

## Research Article

# Melanogenesis inhibitory effect of *Perilla Frutescens* (L.) Britt on a hyperpigmentation model in rabbit

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## ABSTRACT

The study aims to evaluate the melanogenesis inhibitory action of the ethyl acetate (EA) extract from *Perilla frutescens* (L.) Britt on a hyperpigmented rabbit model induced by UVA-exposure combined to progesterone injection. The dorsal shaved skin of the experimental rabbits was exposed to UVA radiation (386 nm, 147 uW/cm<sup>2</sup>) in 30 minutes each day for 28 days. Progesterone was intramuscularly injected every other day at the dose of 5 mg/kg. EA extract was topically applied daily from the first day of melasma appearance until the end of the experiment. Morphometric observation based on color thresholder application on MATLAB software. Moreover, melanin determination and histological analysis were also performed. The morphometric observation on the surface of melasma areas as well as the histological analysis from 5% EA-treated group are similar to these of 4% Hydroquinone-treated group at day 14, 21, 28. Melanin concentration in 5% EA-treated group was significantly decreased as compared to the hyperpigmentation group. In conclusion, EA extract of *Perilla* leaves clearly exhibited the inhibitory effect on melanogenesis in the hyperpigmented rabbit-induced by UVA exposure combined to progesterone injection.

### Keywords:

Hyperpigmentation, Melasma, Melanin, *Perilla* leaves

## 1. INTRODUCTION

Melasma is a chronic acquired hyperpigmentation of the skin due to melanogenesis dysfunction and has an impact on the quality of life of patients. It presents as irregular brown macules symmetrically distributed on sun-exposed areas of the body<sup>1</sup>. There are some known factors implicated in the etiopathogenesis such as sun exposure, pregnancy, sexual hormones, inflammatory processes of the skin, use of cosmetics, steroids, and photosensitizing drugs<sup>1</sup>. Although melanogenesis is a complicated process represented by numerous enzymatic and chemical reactions, the pathophysiology of various skin hyperpigmentation disorders is essentially related to genes that encode tyrosinase, the key enzyme in melanogenesis and the tyrosinase-related proteins (TRPs), including TRP-1 and TRP-2<sup>2</sup>. The treatment of melasma is challenging and requires long-term treatment because

the available therapies are insufficient, usually less effective than expected and the reoccurrence is common. Multiple options for topical treatment are available, of which hydroquinone (HQ) is the most commonly used agent<sup>3</sup>. HQ inhibits the conversion of DOPA to melanin by mainly inhibition of the tyrosinase enzyme and decreasing the number of melanocytes. However, the safety of daily long-term use of cosmetic products containing high concentrations of HQ has been raised questions<sup>4</sup>. Patients commonly experience irritation, erythema, stinging, scaling and occasional paradoxical post-inflammatory hypermelanosis as well as exogenous ochronosis characterized by progressive pigmentation of the area to which the agent is applied. Therefore, a number of natural sources including plants, bacteria and fungi have recently become of increasing interest for their whitening effect<sup>5</sup>.

*Perilla frutescens* (L.) Britt., is an annual herb, belonging to the family Lamiaceae, widely cultivated in

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some areas in Vietnam<sup>6</sup>. *Perilla* usually uses as traditional medicine, but it has been recently investigated as a fundamental cosmetic ingredient because of the wide range of potent antioxidants, including, caffeic acid, coumaroyl tartaric acid, rosmarinic acid, catechin, and apigenin. Among these compounds, rosmarinic acid, a major polyphenol in *Perilla* leaves, exhibits antioxidant and anti-tyrosinase activities-the important biological activities for the inhibition of the melanogenesis process<sup>7</sup>. Indeed, the results from studies of Preedalikit et al (2020) and Mungmai et al (2020) proved the melanogenesis inhibition of extracts from *Perilla* leaves and seeds on B16F10 cell line in a dose-dependent manner without cytotoxicity<sup>7-8</sup>. Moreover, twice daily application of *Perilla* leaves extract which was formulated as an underarm serum on 30 Thai women for 4 weeks lowered the melanin and the erythema index compared with initial values and improved the skin tone without any skin irritation<sup>7</sup>. Therefore, in this study, we evaluated the melanogenesis inhibitory action of *Perilla* leaves on hyperpigmented rabbit model induced by UVA-radiation combined to progesterone injection.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and reagents

Progesterone 25 mg/ml was purchased from Rotex-medica, Germany; 4% Hydroquinone cream from Acella Pharmaceuticals, Korea. Synthetic melanin and bovine serum albumin were purchased from Sigma-Aldrich, USA; Pronase and Tris-HCl from Merck, Germany; Penicillin/Streptomycin from Invitrogen Corp., USA. All chemical reagents including ethanol, methanol, chloroform, ethyl acetate was purchased from Guangdong, China.

