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Research Article

IN VITRO BIOLOGICAL PROPERTIES OF CRUDE METHANOL EXTRACT FROM MUSHROOM; *FLAMMULINA VELUTIPES* (GOLDEN NEEDLE MUSHROOM)

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ABSTRACT

Objectives: The objectives of this study were to investigate the *in vitro* biological effect of the methanolic crude extract received from edible mushrooms; *Flammulina velutipes* on anti-inflammatory, antioxidant activity, anti-lipid peroxidation (LPO), antimicrobial activity, and anticancer activity. The scientific result obtained here was possibility of developing this variety as an ingredient in health/medical food purposes as well as a functional food.

Methods: In this study, the methanol crude extract of *F. velutipes* has been performed and *in vitro* pharmacological properties such as anti-inflammatory, antioxidant activity, anti-LPO, antimicrobial activity, and anticancer activity were evaluated.

Results: The results revealed that anti-inflammatory activity of crude extract was very significant with 79.81±0.87% of the dose-dependent manner. Antioxidant and anti-low-density lipoprotein (LDL) peroxidation activity of the extract showed its capacity of free radical scavenging with high-level antioxidant compounds and lipid preventive effects (2, 2-diphenyl-1-picrylhydrazyl: 79.81±0.01%, total phenol: 991.10±0.01 µg gallic acid equivalents/0.1 g dry matter, H_2O_2: 93.27±0.05%, LPO: 33.33±0.00%, and LDL: 57±0.03%, respectively). Analysis has also demonstrated that the methanolic extract of *F. velutipes* provided an antimicrobial activity to ESBL-producing *Klebsiella pneumoniae*. Moreover, *F. velutipes* extract showed a growth inhibitory effect in HepG2 cells with the 50% inhibitory concentration (IC_{50}) value IC_{50}=8.25±7.59 µg/ml.

Conclusion: This indicated that crude extract from *F. velutipes* was scientifically proved as a potential source of therapeutic agent and be able to utilize as a functional ingredient for healthy or supplement food.

Keywords: *Flammulina velutipes*, Methanol crude extract, Pharmacological properties, Antioxidant, Antimicrobial, Antitumor.

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INTRODUCTION

Recently, there has been the most considerable in the use of natural antioxidants as food supplements and scientific interest in fungal biomolecules as antioxidants [1-4]. Although the chemical nature of such compounds is still unclear, phenolic compounds and non-phenolic compounds including terpenoids and polysaccharides have also been designated as major naturally occurring antioxidant components found in medicinal mushrooms [5-7].

Edible mushrooms, *Flammulina velutipes* is one of the most popular edible mushrooms in Asia. It contained a lot of bioactive compounds such as polysaccharides, protein-glucan complex, and sterols and lectins of medicinal and pharmaceutical properties [8]. *F. velutipes* is well known for promoting human health and scientifically proved for its important natural resources of immunomodulating, antitumor, antioxidant, thrombolytic, fibrinolytic, antibacterial, antifungal, antiviral, haploidic, and mitogenic agents [9]. However, drug action and dose consumption of the extracts were varied since the differentiation of extraction technique and active ingredients consisting in it.

Our objective was to investigate the *in vitro* biological effect of the methanolic crude extract received from *F. velutipes* on anti-inflammatory, antioxidant activity, anti-lipid peroxidation (LPO), antimicrobial activity, and anticancer activity. The scientific result obtained here was possibility of developing this variety as an ingredient in health/medical food purposes.

MATERIALS AND METHODS

Mushroom material

Fruit bodies of *F. velutipes* (Curtis: Fries) were purchased from the local market Pathum Thani, Thailand. The botanical identity of the plant specimen of *F. velutipes* was confirmed by a taxonomist at Sun Herb, Thai, Chinese Manufacturing, Rangsit University. It was authenticated to be *F. velutipes* belonging to family Physalacriaceae. Thereafter, the mushroom was cleaned, cut into small pieces and was subjected to dry at 40°C for 15–20 h. The dry material was ground into powder and stored in air-tight plastic bag in a desiccator at room temperature for further analysis.

