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Evaluation of ethanol concentration, temperature and shaking time of extracted Thai Jasmine rice on cholinesterase enzyme activity

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Abstract

The objective of this study was to investigate the effect of ethanol concentration, temperature and shaking time of extracted Thai Jasmine rice on cholinesterases, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzyme activities. These enzymes were believed to have activities in termination of neurotransmitters in Alzheimer's disease occurrence. The rice samples used in this experiment included parboiled germinated brown rice (PGBR), germinated brown rice (GBR), brown rice (BR), and white rice (WR). The enzyme assays were performed as a colorimetric reaction between a sulfhydryl product from cholinesterase reaction and 5,5'–dithiobis(2–nitro benzoic acid), and were analyzed using a 96–well plate reader. Results found that the best extraction conditions for the highest activity of AChE and BChE inhibitory activities were found at 40% v/v ethanol concentration, shaked for 2 hours at 50°C. The inhibitory activity of both enzymes showed significantly highest by PGBR compared with other types of rice with the values of 6% AChE inhibition and 39% BChE inhibition. These data suggested that PGBR could be used as a natural prevention of Alzheimer's disease.

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Introduction

In healthy brain, neuron cells always communicate to each other in one-way direction via electrical charges through axon. Once the impulses reach the end of axon, depolarization causes calcium ion to enter the neuron. High concentration of intracellular calcium ion leads to release of neurotransmitters into the synaptic space (Lodish *et al*., 2013). The neurotransmitters then bind to their receptor of next target cell or a postsynaptic neuron. The metabolisms are followed the neurotransmitter's transmission, which are particularly interacted with their target receptors, either ligand–gated channel receptors or messenger–linked receptors. The interaction of neurotransmitter and receptor can activate gate channels to open, which allow charges particles to flow through the membrane (Lodish *et al.*, 2013). Residue neurotransmitters, however, are hydrolyzed by neurotransmitter enzymes and converted to choline and re-use for other synapses.

The enzymes responsible for neurotransmitter degradation are cholinesterases (ChEs), consisting of acethylcholinesterase (AChE, EC 3.1.1.7) (Soreq *et al*., 1990) and butyrylcholinesterase (BChE, EC 3.1.1.8) or pseudocholinesterase (Prody *et al*., 1987). Both enzymes are mostly found in vertebrate cells and presented in several tissues. AChE is discovered in brain, nerve cells, muscle and erythrocyte membrane, while BChE is presented in liver, intestine, heart, kidney and lung. Indeed, AChE and BChE share structural similarity (65%), thus BChE can hydrolyze AChE substrate. Nevertheless, the activity of ChEs is depend on metabolic rates and affinities of neurotransmitters (Lodish *et al.*, 2000).

In cholinergic hypothesis of Alzheimer's disease (AD) development described that concentrations of acetylcholine or ACh and choline are rapidly declines, whereas AChE is excessively increased, causing nerve cell dysfunction (Monczor, 2005). Thus, cholinergic activities or neurotransmitters are decreased in the central nervous system, which is a hallmark on brain memory and cognitive function. Inhibition of cholinesterase reactions by cholinesterase inhibitor (ChEI), leading to hindrance of the breaking down of cholinergic synapse and possibly be applied for AD prevention and treatment (Monczor, 2005).

Currently, natural products are widely investigated as functional foods to promote health benefits (Nicoletti, 2012). Rice is one of the major sources of staple food in most countries accounting for approximately 23% of calories consumed by the world population (Khush, 2003). According to the Food and Agricultural Organization of the United

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Nations (FAO, 2001) statistical database in 2001, rice intake accounts for 43% of the total caloric intake by Thai population in 1999. Even though rice is mostly consumed in the form of white (polished) rice, brown rice with intact germ and bran layer possesses higher nutritional values of vitamins E and B, phytic acids and γ–amicobutyric acid (GABA). Germination of brown rice eliminates its undesirable properties of dark color and rough texture and also leads to an increase in quantity of various nutrients such as amino acids, peptides, simple sugars and several bioactive compounds (Frias *et al*., 2005; Fernandez-Orozco *et al*., 2008).

