Antioxidant activity, β-amyloid aggregation inhibition, and neuroprotective effect of cricket (*Gryllus bimaculatus*) ethanol extract

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Abstract

Alzheimer's disease (AD) is a neurodegenerative illness associated with dementia and is the most prevalent among the elderly population. The cricket (*Gryllus bimaculatus*), a food and pharmaceutical insect, has been used as an antihypertension, antipyretic or anti-diarrhea. To evaluate the biological activities related with Alzheimer's disease, the cricket was extracted with ethanol and then tested for antioxidant activity, neuroprotective effect, acetyl-cholineesterase inhibition and anti-beta-amyloid aggregation. The antioxidant activity of cricket extract was assessed by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis-[3-ethylbenzthiazoline-6-sulphonic acid] (ABTS) assays. The cricket ethanol extract possessed antioxidant activity with IC_{50} values of 1.15 ± 2.8 and 1.70 ± 5.7 mg/ml by the DPPH assay and ABTS assay, respectively. The MTT assay and phase contrast microscope results showed that the extract significantly inhibited H_2O_2-induced SH-SY5Y neuronal cell death. In addition, it exhibited significant inhibitory effects on β-amyloid aggregation, but there was no inhibitory effect on acetylcholinesterase enzyme function. This study suggested that ethanol extract of cricket may be developed and applied for neuronal damage prevention.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder which causes significant dementia in elderly (Ferri *et al.*, 2005). The neuropathological hallmarks of AD include deposit of amyloid β fibrils, presence of neurofibrillary tangles, reduction of acetylcholine and oxidative stress which play a significant role in the onset and progression of AD (Floyd *et al*., 2002; Wilkinson *et al*., 2004; Ohnishi *et al*., 2004; Goedert *et al*, 1991). The molecular mechanisms underlying the progression of the disease remain unknown. This incomplete understanding of the etiology of AD has limited the development of effective therapeutic agents. Currently, there are only five drugs approved by US Food and Drug Administration (FDA) for AD treatment including acetylcholinesterase (AChE) inhibitors (rivastigmine, galantamine and donepezil), N-Methyl-D-aspartate receptor (NMDA) antagonist (memantine) and a combined drug between AChE inhibitor and NMDA receptor antagonist (Namzaric) (Kumar *et al.*, 2015). However, these drugs that modulate such a single target could only enable a palliative treatment instead of curing or preventing AD. Due to the multi-pathogenesis of AD, the classical approach modulating at one target may be inadequate in this complex disease. Therefore, searching the candidates acting at multiple sites of

pathologic cascade has become a new strategy for the design of new drugs for AD. Presently, natural products including animals and plants are remarkable source for potential therapy in various complex diseases. Thus, it should be great interest in finding new and better potentials from natural products for AD treatments.

The cricket (*Gryllus bimaculatus*) is commonly consumed as food in different parts of the world. In southeastern part of Asia, the cricket is used as traditional medicine for fever, diarrhea, kidney stone or hypertension as well as a food source (Park, 2001; Ahn et al., 2005; Lee *et al*., 2015). General contents of crickets include fat, protein, polyunsaturated fatty acid, mineral, and fiber. Cricket is high in essential amino acids such as lysine, leucine, valine, and isoleucine (Belluco *et al*, 2013). Crickets also contain high concentrations of fatty acids especially unsaturated fatty acids (68.6 %) such as linoleic and oleic acid (Wang et al., 2004; Kim and Jung, 2013). Several studies showed that the crickets possess several biological activities including anti-coagulation, antioxidant, anti-inflammation, anti-aging, anti-high blood fat and Anti-obesity (Ahn *et al*., 2014; Lee *et al.,* 1997; Hwang *et al*., 2004). In addition, cricket ethanol extract showed an ability to inhibit adipose tissue accumulation in high phosphate dieted Wister rats. However, the effect of the cricket on neurodegenerative diseases especially AD have not been reported. Therefore, the aim of our study was to evaluate the effects of the cricket extract on the biological activities related AD pathological cascade, namely antioxidant, AChE activity and Aβ aggregation. In addition, the neuroprotective effects against oxidative stress of the extract was also investigated in a cell culture model.

Methodology

Materials

Analytical grade reagents were purchased from Sigma-Aldrich (SM Chemical supplies Co., Ltd, Thailand), Merck (Merck, Thailand) and Fluka (SM Chemical supplies Co., Ltd, Thailand) and were used as supplied.

Preparation of criket extracts (CRET)

The crickets were collected from Ubon Ratchathani province, Thailand. Dried and finely powdered whole part of cricket (140 g) was macerated in ethanol at room temperature for 7 days. After that, the extract was filtered and then concentrated under reduced pressure at 40 °C yielding a crude extract, stored in an air-tight container at 2-8 °C in a refrigerator until use.