Preparation of *F. velutipes* crude extract

For extraction, methanol extraction has been conducted with the ratio of sample of mushroom powders: Methanol equal to 1:10 under 37°C for 5 days. Solid has been discarded by filtered through Whatman No. 1 filter paper. The filtrate was subjected to rotary evaporator for ethanol removal, then crude extract of *F. velutipes* has been received. The crude was redissolved in dimethyl sulfoxide (DMSO) at the concentration of 1 g/ml. Crude extract was kept in glass bottle under −20°C along this experiment.

Microorganisms and chemicals

Microorganisms used in this study were both Gram-positive and Gram-negative, for example, two strains of multidrug-resistant *Acinetobacter baumannii* (MDR I and MDR II), vancomycin-resistant *Enterococcus faecalis*, ESBL-producing *Escherichia coli*, ESBL-producing *E. coli* P174, ESBL-producing *Klebsiella pneumoniae*, two strains of multidrug-resistant *Pseudomonas aeruginosa* (MDR I and MDR II), and two strains of methicillin-resistant *Staphylococcus aureus* (MRSA I and MRSA II). All chemicals used were analytical grade.

Anti-inflammatory assay of *F. velutipes* crude extract

Anti-inflammatory was determined as the inhibition effect on protein denaturation using modified Mizushima and Kobayashi (1968) method [10]. Crude extract of *F. velutipes* has been serial 2-fold diluted to the concentration of 625, 1250, 2500, 5000, and 10,000 µg/ml in distill

water. 100 µl of each concentration mixed with 500 µl of 1% bovine serum albumin, then let the mixtures stand at ambient temperature for 10 min. The mixture was heated at 51°C for 20 min and cooled at room temperature. The absorbance was measured at 660 nm. Acetylsalicylic acid was used as positive control. Inhibited percent was calculated as follows:

$$\% \text{ inhibition } = 100 - \frac{(A1 - A2)}{A0} \times 100$$

Where, A1, A2, and A0 were absorbance of sample, product control, and positive control, respectively.

Antioxidant assay of *F. velutipes* crude extract

2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

The free radical scavenging activities of the extracts were measured using DPPH [11]. Briefly, extract concentration of 625–10,000 µg/ml in 100 µl methanol was mixed with 100 µl ml of methanolic solution containing 0.2 mM of DPPH (DPPH, Sigma). The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank. The calibration curve was also established using Vitamin C equivalent antioxidant capacity by solution of L-ascorbic acid in methanol. The capability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = $[1-(A_1/A_0)] \times 100$

Where, A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the sample.

Folin–Ciocalteu colorimetric assay

Total phenolic compounds were determined using Folin–Ciocalteu's method [12]. Briefly, extract concentration of 625–10,000 µg/ml in 20 µl ethanol was mixed with 100 µl ml of Folin–Ciocalteu's reagent (100 µl, previously diluted with water 1:10, v/v). From the resulting mixture, 80 µl of 2% aqueous sodium carbonate was added. The mixture was incubated for 30 min; absorbance was measured at 765 nm against the blank. The content of total phenol was calculated on the basis of the calibration curve of gallic acid, and the results were expressed as µg of gallic acid equivalents (GAEs) per mg of extract.

Hydrogen peroxide scavenging activity

The ability to eradicate hydrogen peroxide was determined according to Halliwell *et al.* [13]. An aliquot (850 µl) of *F. velutipes,* crude extract at different dilutions (625–10,000 µg/ml), was added to 150 µL of 4 mM hydrogen peroxide solution in phosphate buffer (0.1 M, pH 7.4). The solution was incubated for 10 min and the absorbance measurement was performed at 230 nm where butylated hydroxytoluene was used as positive control.

Anti-LPO

Thiobarbituric acid (TBA), reactant substances assay, was used to analyze the inhibition of lipid peroxidase as described by Ruberto *et al*. [14], with some modification. Briefly, diluted egg yolk to 10% (w/v) with KCL solution was added to 50 µl of different concentrations *F. velutipes* crude extract. The addition of 20% acetic acid (pH 3.5) 300 µl and TBA 300 µl into the previous solution then mixed with vortex mixer. The mixtures were incubated at 95°C for 1 h and left them to cool at ambient temperature. Adding 750 µl of butanol then centrifugation at 3000 rpm for 10 min. Supernatant was absorbance measurement using an ELISA reader at 532 nm.