At present, *in vitro* information regarding anti–AD properties of rice after polishing, germinating and parboiling processes is not available. Several researchers reported that Thai Jasmine rice contains various types of water-soluble antioxidants (i.e., feruric acid, p-coumaric acid and other phenol derivatives) (Tewaruth *et al*., 2012; Yodpitak *et al*., 2013). The effect of compounds extracted from rice samples on brain function has previously been investigated; however, most reports had focused on GABA, the primary inhibitory neurotransmitter in the central nervous system (Roohinejad *et al*., 2009; Wunjuntuk *et al.*, 2016). Thus, the effect of Jasmine rice on the brain in other perspectives i.e., cholinergic hypothesis (termination of physiological role of cholinergic synapses) is of interest. It is possible that Thai Jasmine rice could prevent the action of ChEs, leading to the prevention of AD occurrence. Thus, the objectives of this study were to determine the optimum extraction conditions of extracted Thai Jasmine rice on the activity of cholinesterase enzymes.

Materials and Methods

Sample preparation

Four types of raw Thai Jasmine rice (variety Khao Dok Mali 105), which are parboiled germinated brown rice (PGBR), germinated brown rice (GBR), brown rice (BR), and white rice (WR), were obtained from RCK Agri Marketing Co., Ltd. (Thailand). Parboiling process of germinated brown rice consists of two steps; germination process of BR with husk followed by dehusking process. Initially, rice paddy (80 kg) was soaked in water (160 L) for 18 hours. Water was then changed every 4 hours in order to prevent the increase in temperature and also to prevent the foul smell that may occur during the process of germination. The soaking process was continued until the moisture content of the paddy increased by 30%, followed by separation of rice paddy from water to produce steeped paddy. This steeped paddy was then germinated for 42 to 48 hours, followed by steaming for 60 minutes in a vacuum environment for parboiling process to produce PGBR. Then, PGBR was dried at 70 to 75°C for 2 hours.

In order to reduce moisture content, PGBR was left in a hot air dry oven at 40°C until the moisture content was reduced to 13%. Finally, rice sample was left for 7 days, allowing the moisture to be evenly distributed throughout the rice grain before the dehusking process. The samples were freeze-dried (Heto PowerDry PL9000, Thermo Fisher Scientific, Waltham, MA, USA) before being grounded into fine powder by a cyclotec sample mill (series 1903 with 200–240V and 50/60 Hz; FOSS, Höganäs, Sweden). The moisture content of rice sample after freeze drying process was determined using Association of Official Analytical Chemists (AOAC) method (AOAC, 2005) until the range was found between 4 to 6%. All samples were kept in vacuum bags and stored at –20°C before further analysis.

Preparation of rice extract

Rice powder (4 g) was dissolved in 20 mL water and extracted using a bath sonicator (model B1510, 40 KHz; Bransonic Bransonic® Ultrasonic, Danbury, CT) for 10 minutes before being shaken in a water bath shaker (Memmert GmbH, Wisconsin, USA) at temperature (30°C, 50°C, and 70°C), and time (0.5, 1, 2, 4, and 8 hours). The mixture was then centrifuged (model Z 400K; HERMLE Labortechnik GmbH, Wehingen, Germany) at 1190 x g for 5 minutes. The supernatant was filtered through Whatman No. 1 filter paper (GE Healthcare, Bangkok, Thailand). The filtrate was then kept at 4°C for analysis.

Cholinesterase inhibitory assay

The enzymatic assay for cholinesterase activity was done according to the method of Jung *et al.* (2009) with some modifications. The inhibitory enzymatic assay consisted of cholinesterase (5 to 20 ng Electrophorus electricus AChE (1,000 units/mg) or 10 to 50 ng equine serum BChE (≥10 units/mg protein), thiocholine (0.08 mM acetylthiocholine (ATCh) or 0.1 mM butyrylthiocholine (BTCh), DTNB (16 mM) and rice extracts (40 mg/mL). All the chemicals were from Sigma–Aldrich (St. Louis, MO). Next, all mixture was pipetted into 96–well plates and the enzymatic activity was then monitored at a wavelength of 412 nm by a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA), followed by analysis using the Gen5 data analysis software. The initial rate was fitted by the Michaelis–Menten equation with least squares fit parameter by

the GraphPad Prism software version 5.00 (GraphPad Software, Inc., La Jolla, CA, USA). The enzymatic inhibitory activity was calculated as % inhibition according to the following equation.

$$\% \text{ inhibition} = 100 \times (1 - ((B-b)/(A-a)))$$

$A = V_i$ of the control reaction (without the rice extract) with the enzyme
$a = V_i$ of the control reaction without the enzyme
$B = V_i$ of the enzyme reaction with the rice extract
$b = V_i$ of the reaction with the rice extract but without the enzyme
V_i = Initial velocity

Eserine (Sigma–Aldrich, St. Louis, MO), a reversible anti–ChE drug, was used as a standard inhibitor for both AChE and BChE assays.