### 2.2. Animals

Healthy female British giant rabbits (4-5 months old), weighing 2.0-2.5 kg purchased from the Pasteur Institute in Ho Chi Minh city. The animals were acclimatized for at least 5 days prior to the experiments. One day before the experiments, an area of approximately 5 cm × 7.5 cm on each side of the dorsal skin was shaved using an animal clipper. All animal experiments were carried out in the animal house of the Department of Pharmacology, Faculty of Pharmacy of University of Medicine and Pharmacy at Ho Chi Minh city, Vietnam and in strict accordance with the guidelines for care and use of laboratory animals. The animal experiments were approved by the scientific committee, speciality of Pharmacology and Clinical pharmacy, Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh city, Vietnam (Number 4380/QĐ-ĐHYD).

### 2.3. Plant materials and extraction process

*Perilla* plants were harvested in Ha Noi, Vietnam in June, 2019. The botanical authentication of *Perilla* was enrolled and identified under scientific name *Perilla frutescens* var. *crispa* (Benth.) Deane ex Bailey, Lamiales<sup>9</sup>. The *Perilla* leaves were then picked, washed, dried, and grounded into crude powder with the size of 3 mm cube. The total extract from leaf powder was obtained thanks to the exhausted percolation process with 50% ethanol (no reaction with 5% FeCl<sub>3</sub>). The resulting total extract was shake with ethyl acetate through liquid-liquid partition process. The settled ethyl acetate layer was then collected and evaporated under vacuum to obtain fractioned ethyl acetate (EA) extract with the humidity lower than 20%. The EA extract was kept in the refrigerator for further experiments in animals.

### 2.4. Animal experimental design

#### 2.4.1. Hyperpigmentation induced by UVA radiation combined to progesterone injection

At the commencement of the experiments, the dorsal skin areas of the rabbits were exposed to UVA radiation (Cnlight ZW36D12W-H386) at the wavelength of 386 nm and the power density about 147 uW/cm<sup>2</sup> with a distance of 20 cm between the target skin and the light source within 30 minutes every day. The rabbits were then intramuscularly injected progesterone every other day at the dose of 5 mg/kg within 28 days of the experiment.

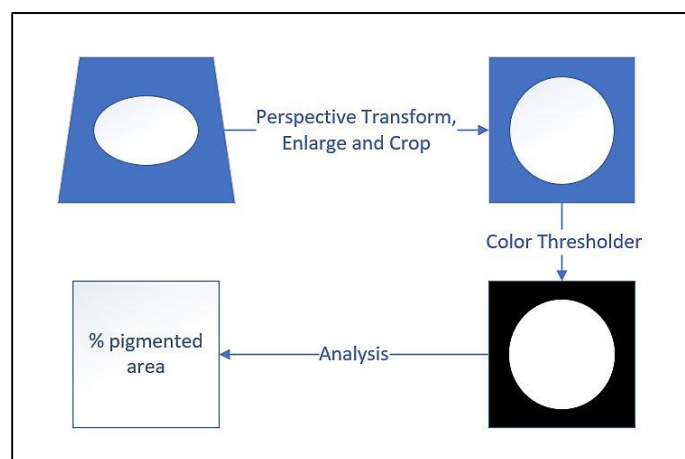
#### 2.4.2. Investigation of the therapeutic effect of *Perilla* extract

The EA extract at the concentration of 5% or 4% HQ cream were topically applied once daily to the dorsal shaved skin areas of the rabbits (after UVA radiation completion) once daily from the first day of melasma appearance to the end of the experiment (Day 28). The hyperpigmented group did not receive any treatment. Moreover, a physiological group (Sham group) that rabbits were not exposed to UVA at the dorsal shaved skin nor injected progesterone were also included.