In vitro antioxidant activity assays (Arnao *et al*., 2001, Pichaiyongvongdee *et al*., 2014)

The radical scavenging activity of the ethanol extract of cricket was measured by both the 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) method.

The ABTS was dissolved in water to obtain a 7 mM concentration of ABTS stock solution. ABTS radical cation ($ABTS^{\bullet+}$) was generated by adding 2.45 mM potassium persulfate to the ABTS stock solution and keeping it in the dark at room temperature for 12–16 h. The $ABTS^{\bullet+}$ solution was diluted with ethanol to give an absorbance of 0.70±0.02 at 734 nm. The 50 µL of the extracts were allowed to react with 100 µL of $ABTS^{\bullet+}$ solution. The absorbance was taken 15 min after initial mixing. Trolox was used as a standard.

The DPPH assay was performed as described by Pichaiyongvongdee. One hundred µL of the test compound was added to 100 µL of 0.2 mM DPPH solution. After a 30 min of reaction at room temperature, the absorbance of the solution was measured at 517 nm. The free radical scavenging activity of each fraction was determined by comparing its absorbance with that of a control.

The ability to scavenge the DPPH and $ABTS^{\bullet+}$ radical was calculated using the following equation:

$$\text{Free radical scavenging activity (\%)} = (A0 - A1)/A0 \times 100$$

Where A0 is the absorbance of the control and A1 is the absorbance of the sample

In vitro AChE inhibitory activity assay (Ellman et al, 1996)

The ethanol extract of cricket was investigated for their acetylcholinesterase (AChE) inhibitory activity through the modified Ellman's spectrophotometric method. The assay was performed in 96-well plate by adding 25 µL of 1 mM acetylthiocholine iodide used as substrate in the assay, 125 µL of 1 mM 5, 5'-dithiobis-(2 nitrobenzoic acid) (DTNB), 25 µL of 0.1 M phosphate buffer pH 7.4, 25 µL of the extracts in various concentrations and 50 µL of 0.2 Units/ml AChE from an electric eel (type VI-S), respectively. At least five concentrations of the test compounds were assayed. The absorbance changes at 405 nm were detected every 30 s over the period of 5 min with a microplate reader. The enzyme activity and the percent inhibition were determined.

In vitro assay for Aβ aggregation inhibition by thioflavin T assay (Levine, 1993)

Thioflavin-T (ThT) fluorescence assay was used to monitor the aggregation state of Aβ1–42. The assay was performed as described by Levine with minor modification. Briefly, twenty-five µM of Aβ1–42 in 50 mM phosphate buffer, pH 7.4, was incubated at 37 °C with various concentrations of the extract for 48 h. After incubation, the samples were mixed with 50 µM glycine/NaOH buffer (pH 9.2) containing 5 µM ThT. Fluorescence intensities were measured at an excitation wavelength of 446 nm and an emission wavelength of 490 nm. The fluorescence intensities were recorded, and the percentage of inhibition on aggregation was calculated by using the following equation: (1 - IFi/IFc) * 100% in which IFi and IFc were the fluorescence intensities obtained for absorbance in the presence and absence of the test compound, respectively, after subtracting the background fluorescence of 5 µM ThT in the blank buffers.

Effect on H_2O_2-induced oxidative cell damage in neuroblastoma cells (Park et al., 2015)

Neuroblastoma cells (SH-SY5Y) were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 containing 50 IU/ml penicillin, 50 g/ml streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum. Cultures were maintained at 37ºC in a humidified incubator in an atmosphere of 5% CO_2. For assays, the SH-SY5Y cells were seeded in a 96-well plate and incubated for 48 h. After this incubation period, the cells were treated with various concentrations of test compounds for 2 h. After removing the unabsorbed test compounds, the cells were treated with hydrogen peroxide (H_2O_2) for 2 h to induce oxidative stress. The cell viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetry. The optical density of each well was measured at 550 nm in a microplate reader.

Effect on the protein expression induced by H_2O_2 by Western blotting analysis (Waiwut et al., 2011).

Human neuroblastoma cells (SH-SY5Y) were cultured in Dulbecco's modified Eagle's medium (DMEM) /F12 containing 50 IU/ml penicillin, 50 g/ml streptomycin, 2mM L-glutamine, and 10% fetal bovine serum. Cell cultures were maintained at 37ºC in an atmosphere of 95% humidified air and 5% CO_2. Cells were cultured in 6 well plates for 3 days. Then, the cells were pretreated with various concentrations of extract for 24 h. After removing the unabsorbed extract, the cells were treated with hydrogen peroxide (H_2O_2) for optimum time.