In order to determine the optimum of AChE and BChE inhibitory activities, five concentrations of ethanol (0%, 20%, 40%, 60%, 80% and 100% (v/v) in deionized water, by means of a Millipore water purification system with resistivity 18.2 $M\Omega cm^{-1}$, Millipore RiOs-DITM134, Bedford, MA, USA), heating temperatures (30°C, 50°C, and 70°C), and shaking times (0.5, 1, 2, 4, and 8 hours) were evaluated in this study.

Statistical analysis

All data were expressed as mean ± standard deviation (SD) of triplicate assays. One way analysis of variance (ANOVA) and Duncan test were performed to determine the significant differences between values at $p<0.05$. All statistical analyses were performed using IBM SPSS Statistics version 19.0 (IBM Corp, Armonk, NY).

Results and Discussion

Effect of different ethanol concentrations on AChE and BChE inhibitory activities

Results of AChE and BChE inhibitory assays (Table 1) suggested that the highest percentage of inhibition activity of extracted WR, GBR, and PGBR were mostly found in extraction of 40% ethanol in water (v/v). This result can be related to the previous study, which investigated the effect of ethanol concentration on antioxidant activities and phenolic compounds of three pigmented rice bran (black, red, and green) and brown rice (Jun *et al.*, 2012). The results of the study suggested that 40% (v/v) aqueous ethanol extracts of all rice samples significantly contained the highest phenolics content (Jun *et al.*, 2012). It was found that phenolic acids including ferulic acid and p-coumaric acids are major bioactive compounds found in our rice samples as being analyzed by HPLC (data not shown). These results were corresponded to the work of Tian et al. (2004) that reported high contents of free phenolic acids including ferulic acid, p-coumaric acid and sinapic acid. The results were confirmed by Yodpitak *et al.* (2013), which reported that rice contains high quantity of phenolics that can function as antioxidants. These compounds were well-dissolved in a mixture of water and ethanol, which might be due to its hydroxyl moieties and aromatic groups.

Table 1. Results of AChE and BChE inhibitory activities of rice samples extracted with various ethanol concentrations

Rice samples*	Ethanol concentration (% v/v in deionized water)	% Inhibition	
		AChE	BChE
WR	0	2.14 ± 2.14^{b}	3.44 ± 0.86^{d}
	20	1.05 ± 0.46^{b}	9.38 ± 2.41^{c}
	40	7.16 ± 1.54^{a}	42.78 ± 0.24^{a}
	60	NA	23.83 ± 1.71^{b}
BR	0	9.23 ± 3.41^{a}	6.95 ± 2.83^{b}
	20	7.02 ± 0.70^{a,b}	7.04 ± 0.24^{b}
	40	8.77 ± 0.90^{a,b}	25.67 ± 0.50^{a}
	60	4.82 ± 2.32^{b}	9.66 ± 0.81^{b}
GBR	0	2.04 ± 1.90^{b}	13.29 ± 2.48^{b}
	20	1.49 ± 1.08^{b}	3.21 ± 1.11^{c}
	40	5.89 ± 1.37^{a}	29.40 ± 2.03^{a}
	60	NA	11.78 ± 1.19^{b}
PGBR	0	2.01 ± 1.67^{b}	3.98 ± 1.62^{d}
	20	2.95 ± 0.96^{b}	9.92 ± 2.37^{c}
	40	11.33 ± 1.95^{a}	45.06 ± 2.44^{a}
	60	5.29 ± 1.98^{b}	26.54 ± 2.24^{b}

*Concentration of extraction solvent was determined when heating at 30°C and shaking for 2 hours. The final concentration of rice sample = 40 mg/mL. Each value was presented as mean ± SD (n = 3). For each enzymatic inhibitory activity, values with different superscript letters (a-d) within the same type of rice were significantly different ($P < 0.05$). NA = not available.

