* Murr. extracts led to decreased calpain but increased calpastatin protein levels.

Conclusion: *S. acmella* Murr. extracts exerted neuroprotective effect, via an alteration of calcium homeostasis, against pirimicarb induced neurotoxicity. The *S. acmella* Murr. might be a potential natural candidate with neuroprotective activity.

1. Introduction

Pesticides are widely used for crops protecting against insects, fungi and pests. However, pesticides are potentially toxic to human nervous, cardiovascular, respiratory, and reproduction systems. In addition, toxicity of pesticides may be the cause of cancer. It was estimated that over 3 million cases of acute and chronic pesticide poisonings were reported in most developing countries [1,2]. Exposures to pesticides such as organophosphates, carbamates, pyrethroids and organochlorines might result in long term health problems [3]. Therefore, contaminated pesticides in food are controlled in a certain level such as an acceptable daily intake of pirimicarb, a carbamate insecticide, is 0.02 mg/kg/day [4].

Carbamates cause neurotoxic effects by an inhibition of acetylcholinesterase activity in the synaptic cleft, which lead to an increase cholinergic activity. An excessive release of acetylcholine contributes to dizziness, cramps, nausea, vomiting, abdominal pain, numbness, fatigue, headaches, salivation, diarrhea, generalized weakness, respiratory problems and blurred vision [5]. Furthermore, carbamates may cause a long term adverse health effects including neuropsychological and neurobehavioral changes [6], which lead to increased risk of neurodegenerative development such as Alzheimer's, Parkinson's and Huntington's diseases [7].

A homeostasis of calcium, in the brain, regulates neuronal plasticity underlying learning – memory and neuronal survival. Under physiological condition, cytosolic calcium concentration is produced by an opening of calcium-permeable channels in the plasma membrane, mitochondria and endoplasmic reticulum. An excessive cytosolic calcium in the mitochondria can release apoptogenic factors into the cytosol, which ultimately cause dynamic alterations such as fission and fusion [8]. Calpains are a family of intracellular calcium-dependent cysteine protease. In the central nervous system, μ- and m-calpains are ubiquitously

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expressed in neuron and glia. Both calpains require different calcium concentrations, 3–50 μM for μ-calpain and 0.4–0.8 mM for m-calpain [9]. Calpain activation has been implicated in various neurodegenerations, thus, the calcium influx and oxidative stress caused by carbamate pesticides may lead to the neuronal cell death [10]. A number of natural antioxidants i.e., flavonoid and phenolic compounds have been shown to exhibit neuroprotective activity [11]. To search for novel neuroprotectants, it is of interest to explore medicinal plants with antioxidant property such as *Spilanthes acmella* (*S. acmella*) Murr. [12].

S. acmella Murr., known in Thai as Phak-Krad Hauwaen, has been used in traditional medicines [13] for treatment of fever [14,15], flu, cough, rabies diseases [16], tuberculosis, malaria [17], bacterial infection [18], skin disease [19], scurvy [20] and gastric ulcer [21]. *S. acmella* Murr. also has been noted for its potential to control obesity [22], stimulate digestion [23], and modulate immune response [20]. Furthermore, the plant species has been widely used to relief dental pain regarding its local anesthetic property [24]. Pharmacologically, *S. acmella* Murr. exerted diverse bioactivities such as local anaesthetic [25], antipyretic [26], anti-inflammatory [27], antifungal [28], diuretic [29,30], vasorelaxant, antioxidant [31,32], antiplasmodial [17] and antimicrobial [33] activities. Moreover, the extracts of *S. acmella* Murr. were isolated to give diverse biologically active compounds i.e., 3-acetylaleuritolic acid, vanillic acid, β-sitostenone, scopoletin, ferulic acid and isoferulic acid [12].

However, the neuroprotective effect of *S. acmella* Murr. against pesticide induced neurotoxicity has not been investigated. This herbal medicinal plant might be a source of novel neuroprotectant approached in neurodegenerative diseases. Herein, the neuroprotective activity of *S. acmella* Murr. against pirimicarb induced neurotoxicity in neuronal, SH-SY5Y, cells is reported.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL (Gaithersburg MD, USA). The mouse monoclonal anti-β actin was obtained from Chemicon International (Temecula, CA, USA.). The rabbit polyclonal anti-calpain, anti-calpastatin and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody were supplied with Cell Signaling (Beverly, MA, USA). Enhanced chemiluminescence (ECL) Plus Western Blotting Reagent was purchased from Amersham Biosciences (Piscataway, NJ, USA). Nunclon™ culture flasks and Corning culture plates were obtained from Corning Incorporated (Acton, MA, USA). The pesticide, pirimicarb, was obtained from Dr. Ehrenstorfer GmbH (Augburg, Germany). Human dopaminergic neuroblastoma (SH-SY5Y) cell line was provided by American Type Culture Collection (Manassas, VA, USA). Other chemicals used in this study were analytical grade, and obtained essentially either from Sigma Aldrich or Labscan analytical science (Dublin, Ireland).