Whole cell lysates were prepared with lysis buffer (25 mM HEPES pH 7.7, 0.3 mM MgCl2, 0.2 mM EDTA, 10% Triton X-100, 20mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Cell lysate was collected from supernatant after centrifugation at 14,000 rpm for 10 min. Cell lysate was resolved by SDS-PAGE and transferred to an Immobilon-P-nylon membrane (Millipore). The membrane was treated with BlockAce (Dainippon Pharmaceutical Co. Ltd, Suita, Japan) and probed with primary antibodies. The antibodies were detected by the use of horseradish peroxidase-conjugated anti-rabbit, antimouse, and anti-goat IgG, and visualized by the enhanced chemiluminescence system (Amersham Biosciences).

Statistical analysis

Data were expressed as mean ± standard deviation. Significant differences were determined using Student's t test, where differences were considered significant if $p < 0.05$and < 0.01.and 50% inhibitory concentration (IC_{50}) values

Results and discussion

Antioxidant activity of cricket extract

The antioxidant activity of the ethanol extract of cricket (CRET) was determined by using the ABTS and DPPH radical scavenging method. The results showed that CRET had the ability to scavenge both ABTS and DPPH radicals with IC_{50} of 1.15 ± 2.8 and 1.67 ±2.6 μg/mL, respectively, as shown in Table 1.

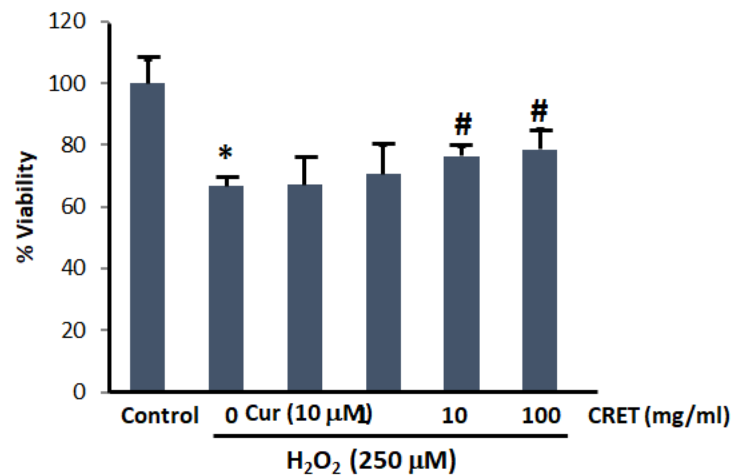
Table 1 Antioxidant activities of Cricket (*Gryllus bimaculatus*) extracts.

Index	Cricket (*Gryllus bimaculatus*) ethanol extract
DPPH (IC_{50})	1.15 ± 2.8 μg/ml
ABTS (IC_{50})	1.67 ±2.6μg/ml

Effect of CRET on H_2O_2 induced neurotoxicity of SH-SY5Y neuron cells

To assess the neuroprotective effects against cell damage induced by H_2O_2 in SH-SY5Y cells**,** the SH-SY5Ycells were treated with CRET at concentrations of 1, 10, 100 μg/ml and then added 250 μM H_2O_2 for 4 hours. The result showed in Figure 1A. The cells treated with H_2O_2 alone markedly toxics to neuron cells. The CRET significantly increased cell viability at the concentrations of 10 and 100 mg/ml which is correlated with the cell morphology result (Figure 1B)