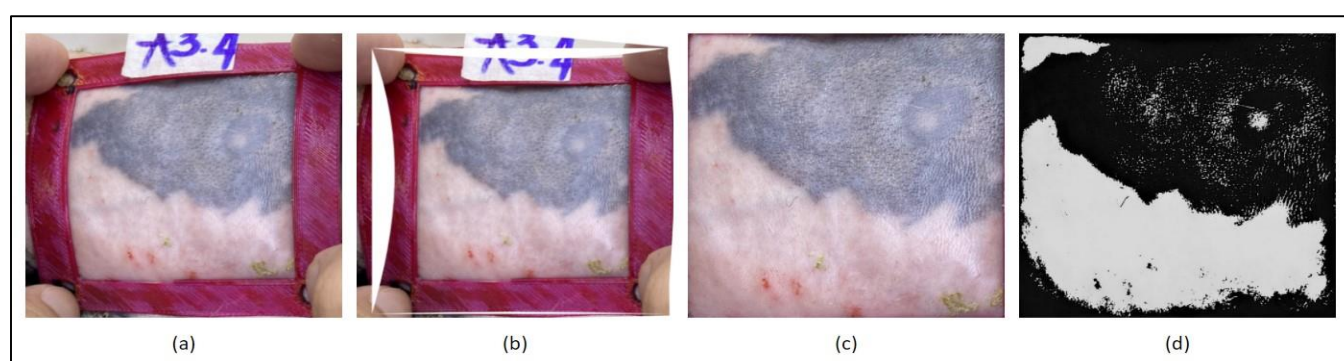
At Day 14 and Day 28, 10-15 minutes after applying the 2.5% Lidocaine cream to the dorsal shaved skin for local anesthetic, an area of approximately 2 cm × 2 cm was removed using a skin punching tool. The tissue fragments were then washed in 0.9% NaCl solution, removed the adipose tissue and dried on a filter paper.

#### 2.4.3. Morphometric observation

The appearance of the dorsal shaved skin (skin color, flaking, roughness and thickening of the epidermis)



**Figure 1.** The overall handling process of digital images for dorsal shaved skin from raw image to calculated % pigmented area.



**Figure 2.** Samples from the image handling process: (a) Raw image of dorsal skin; (b) Image without removed bird-eye effect; (c) Enlarged and cropped image with resolution 0.1 mm/pixel; (d) Image after thresholding with white area represents unpigmented area and black area represents pigmented area.

and the latency of melasma formation was observed every week. The dorsal shaved skin was captured by an electronic camera in digital images with a distance of 15 cm from the target skin to the camera. Figure 1 illustrates the handling process of the obtained digital images for dorsal shaved skin. First, the captured image was pre-processed by perspective transform to remove the bird-eye effect. Then, it was enlarged and cropped to obtain size uniformity. After that, elements of interest were segmented by applying Color Thresholder application in MATLAB software. The Color Thresholder application segmented color images by thresholding the color channels based on different color spaces. Using this application, we transferred a color image to a binary segmentation of hyperpigmented skin where white represents unpigmented area and black area represents pigmented area, as in Figure 2. The pigmented area was measured in the pixel unit, the percentage of pigmented area was calculated using the following formula:

$$\% \text{Pigmented area} = \frac{\text{pixel units of pigmented area}}{\text{total pixel units of examined area}}$$

#### 2.4.4. Histopathological processing

A quarter of the tissue fragment was embedded in formol 10%, sectioned with microtome and stained with

Hematoxylin and Eosin (H&E) stain. The stained sections were analyzed by using light microscopy at 40x and 100x magnification. The density of melanin in the dermis layer was scored by 4 levels: 0 (+/-) no melanin granules, 1 (+) few melanin granules, 2 (++) several melanin granules, 3 (+++) many melanin (several appear as melanin clumps)<sup>10</sup>.

#### 2.4.5. Melanin concentration assay

Standard curve of melanin: Melanin was dissolved in NaOH 0.1 N and was tested at the concentrations ranging from 0 µg/mL to 14 µg/mL. The absorbance of mixture was then measured at 405 nm.