Besides, it was previously reported that some phenolics exhibited anti-AChE and anti-BChE activities (Murray *et al*., 2013). Main phenolic acids in rice, ferulic acid and p-coumaric acid, were found to act as non-competitive inhibitors against AChE with K_i of 22±1.2 and 13±1.1 mM, respectively and against BChE with K_i of 21±2.2 and 16±1.6 mM, respectively (data not shown). Therefore, suitable solvent for extracting phenolics from rice samples can lead to high extracted anti-ChE agents.

Interestingly, anti-AChE activities of rice samples were relatively low (<10% inhibition), comparing to BChE (3–45% inhibition). These results were similar to a study conducted with rice bran extracts that also revealed a more prominent inhibition of BChE than AChE inhibition (Abeysekera et al., 2015). It has been previously found that BChE has a lower substrate specificity than AChE and has a wider range of substrates as it acts on both ACh and BCh substrates (Chatonnet and Lockridge, 1989). This matter can be explained regarding the sizes of substrate binding

Table 2. Results of AChE and BChE inhibitory activities of rice samples extracted at various heating temperatures

Rice samples*	Extraction temperatures (°C)	% Inhibition	
		AChE	BChE
WR	30	1.55 ± 1.28^{a}	36.86 ± 0.01^{c}
	50	1.23 ± 0.70^{a}	39.99 ± 0.24^{a}
	70	NA	38.00 ± 0.09^{b}
BR	30	6.32 ± 0.66^{b}	32.22 ± 0.05^{b}
	50	8.41 ± 0.60^{a}	35.38 ± 1.29^{a}
	70	1.67 ± 1.04^{c}	26.09 ± 1.02^{c}
GBR	30	2.01 ± 0.15^{c}	27.46 ± 0.93^{b}
	50	6.02 ± 1.28^{b}	33.81 ± 1.37^{a}
	70	9.29 ± 1.53^{a}	33.64 ± 0.99^{a}
PGBR	30	5.96 ± 2.08^{b}	44.01 ± 2.89^{a}
	50	10.38± 1.93^{a}	43.66 ± 0.64^{a}
	70	9.27 ± 0.48^{a,b}	41.69 ± 0.21^{a}

*Extraction temperature was determined when extracting with 40% (v/v) aqueous ethanol and shaking for 2 hours. The final concentration of rice sample = 40 mg/mL. Each value was presented as mean ± SD (n = 3). For each enzymatic inhibitory activity, values with different superscript letters (a-c) within the same type of rice were significantly different ($P < 0.05$). NA = not available.

cavities in AChE and BChE. Comparing to the acyl pocket of AChE that consists of two larger Phe residues, the acyl pocket composing of small aliphatic Leu and Val residues is responsible for catalysis and substrate specificity in BChE (Çokuğraş, 2003; Bajda *et al*., 2013). Therefore, substrates binding at the active site of AChE are more selective than those in the larger active site of BChE, leading to wider varieties of substances that can inhibit BChE as being observed in our experiment (higher percentage of inhibition).

Effect of different heating temperatures

Effect of heating temperatures on AChE and BChE inhibitory activities found that the highest activity was found at 50°C for all of the rice samples in comparison to the other heating temperatures (Table 2). High temperature could cause a better cell wall disruption than low temperature; however, some heat sensitive compounds that leaks into solution after cell breaks might be, as well, degrade under this condition. This thermodegradation could be observed for almost all plants that undergone high temperature for long time period. Low temperature, however, suffers from low extraction yield. Thus, suitable extraction temperature, in this case, was chosen as 50°C for further investigation on extraction time.