2.2. Plant extracts preparation

Plant materials (*S. acmella* Murr.) were collected from Nakornsrithammarat, Thailand. A voucher specimen [31] has been deposited at the department of Chemistry, Faculty of Science, Srinakharinwirote University, Bangkok, Thailand. The dried aerial parts of *S. acmella* Murr. were extracted as previously described [31] using hexane, chloroform, ethyl acetate and methanol to give the corresponding plant extracts.

Chemical profiles of the plant extracts (hexane, chloroform, ethyl acetate and methanol) include thin layer chromatography as well as ^{1}H NMR and HPLC [31]. Analytical TLC was performed on silica gel 60 F_{254} aluminum sheets and visualized under UV at 280 nm. ^{1}H NMR spectra were recorded on a Bruker AVANCE 300 NMR spectrometer operating at 300 MHz using $CDCl_3$ and CD_3OD as solvents.

2.3. Cell lines

Human neuroblastoma (SH-SY5Y) cells were maintained and cultured as previously described [34]. To perform the experiments, cells were seeded in 96 wells and 6 wells plates, and grown to 70–80% confluence. Before the start of treatment, the medium was replaced with fresh media. The above conditions were applied to all of the experiments performed in this study.

2.4. Cell viability

Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the conversion of MTT from yellow to dark blue formazan crystals by mitochondrial dehydrogenases as previously described [35]. Briefly, cells (1×10^5 cells/mL) were seeded in 96 well plates. After 24 h, the cells were treated with different concentrations of pirimicarb (0.125, 1.25 and 12.5 ppm) for 24 h. To investigate the neuroprotective effect, *S. acmella* Murr. extract was added to the medium for 24 h prior to the incubation with 100 μM H_2O_2 or with pirimicarb for 24 h. Control-untreated cells were incubated with the culture medium. MTT in 0.1 mM phosphate buffer saline (PBS) were added to each well and incubation at 37 °C for 3–4 h. The solution was discarded then the extraction buffer (0.04 N HCl in isopropanol) was added. The optimal densities were measured at 570 nm spectral wavelength using a microtiter plate reader.

2.5. Protein expression

Cellular proteins were detected mainly by Western blotting as previously described [36] using the following antibody: anti-calpain and anti-calpastatin antibody (1:2000) and anti-β actin antibody (1:10000). The blots were developed with ECL Plus Western Blotting detection reagents through an exposure of the membrane to X-ray film. The specifically labeled protein bands were scanned and quantified by densitometry using the image J program. Data were normalized to β-actin.

2.6. Immunocytochemistry

Cells were grown in 24 well plate containing glass coverslips at 37 °C for 24 h, then exposed to pirimicarb for 24 h. The cells were incubated using MitoTracker®Red CMXRos for 30 min. The medium was removed, and then the cells were washed with ice-cold PBS. The cells were fixed with 4% paraformaldehyde in PBS for 30 min at 4 °C, and washed with PBS three times for 5 min each time. Cells were permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature and rinsed with PBS

three times. The cells were blocked by non-specific antibody binding sites by incubating with 10% donkey serum in PBS, containing 0.3% Triton X-100 and 1% bovine serum albumin (BSA), for 10 min at room temperature. The cells were incubated with primary antibody against calpain (1:1 000 in PBS containing 0.3% Triton X-100 and 0.25% BSA) overnight at 4 °C, followed by incubation in fluorescein isothiocyanate (FITC)-conjugated with donkey anti-rabbit IgG (1:200 in PBS containing 0.3% Triton X-100 and 0.25% BSA) for 2 h at room temperature. The cells were washed three times with PBS. The slides were mounted using antifade reagent in glycerol buffer (Vector Laboratories, Burlingame, USA), and visualized under fluorescence microscopy (Olympus, Tokyo, Japan).