#### 2.4.6. Melanin homogenous preparation and quantification

The melanin homogenates were prepared as in accordance to Watts KP et al.<sup>11</sup>. Briefly, the remaining tissue fragments after cutting for histological analysis was chopped with scissors and homogenized in 5.0 mL of distilled water by using a warring blender OV5, Italy. The homogenate was centrifuged for 15 minutes at 13,000 rpm. After removing the supernatant, 5 mL of 0.1 M sodium phosphate buffer pH 6 was added to the

obtained precipitate and shake well. The mixture was centrifuged for 15 minutes at 13,000 rpm. The obtained precipitate was soaked with chloroform: methanol (2:1) for 1 minutes and removed the solvent. The precipitate was then soaked with ethanol: ether (3:1) and removed the solvent. The residue was shake with 7 mL of 5 mM  $\text{MgCl}_2$  in 0.15 M NaCl and centrifuged for 15 minutes at 13,000 rpm. The residue after centrifugation was then treated with a pronase solution (2 mg/mL pronase in 0.05 M Tris buffer pH 8.0) and the mixture was incubated at 30°C for 48 hours. After centrifugation for 15 minutes at 13,000 rpm, the residues were washed with 6.0 mL of 0.9% NaCl solution; 5 mL of distilled water was then added. The mixture was then centrifuged for 15 minutes at 13,000 rpm. The remaining residue was mixed in 0.5 mL of distilled water and 1 mL of 0.1 N NaOH and heated in a boiling water bath at 90°C for 90 minutes. The obtained mixture was then determined the absorbance at 405 nm. The melanin quantification was calculated from the curve of the standard melanin concentrations and the corresponding absorbances.

Data were collected and presented as mean $\pm$ standard errors of mean (SEM). The data was evaluated by T-test and Mann Whitney test using SPSS 20. Differences between groups were considered significant when  $p < 0.05$ .

### 3. RESULTS

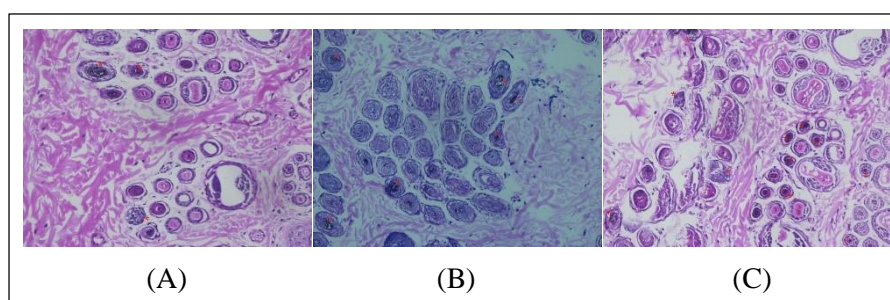
#### 3.1. Morphometric observation

On Day 7, the rabbit skin areas exposed to UVA radiation were flushing with the flaking epidermis whereas the rabbit skin of the sham group was smooth during the experiment. The pigmented spots were appeared from Day 12; the treatments were initiated from this day in 5% EA extract-treated group and 4% HQ cream-treated group. The hyperpigmentation gradually expanded with the increased intensity of pigments on Day 14 and there was not significantly different in the percentage of calculated pigmented areas among these groups (Table 1). From Day 21, the percentage of pigmented areas in treated groups was markedly decreased, especially on Day 28. Indeed, the reduction of pigmented areas of 5% EA extract-treated group and 4% HQ cream-treated group on Day 28 was about 59.5% and 44.2%, respectively, compared to Day 14 and significantly different from that of hyperpigmented group. However, there was no significant difference between these two treated-groups at the different time-points of the experiments (Table 1).