Antioxidative low-density lipoprotein (LDL) ability was also determined. Briefly, 9 µl of human LDL were mix with 191 µl of 10 mM ferrous sulfate and 100 µl of *F. velutipes* crude extract at different dilution (625–10,000 µg/mL). After that, 15% trichloroacetic acid 500 µL and 1% TBA 1 ml were added. The mixtures were incubated at 100°C for 10 min. Then, cool by left them to stand at ambient temperature. Pipette 300 µl of the mixtures into microtiter plate and measured the absorbance at 532 nm using an ELISA reader, and $CuSO_4$ (pH 7.4) has been applied as blank.

Antimicrobial test of *F. velutipes* crude extract

Antimicrobial activity was tested by agar well diffusion method. Gram-negative and Gram-positive bacteria mentioned above were used for antimicrobial test. Reculture those respective bacteria from freezing vials on nutrient agar and adjusted its turbidity using spectrophotometer measurement at 625 nm to achieve concentration of 1.5×10^8 CFU/ml in a sterile 0.85% NaCl solution. 100 µl of test organism was applied to the surface of Muller-Hinton agar using sterile cotton swab. 6 mm diameter wells in agar were made using a sterile cork borer. 30 µl of different dilutions of *F. velutipes* crude extract (625–10,000 µg/ml) were added to the wells. Similarly, 30 µl of nutrient broth and gentamycin were used as negative control and positive control. The plates were incubated at 35±2°C for 24 h, and the diameter of inhibition zone around the well was measured using a scale. All experiments were repeated 3 times.

Cell lines and culture medium

Chemicals

3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), and trypsin were obtained from Sigma-Aldrich Co., St. Louis, USA. Ethylenediaminetetraacetic acid (EDTA), DMSO, and propanol were obtained from Merck Ltd., USA.

Human, liver hepatocellular cells (HepG2) stock cells were cultured in DMEM supplemented with 10% inactivated FBS, penicillin (100 IU/ml), in a humidified atmosphere of % CO_2 at 37°C until confluent. The cells were dissociated with a trypsin phosphate solution (0.2% trypsin, 0.02% EDTA in PBS). The stock cultures were grown in 25 cm^2 culture flasks, and all experiments were carried out in 96 microtiter plates (Nunc. Ltd., USA).

Preparation of test solutions

For cytotoxicity studies, the crude extract from *F. velutipes* was dissolved in DMSO and volume was made up with non-supplemented DMEM and sterilized by filtration. Serial dilutions were prepared for this for carrying out cytotoxic studies.

Cytotoxicity assay

The MTT assay was performed as described by Cardile *et al.* [15]. The viability of the cell was assessed by MTT assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Cells were seeded in 96-well microtiter plates (5×10^3cells/well in 100 µl medium) and routinely cultured in a humidified incubator at 37°C in 5% CO_2 for 24 h. The extracts were added in serial concentrations such as 7.81, 15.63, 31.25, 62.5, 125, 250, 500, 1000, 10,000, and 100,000 µg/ml and Triton X (1 and 2%) was used as positive control. The plate was reincubated for 24 h. Then, the medium was discarded and 30 µl of MTT dye solution (5 mg/ml in PBS) was added to every well and reincubated for 4 h. After removing untransformed MTT reagent, 100 µl of DMSO was added to dissolve the formed formazan crystals. Amount of formazan was determined by measuring the absorbance at 570 nm using an enzyme-linked immunosorbent assay plate reader. All experiments were carried out 3 times.

Statistical analysis

All experimental results were carried out in triplicate and were expressed as average of three analyses ±SD (standard deviation) using the SPSS version 22.

RESULTS AND DISCUSSION

The need for a new source of natural bioactive compounds, in general, comes from the increasing health promotion regimen. The present study was pointing out to *F. velutipes* due to their wide ubiquity in nature compound, leading the production of various therapeutic properties,

i.e.,T anti-inflammatory, antioxidant, antimicrobial, and antitumor.