2.7. Statistics

All results are presented as mean ± standard error of the mean (SEM). Significance was assessed by one-way analysis of variance (ANOVA) followed by a Tukey-Kramer test using the scientific statistic software PASW version 18. Probability (P) values of less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of pirimicarb on cell viability in human dopaminergic SH-SY5Y cells

To examine the effect of pirimicarb on cultured SH-SY5Y cells, the cells were incubated with pirimicarb at various concentrations of 0.125, 1.25 and 12.5 ppm for 24 h. Cell viability (Figure 1) was evaluated using the MTT assay, and results were expressed as percentage of control cells which represented cells incubation for 24 h in the culture medium (100% cell viability). Pirimicarb induced significant cell death with dose dependent manner at 1.25 ppm (81.50 ± 3.16%, $P < 0.05$) and 12.5 ppm (79.50 ± 3.56%, $P < 0.01$).

3.2. Neuroprotective effect of S. acmella Murr. on pirimicarb induced reduction of cell viability

To determine the neuroprotective effect of *S. acmella* Murr. (hexane, chloroform, ethyl acetate and methanol) extracts, the SH-SY5Y cells were exposed to different concentrations of *S. acmella* Murr. extracts (1, 10, 100 and 1 000 µg/mL). These *S. acmella* Murr. extracts decreased cell viability at 1 000 µg/mL. Antioxidant activity of *S. acmella* Murr. extracts, at the 4 different concentrations, on the cells with oxidative stress (100 µM H_2O_2) was studied. *S. acmella* Murr. hexane extract at 1 µg/mL significantly decreased the loss of cell viability induced by treatment with 100 µM H_2O_2. Therefore, *S. acmella* Murr. hexane extract was selected for the following experiments.

The SH-SY5Y cells were exposed to different concentrations of pirimicarb with or without pretreatment with *S. acmella* Murr. extracts. It was observed that cell viability reduction induced by pirimicarb was attenuated by pretreatment the cells with 1 µg/mL of *S. acmella* Murr. extracts for 24 h. Significantly, the increased survival of cells exposed to pirimicarb at 1.25 ppm was noted from (81.50 ± 3.16)% to (94.75 ± 4.96)% ($P < 0.05$) (Figure 2A). However, treatment of the cells with *S. acmella* Murr. extract alone had no effect on the cell viability (Figure 2 A). Cell shrinkage and less number of cells were noted for the cells treated with pirimicarb. Pretreatment of the cells with *S. acmella* Murr. (hexane extract) prior to and during exposure

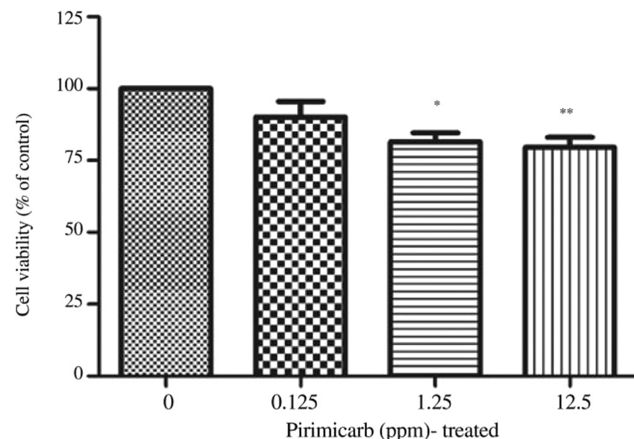

Figure 1. Effect of pirimicarb-induced reduction in cell viability.