Similarly to the effect of ethanol concentrations, AChE inhibitory activity (<10% inhibition) was found relatively lower than those of BChE reactions (32–44% inhibition). This matter might be due to substrate specificity of the enzymes as mentioned previously that the catalytic pocket of AChE consists

Table 3. Results of AChE and BChE inhibitory activities of rice samples extracted at various shaking times

Rice samples*	Extraction times (hours)	% Inhibition	
		AChE	BChE
WR	0.5	NA	33.12 ± 1.73^{c}
	1	3.52 ± 2.24^{a}	35.31 ± 1.13$^{b, c}$
	2	3.18 ± 1.73^{a}	32.89 ± 0.88^{c}
	4	3.95 ± 2.75^{a}	39.78 ± 3.37^{a}
	8	0.99 ± 0.63^{a}	36.83 ± 0.54^{a,b}
BR	0.5	1.48 ± 0.49^{c}	20.60 ± 1.37^{c}
	1	5.14 ± 0.91^{b}	25.04 ± 0.80^{b}
	2	6.91 ± 1.12^{a}	25.47 ± 0.26^{b}
	4	7.18 ± 1.22^{a}	26.56 ± 1.78^{a,b}
	8	6.79 ± 0.00^{a}	28.22 ± 1.29^{a}
GBR	0.5	4.00 ± 2.16^{a}	26.43 ± 0.61^{b,c}
	1	0.22 ± 0.06^{b}	25.82 ± 1.91^{c}
	2	6.49 ± 3.36^{a}	30.21 ± 1.33^{a,b}
	4	3.17 ± 1.73^{a,b}	30.02 ± 2.91^{a,b}
	8	4.91 ± 1.87^{a}	31.53 ± 2.93^{a}
PGBR	0.5	NA	34.52 ± 2.05^{c}
	1	NA	38.31 ± 0.84^{a,b}
	2	6.37 ± 1.77^{a}	39.35 ± 1.71^{a}
	4	7.63 ± 0.41^{a}	38.49 ± 1.24^{a,b}
	8	0.39 ± 0.21^{b}	36.35 ± 0.87^{b,c}

*Extraction time was determined when extracting with 40% (v/v) aqueous ethanol and heating at 50°C. The final concentration of rice sample = 40 mg/mL. Each value was presented as mean ± SD (n = 3). For each enzymatic inhibitory activity, values with different superscript letters (a-c) within the same type of rice were significantly different ($P < 0.05$). NA = not available.

of larger residues than those of BChE; therefore, AChE is more specific to the substrates than BChE (Chatonnet and Lockridge, 1989; Çokuğraş, 2003; Bajda *et al*., 2013). Thus, BChE can accept wider varieties of inhibitors as being observed in our experiment.

Effect of different shaking times

The AChE inhibitory activities of rice samples found that 2 hours of shaking time was the most suitable extraction time (Table 3), even though 1 hour shaking time should be enough for WR extract, while BR required at least 2 hours for extraction of anti-ChE agents. For GBR, the statistical analysis suggested that 0.5, 2 and 8 hours of shaking time were optimum, but the results suggested that 2 hours provided the highest AChE inhibitory activity. Besides, since PGBR exhibited the highest inhibitory activity under 2 and 4 hours of shaking time, lower time period was preferable for efficiency. Overall, 2 hours of shaking time was provided significantly higher AChE inhibitory activities than those extracted under 0.5 and 1 hour in most samples, thus 2 hours of shaking time was chosen in this experiment.

As for BChE inhibitory activity, WR and BR exhibited the highest inhibitory activity with at least 4 hours of shaking time, while GBR and PGBR preferred at least 2 hours. The raw data of BR also suggested that 1-2 hours of shaking time also provided comparable inhibitory activity to that of 4 hours. This could be explained by second diffusion

Table 4. Results of AChE and BChE inhibitory activities of rice extracts and standard, serine.

Samples	% Inhibition	
	AChE	BChE
Eserine*	19.27 ± 2.25	15.80 ± 1.13
WR**	5.05 ± 1.24$^{a, b}$	34.09 ± 3.83^{b}
BR**	4.71 ± 0.22$^{a, b}$	32.94 ± 1.06^{b}
GBR**	3.32 ± 1.54^{b}	32.09 ± 1.04^{b}
PGBR**	5.79 ± 1.57^{a}	38.80 ± 1.18^{a}

*Final concentration of eserine = 0.3 µM and 0.03 µM for BChE and AChE assays, respectively

**Final concentration of rice sample = 40 mg/mL

Each value was presented as mean ± SD (n = 3). For each enzymatic inhibitory activity, values with different superscript letters (a-b) were significantly different ($P < 0.05$).

law of Fick, which states that after a certain duration, the equilibrium of the compounds dissolved in the solvent will be reached between the solid matrix and the solvent (Pinelo *et al*., 2006). Therefore, the optimum extraction condition of 40% (v/v) aqueous ethanol at 50°C for 2 hrs was then utilized for further analysis of all rice samples.