In this study, *F. velutipes* crude extract has been subjected for its potent of protein denature inhibition. BSA was denatured by heat treatment, and different doses of the extract have been tested for preventing BSA denaturation which presented in Fig. 1. From the data, it was shown that the inhibition of BSA denaturation was dose-dependent. At the initial concentration (625 µg/ml), *F. velutipes* demonstrated inhibition effect of BSA denaturation more than 50% and increased to 79.81±0.01% when the dose reaches 10,000 µg/ml. Denaturation of proteins is the main cause of inflammation [16]. Therefore, ability of preventing protein denaturation suggesting that *F. velutipes* crude extract possessed anti-inflammatory activity undoubtedly. The ability to inhibit BSA denaturation which composed of two binding sites which are aromatic tyrosine-rich and aliphatic threonine and lysine residue region, those therapeutic compounds existing in the extract might activate the tyrosine motif rich receptor dually with threonine that regulates signal transduction biological pathways for their overall biological action [17,18]. It is also noteworthy that those compounds which react to aliphatic lysine residue on BSA may possess antioxidant or anticancer properties the same as polyphenol substances [18-20].

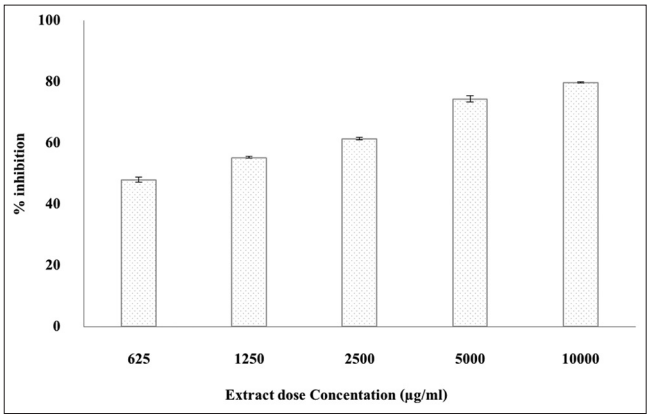

Fig. 1: Effect of *Flammulina velutipes* crude extract on preventing of BSA denaturation

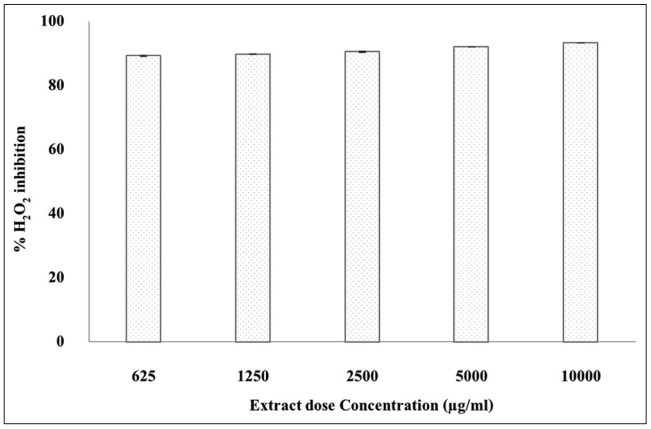

Fig. 2: Effect of *Flammulina velutipes* crude extract on hydroxyl radicals scavenging ability

To determine *F. velutipes* crude extract for its antioxidant activity, numerous methods were used to yield the complete antioxidant profile of respective sample, and the result shown in Table 1 and Figs. 2-3.

Total phenolic compound of crude extract shown high value with 991.10±0.01 µg GAE/0.1 g dry matter. This result was similar to Sławińska *et al*. who revealed the same finding that *F. velutipes* extract with ethanol showed the highest value as 4.73±0.13–6.31±0.34 mg GAE/g of extract [21]. Moreover, *F. velutipes* crude extract possessed DPPH free radical scavenging activity of 68.42±3.03%, which slightly higher than the finding reported by Sławińska *et al*. [21] and Milovanovic [22].