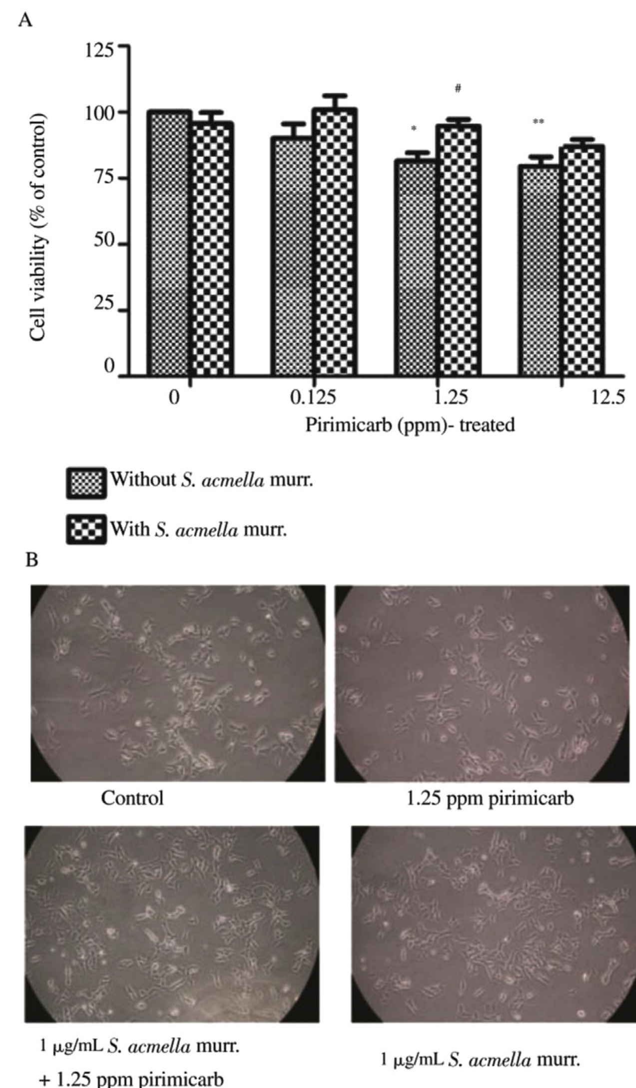

Figure 2. Effect of *S. acmella* Murr. hexane extract on pirimicarb-induced reduction in cell viability. (A) $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control and $^{\#}P < 0.05$ compared with pirimicarb-treated cells. (B) Cell images were visualized under phase contrast microscope at 20× magnifications.

to pirimicarb, the cells did not shrink compared with the control (Figure 2B).

Thus, the neuronal cell death induced by pirimicarb was reversed by pretreatment with *S. acmella* Murr. extracts. The hexane plant extract was shown to be the most effective one, and was further explored for protein expressions.

3.3. Effect of S. acmella Murr. on pirimicarb induced calpain expression in SH-SY5Y cells

Effects of pirimicarb on calpain and calpastatin protein expressions in SH-SY5Y cells were investigated. The cells were incubated with 1.25 ppm pirimicarb for 24 h in the presence or absence of 1 μg/mL *S. acmella* Murr. hexane extract. Incubation of the cells with 1.25 ppm pirimicarb for 24 h, the level of calpain significantly increased to (115.40 ± 4.95)% ($P < 0.01$) compared with the control value. The pirimicarb induced calpain expression was significantly attenuated by pretreating the cells with the *S. acmella* Murr. hexane extract (1 μg/mL), and the calpain level was decreased to (102.60 ± 0.73)% ($P < 0.05$) (Figure 3). Conversely, the level of calpastatin was decreased to (85.63 ± 1.41)% ($P < 0.05$) after the cells were treated with 1.25 ppm pirimicarb (Figure 4). These results suggested that the hexane extract of *S. acmella* Murr. attenuated calpain expression in SH-SY5Y cells during pirimicarb treatment. Consistently, *S. acmella* Murr. hexane extract slightly induced calpastatin expression in the cells exposed to pirimicarb.

To confirm the neuroprotective effect of *S. acmella* Murr. in pirimicarb induced toxicity, immunocytochemistry was performed using specific antibody for calpain. The control (untreated) cells showed minimal fluorescence compared with a markedly increased signal for calpain after pirimicarb treatment (Figure 5).

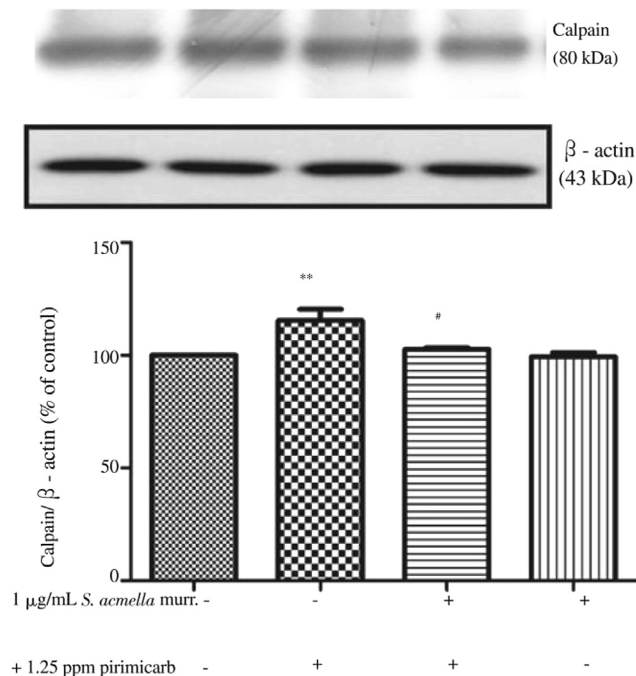

Figure 3. Effect *S. acmella* Murr. on pirimicarb-induced increase in calpain level. $^{**}P < 0.01$ compared with the control and $^{\#}P < 0.05$ compared with pirimicarb-treated cells.