Again, the AChE inhibitory activity was significantly lower (<10% inhibition) than BChE inhibitory activity (20–40% inhibition). The results can be explained in term of substrate binding cavity and substrate specificity of the enzymes as mentioned previously that the catalytic pocket of AChE is smaller than that of BChE (Bajda *et al.*, 2013; Chatonnet and Lockridge, 1989; Çokuğraş, 2003). Thus, wider variety of inhibitors can bind to the active site of BChE, leading to its higher percentage of inhibition as being observed in our experiment.

Comparison of cholinesterase inhibitory activity in rice extracts

Under the extraction conditions of 40% (v/v) aqueous ethanol, heating at 50°C and shaking for 2 hours, PGBR displayed the highest AChE and BChE inhibitory activities among all rice samples, suggesting that parboiling process might be the most effective factor that increases bioactive compounds extraction from rice samples that have AChE and BChE inhibitory activities (Table 4). Parboiling process causes micronutrients to migrate to the core of the grain and then remain there after the starch gelatinization process (Juliano, 1985). The nutrients that were already soluble due to parboiling process may be extracted better in comparison to other rice types including WR, BR and GBR, resulting in extracts with a higher ChE inhibitory activity. On the other hand, the polishing and germinating processes provided less impact on the anti-cholinesterase activity. It was previously suggested that time of germination had an impact on the quantity of bioactive compounds. Germination for 2-4 days exhibited a significant increase of nutrients such as γ-tocopherol, α-tocopherol, α-tocotrienol, γ-oryzanol and phenolic compounds (Fernandez–Orozco *et al*., 2006).

Previously, Tian *et al.* (2004) reported that the contents of free phenolic acids including ferulic acid and sinapic acid had increased significantly during germination. It was also suggested that 24 hours of steeping wheat grains, followed by 7 days of germination were more favorable than 48 hours in terms of antioxidant concentrations (Yang *et al*., 2001). Normally, germination process enhances nutritional components (Frias *et al.*, 2005). The difference in bioactive compounds that usually results from the process of germination was not found to have an effect on ChE inhibitory activity in this study. This could be due to the impact of germination time on the quality and quantity of bioactive compounds.

A study conducted by Yang *et al.* (2001) on the germination of wheat grain found that not only is the number of germination days important but the steeping time of the grains prior to germination is also a significant factor that determines the biochemical changes due to the germination process. Two sets of wheat grains were steeped for 28 hours and 48 hours, both sets had their bioactive compounds at their peak after 7 days of germination. However, the grains steeped for 48 hours showed signs of degradation after 7 days of germination, and also the levels of bioactive compound dropped. Grains steeped for 28 hours, on the other hand, showed peak elevation of ferulic acid, vanillic acid, α−tocopherol and γ−tocopherol contents on day 7 followed by decline in content. Vitamin C and α–carotene contents were optimized on day 8 followed by decline in content on day 9 (Chavan and Kadam, 1989). As for our study, the paddy was steeped for 18 hours followed by approximately 2 days of germination. Cholinesterase inhibitory activities of rice samples observed in our experiments might not be affected by the germination time according to the similar ChE inhibitory activity between BR and GBR.

In addition, our previous experiment in antioxidant activities and total phenolic contents (TPCs) suggested that the optimum extraction conditions were similar to this experiment (40% (v/v) aqueous ethanol, heating at 50°C and shaking for 2 hours) (Sripum *et al*., accepted). Besides, PGBR exhibited the highest antioxidant activities and TPCs, followed by GBR, BR, and WR, respectively. These results supported our hypothesis that some antioxidants and phenolic compounds extracted from rice samples could act as anti-ChEs agents.

Conclusion

The optimum extraction conditions for anti-ChE agents from rice were found at the conditions of 40% (v/v) aqueous ethanol, heating at 50°C and shaking for 2 hours. In addition, BChE inhibitions were greater than AChE inhibitions in all investigated conditions because of larger enzyme substrate binding cavities and less substrate specificity. Under optimized extraction conditions, PGBR significantly ($P<0.05$) exhibited the highest AChE and BChE inhibition activities among all rice samples, suggesting that parboiling process is a significant factor that can maintain anti-ChEs agents in rice samples.

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