A



B

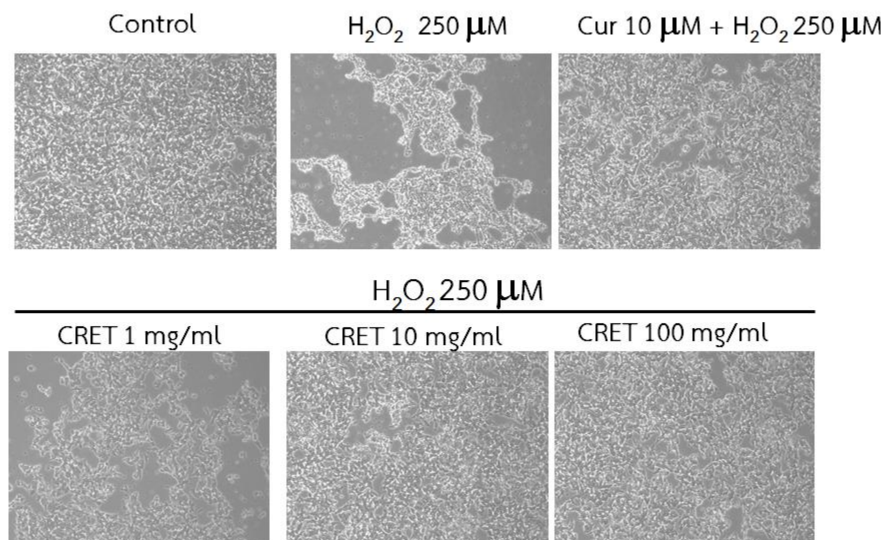


Figure 1. A. The cricket *(Gryllus bimaculatus)* extracts suppressed H_2O_2- induced neurotoxicity of SH-SY5Y cells. The cell viability was determined by MTT assay. Data represented as means + SD (n = 3). The * means P < 0.05, # means P < 0.05. B. The cell morphology of SH-SY5Y cells observed by phase contrast microscopy.

Acetylcholinesterase inhibitory activity of CRET

The Acetylcholinesterase inhibitory activity was determined by using Ellman's method. The substrate acetylthiocholine is hydrolyzed by the acetylcholinesterase to thiocholine, which reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate, detected at 405 nm. The result in Table 2 showed that the CRET showed no effects to acetylcholinesterase inhibitory activity.

Table 2. Effect of CRET on acetylcholinesterase enzyme inhibition.

Experiment groups	% Inhibition
Control	100
AChE	0
AChE+CRET 0.1 mg/ml	ND
AChE+CRET 1.0 mg/ml	ND
AChE+CRET 10.0 mg/ml	ND
AChE+CRET 100.0 mg/ml	ND
AChE+CRET 1000.0 mg/ml	ND

ND: Not detectable

Aβ1-42 aggregation inhibition of CRET

The key hallmark of AD pathogenesis is the formation of toxic Aβ plaques in the brain of AD patients . Therefore, preventing or reducing the aggregation of Aβ has been the primary goal of a number of therapeutic strategies under development or in clinical trials. In the present study, the effect of CRET on the inhibition of Aβ aggregation was examined using the Th-T fluorescence assay. Figure 3 shows the percentage of inhibition on Aβ aggregation of CRET. As shown in Figure 3, the CRET was found to inhibit the aggregation of Aβ1–42 with % Inhibition of 18.90 and 22.92 at the concentration of 1 and 10 mg/mL, respectively.

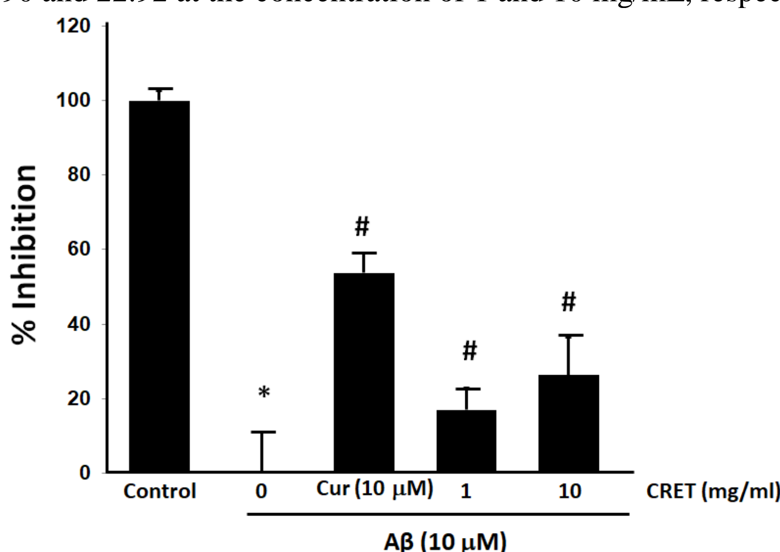


Figure 3. Effect of CRET on Aβ1-42 aggregation. The Aβ1-42 aggregation was determined by ThT Fluorescence assay. Data are means + SD (n = 2). * means $P < 0.05$, # means $P < 0.05$.

Conclusion

Alzheimer's disease risk for persons living into their eighties rises to 20–40% depending on the population. There are millions of AD patients and this number is expected to double and double again with the demographic shift toward a more aged population, leading to over 10 million expected cases, unless preventive measures can be achieved (Brookmeyer 1998). The present study investigated the effect of extract in the prevention of Alzheimer's disease. The results showed that the extract significantly inhibited H_2O_2-induced SH-SY5Y neuronal cell death.. This reduce oxidative stress level in cells (Nicolakakis et al., 2008) (**Figure 2**). In addition, it showed significant inhibitory effects on β-amyloid aggregation(**Figure 4**) This study suggested that ethanol extract of cricket may be developed and applied for neuronal damage prevention.

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