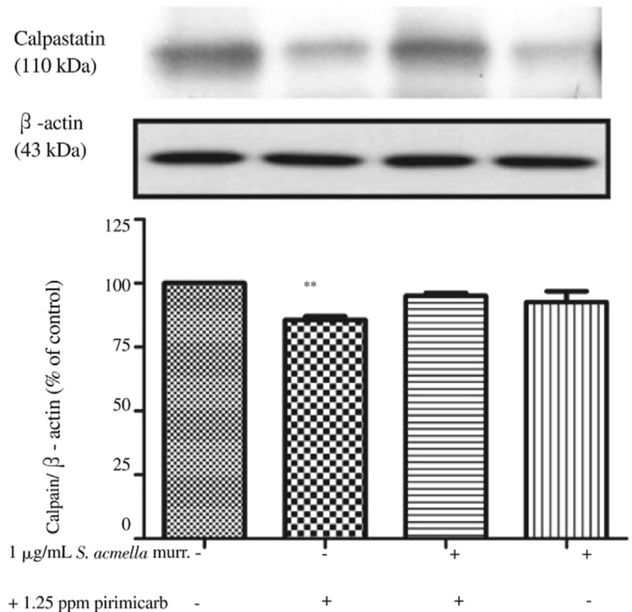

Figure 4. Effect *S. acmella* Murr. on pirimicarb-induced increase in calpastatin level. $^{**}P < 0.01$ compared with the control.

4. Discussion

Pesticides play significant roles in the development and function of nervous systems including central and peripheral nervous systems [37–39]. It is well known that pesticides target on the nervous systems by inhibiting an acetylcholinesterase, which can prolong the excitatory action of acetylcholine [40]. Hallmark features of pesticide toxicity involved the damage of synaptic proteins, synapse formation, and finally resulted in the attenuation of neuronal circuit signaling. Pesticides can bind to various targets such as enzyme, receptor, channel, protein, and membrane that quickly disrupt the neurotransmission processes leading to behavioral alterations [41].

This study revealed for the first time that pirimicarb induced calpain expression in neuronal cells, and this may cause defective cell proliferation and cell death. Therefore, calpain plays an important role in regulating neuronal cell death in the brain damage and neurodegeneration [42,43]. However, an inhibition of calpain promoted neuronal survival in neuronal injury [44].

These results were consistent with the previous studies on other pesticides. For example, pesticide accumulation in the brain can lead to oxidative stress and alter calcium homeostasis [45]. Under pathological condition, excessive levels of intracellular calcium associated with calpain activation were observed prior to a sign of cell degeneration [46]. In human brain, pesticide may directly disturb neurogenesis, proliferation, migration, synaptogenesis, apoptosis and myelination in neuronal development processes [47].

A potential use of natural herbs to improve impairments of neuronal processes is widely discussed [48]. Herbal medicines have been used as dietary supplements that enrich with medicinal ingredients. *S. acmella* Murr. has been used as a traditional medicine for various diseases [12]. Bioactive metabolites were found in aerial parts, leaves, flowers, and whole plants of *S. acmella* Murr. [12]. Its extracts have been shown to exhibit antioxidant activities. Interestingly, the highest cell survival effect of *S. acmella* Murr. was noted for the hexane extract that exerted strong protection in SH-SY5Y cells when treated with H_2O_2.