Hydroxyl radicals, the most reactive radicals generated during aerobic metabolism, are the form of a number of reactive oxygen species such as superoxide anion, hydrogen peroxide, and nitric oxide which damage biological molecules [23-25]. Results on hydroxyl radical scavenging of *F. velutipes* crude extract shown in Fig. 2 and revealed that scavenging activity of the extract was increased when its dose was higher. The scavenging activity of the extract at 10,000 µg/ml was greatly high as over 93.27±0.05%. Two mechanisms of antihydroxyl radicals were proposed; one is to prevent hydroxyl radicals generating through ligate to metal ions and two, scavenge the generated hydroxyl free radical [23]. This might suggest that *F. velutipes* crude extract also carried those mentioned antihydroxyl radical activity. Some similar findings showed a similar trend for DPPH from some selected indigenous wild edible

Table 1: Scavenging effect on DPPH free radical, total antioxidant, and total phenolic compound presented in 0.1 g/ml *F. velutipes* crude extract

Antioxidant assay	Average±SD
Total phenolic compound (µg GAE/0.1 g dry matter)	991.10±0.01
DPPH scavenging activity (%)	68.42±3.03
Total antioxidant (µg of Vitamin C equivalent/0.1 g dry matter)	50.06±5.50

DPPH: 2, 2-Diphenyl-1-picrylhydrazyl, *F. velutipes: Flammulina velutipes*, GAE: Gallic acid equivalents

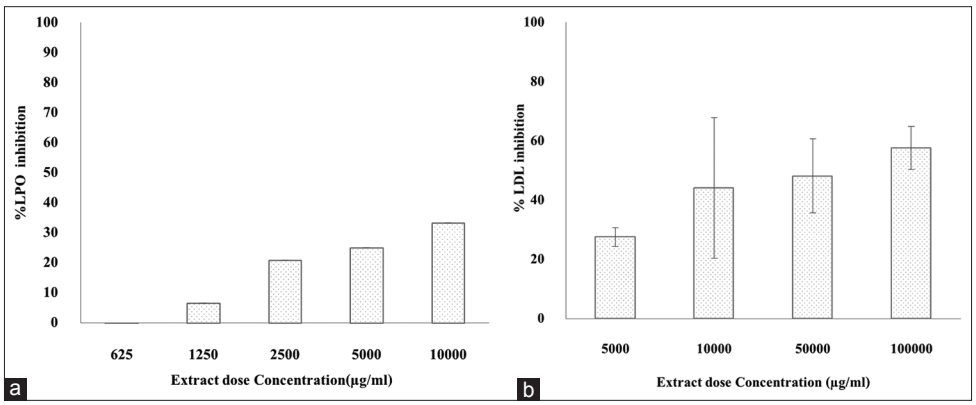

Fig. 3: Effect of *Flammulina velutipes* crude extract as an anti-lipid oxidation; (a) Inhibition ability on lipid peroxidation, (b) Inhibition ability on low-density lipoprotein

Table 2: MIC of *F. velutipes* comparing with gentamicin

Tested crude extract/Drug	Concentration (µg/ml)	Inhibition zone										
		Staphylococcus aureus MRSA	*Staphylococcus aureus*	*Acinetobacter baumannii* MDR1	*Acinetobacter baumannii*	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	*Escherichia coli* P174 ESBL	*Escherichia coli*	*Klebsiella pneumoniae* ESBL	*Enterococcus faecalis* VRE
F. velutipes	100,000	–	–	–	–	–	–	–	–	11±1.00	–
	10,000	–	–	–	–	–	–	–	–	–	–
	5000	–	–	–	–	–	–	–	–	–	–
	2500	–	–	–	–	–	–	–	–	–	–
	1250	–	–	–	–	–	–	–	–	–	–
Gentamicin	100,000	10.67	*9.33*	9	9.67	14.67	14.33	8.33	10.67	10.33	13.33

G: Growth, NG: No growth. MIC: Minimum inhibitory concentration, *F. velutipes*: *Flammulina velutipes*

Fig. 4: Cytotoxicity effect of *Flammulina velutipes* crude extract to HepG2 (liver carcinoma) cell line

mushrooms such as *Termitomyces* group, *Russula,* and *Volvariella* sp. in Eastern India as 61% to as high as 94% [26].