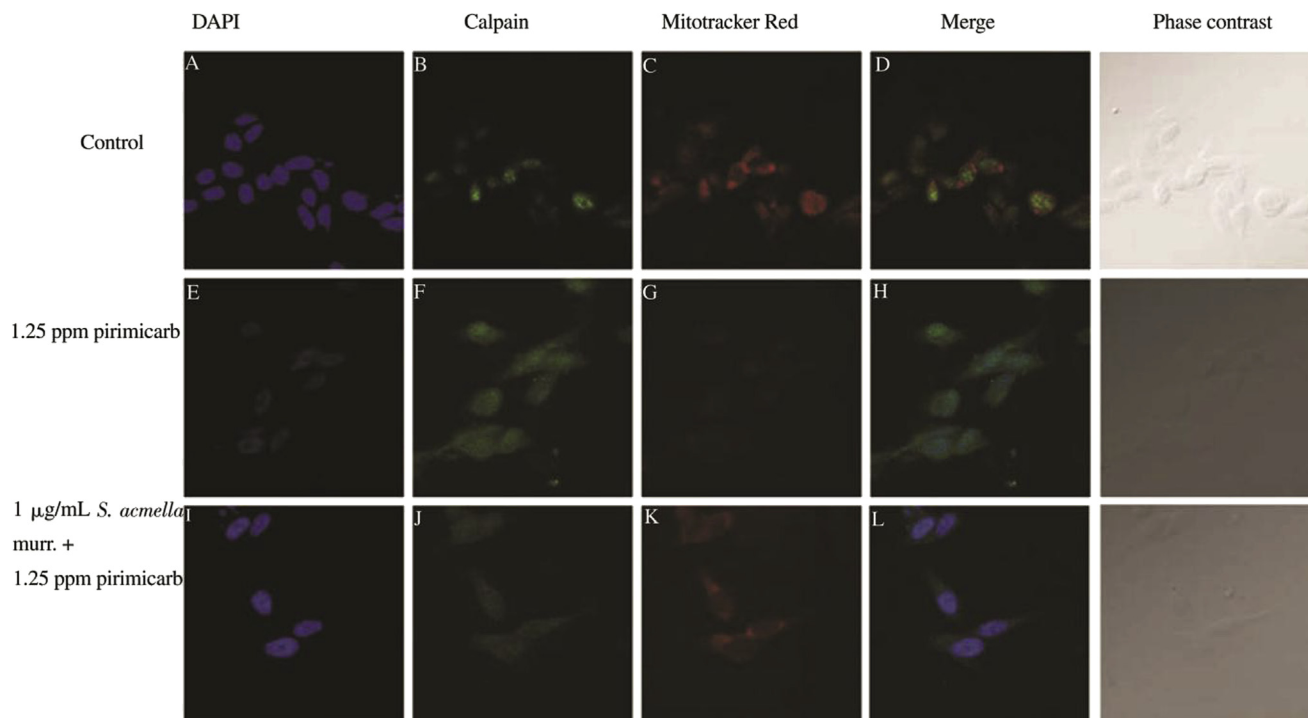

Figure 5. Imaging microscopic analysis of SH-SY5Y cells demonstrating the effect of *S. acmella* Murr. on pirimicarb-induced neurotoxicity in SH-SY5Y cells. The blue color indicated cell nuclei stained with DAPI (A, E and I). The green color indicated calpain immunostaining using fluorescein-5-isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (B, F and J), and the red color indicated mitochondria site using MitoTrackerRed (C, G and K). The fluorescence (merge) images are shown in D, H and L.

Obviously, neuroprotective effect of *S. acmella* Murr. extracts was resulted from their antioxidant properties. Bioactive compounds presented in these plant extracts were triterpenoids (hexane extract), triterpenoids glucosides (chloroform extract), phenolic acid and coumarin derivatives (ethyl acetate and methanol extracts) [12]. Triterpenoids such as β-sitosterol was reported to exert neuroprotective effect [49,50]. Phenolic compound, *tran*-ferulic acid [51,52], and coumarin namely scopoletin [53] displayed neuroprotective activity. The highest neuroprotective effect was noted for the hexane extract of *S. acmella* Murr. It may be due to the nonpolar hexane extract which has better penetration to the cells compared with other polar *S. acmella* Murr. extracts (chloroform, ethyl acetate and methanol).

The present study shows that the decreased calpain expression by *S. acmella* Murr. hexane extract effectively protected pirimicarb induced neuronal cell death. Thus, the neuroprotection was resulted from the decreased oxidative stress and cell death. It remains unclear how the *S. acmella* Murr. extracts ameliorate the neurotoxicity. These results indicate that *S. acmella* Murr. extracts play a crucial role in maintaining calpain and calpastatin levels caused by pirimicarb induced neurotoxicity.

In conclusion, This study revealed that *S. acmella* Murr. may have a potential role in neuroprotection by maintaining calcium homeostasis mediated calpain and calpastatin regulations, which may reduce cell degeneration in pirimicarb induced neurotoxicity.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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