Not only in aqueous matrix has the oxidative reaction but also occurred in lipid layer which LPO has been regarded as the main cause of damaging cellular component and fragmentation of connective tissues [27,28]. From Fig. 3a, it was appeared that *F. velutipes* crude extract contained LPO and LDL inhibition property as dose-dependent manner. An extract dose at 10,000 µg/mL performed 33.33±0.00% of LPO inhibition.

Oxidized LDL is recognized as the cause of lipid accumulated on arterial walls and lead to atherosclerosis-related disease [29,30]. *F. velutipes* crude extract also expressed high potential of LDL oxidative inhibition property which showed the value as 57±0.03% of LDL inhibition was received at an extract dose of 10,000 µg/ml (Fig. 3b). Similar results were found that *F. velutipes* crude extract by methanol can inhibit the formation of thiobarbituric acid reactive substances by use human LDL as a lipid source with value 48.71%, at 1 mg/ml concentration [31].

In the present work, antimicrobial property verified by well diffusion showed the potential of the *F. velutipes* crude extract was characterized against both Gram-negative and Gram-positive microorganisms where gentamicin was used as positive control, and the results were shown in Table 2. Among the tested bacteria, *F. velutipes* crude extract only at 10,000 µg/ml demonstrated as the least concentration to inhibit only ESBL-producing *K. pneumoniae* with the inhibition zone as 11±1.00 mm. Nicolcioiub *et al*. also demonstrated that the *F. velutipes* crude extract by ethanol can inhibit growth of *Bacillus subtilis* subsp. *Spizizenii* ATCC 6633, *S. aureus* ATCC 6538, and *P. aeruginosa* ATCC 9027 with inhibition zone between 10 and 20 mm [32]. Similarly, previously reported showed that the methanolic extract of white button mushroom (*Agaricus bisporus*) displays antimicrobial activity against *P. aeruginosa* and *S. aureus* with a high level of inhibition zone [33].

The biological activity of any phytocompounds depends on the type of chemical composition and the concentration of active constituents as well as types and developmental stages of the cancer [34]. The screening of *F. velutipes* crude extract for their anticancer properties used cell-based assays and established cell lines, in which the cytotoxic effects of mushroom extracts could be measured. MTT assay is a non-radioactive, fast, and economical assay widely used to quantify cell viability and proliferation. MTT is a yellow water-soluble tetrazolium salt. Metabolically active cells are able to convert the dye to water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring [35]. The result of our study revealed that the exposure of crude extract from *F. velutipes* at different concentrations such as 7.81, 15.63, 31.25, 62.5, 125, 250, 500, 1000, 10,000, and 100,000 µg/ml for 24 h resulted in decrease of cell proliferation in a dose-dependent manner. The percentage of inhibitory concentration 50% (IC_{50}) inhibition of cell

proliferation was found to be initiated at the concentration of 8.25±7.59 µg/ml (Fig. 4). It was clearly confirmed that the crude extract from *F. velutipes* was effective and can be used as alternative chemotherapy cancer. Another report from Milovanovic *et al.* was shown that crude extract from *F. velutipes* has a low cytotoxic activity against both HeLa and LS174 cell line with IC_{50} as 259.69±0.70 µg/mL and 338.47±0.97 µg/ml, respectively [22].

CONCLUSION

The results obtained in the present work denote that wild-growing edible mushrooms; *F. velutipes* may constitute a good source of healthy compounds or phenols intake in the diet. The results revealed that the *F. velutipes* crude extract possessed various pharmacological properties (*in vitro* evaluation), for example, antimicrobial, anti-inflammatory, antioxidant, and antitumor, which expressed against both free radicals in aqueous and lipid matrix. Due to these characteristics, *F. velutipes* could be considered as a complement in the diet for the benefits they present and may use as a potential natural ingredient to improve the therapeutic effects of medication or supplement food for some diseases.

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AUTHOR'S CONTRIBUTIONS

All authors contributed extensively to the work presented in this paper. Bajaree Jantrapanukorn designed, performed experiments, analyzed data, and wrote the paper; Pannapa Powthong performed experiments, gave conceptual advice, and revised the manuscript and analyses data; Chitradee Luprasong gave technical support and revised the manuscript. All authors discussed the results and implications and commented on the manuscript at all stages.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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