**Table 1.** Percentage of the pigmented areas (%) of experimental groups.

Day	5% EA extract (n=7)	4% HQ cream (n=10)	Hyperpigmented (n=8)	Sham (n=8)
0	4.66 $\pm$ 1.93	3.27 $\pm$ 3.21	1.72 $\pm$ 2.01	8.78 $\pm$ 4.43
7	7.16 $\pm$ 4.51	18.76 $\pm$ 5.57	14.01 $\pm$ 1.76	13.13 $\pm$ 5.38
14	92.84 $\pm$ 4.49(*)	81.85 $\pm$ 11.81(*)	90.09 $\pm$ 9.11(*)	7.62 $\pm$ 3.27
21	69.04 $\pm$ 13.16(*)	65.01 $\pm$ 19.53(*)	75.12 $\pm$ 21.84(*)	8.72 $\pm$ 4.92
28	33.54 $\pm$ 12.99(*#)	37.61 $\pm$ 21.30(*#)	56.31 $\pm$ 23.60(*)	9.16 $\pm$ 3.95

(\*)  $p < 0.05$ , compared to the sham group; (#)  $p < 0.05$ , compared to the hyperpigmented group



**Figure 3.** Level scores of the density of melanin granules in the dermis layer. Melanin granules was marked by red asterisks. Observation of a few melanin granules is scored as level 1 (A), several melanin granules as level 2 (B) and many melanin granules (presented in melanin clumps) as level 3 (C).

#### 3.2. Histopathological evaluation

The histological analysis on Day 14 demonstrated that melanin granules in the dermis layer of sections from skin areas exposed to UVA radiation were very dense; all sections were at the level 2 and 3 (Figure 3). On the contrary, there was a little of melanin granules observed in the sections from the sham group through-

out the experiment. On Day 28, the melanin granules were significantly decreased and sparsely distributed in the dermis of the skin section of groups treated with 5% EA extract and 4% HQ cream. The level scores of the density of melanin granules in these two groups were comparable and significantly lower than that of hyperpigmented group (Table 2).



**Table 2.** Scores of the density of melanin granules in the dermis of experimental groups.

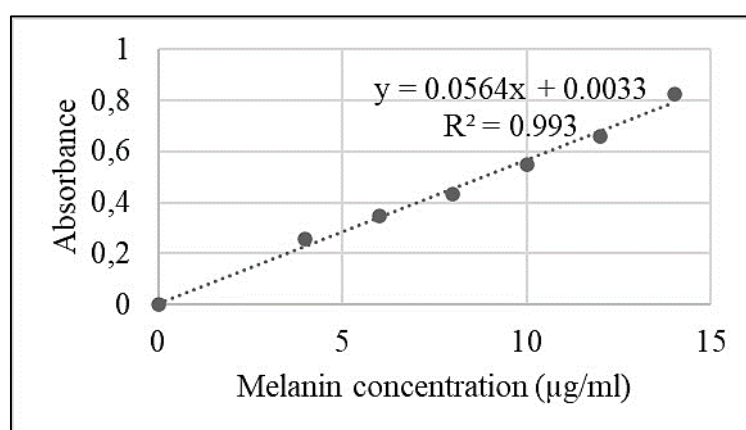
Groups	Day 14	Day 28
5% EA extract	2.83±0.67	1.00±0.37(*)
4% HQ cream	2.67±0.21	1.17±0.31(*)
Hyperpigmented	2.67±0.21	2.17±0.31
Sham	0.75±0.25	0.50±0.29

(\*)  $p < 0.05$ , compared to the hyperpigmented group

**Table 3.** Melanin concentration of experimental groups.

Day	5% EA extract	4% HQ cream	Hyperpigmented	Sham
14	13.369±0.744	13.966±1.093	15.509±0.064	5.438±0.025
28	8.049±0.681*	7.601±0.931*	13.386±1.533	7.778±0.226

(\*)  $p < 0.05$ , compared to the hyperpigmented group.

**Figure 4.** Correlation between the melanin concentration and the corresponding absorbance.

### 3.3. Melanin concentration assay

From the linear equation illustrating the correlation between the standard melanin concentrations and the corresponding absorbances  $y = 0.0564x + 0.0033$  with the value of  $R^2 = 0.993$  (Figure 4), the melanin concentrations extracted from the skin fragments of different experimental groups were presented in Table 3. On Day 14, melanin presented in the tissue fragments from the UVA-exposed skin was markedly increased and not significantly different between the hyperpigmented group and two treated-groups. The topical treatment with EA extract or HQ cream decreased the melanin concentration by 39.9% and 43.2%, respectively, as compared to the hyperpigmented group at the end of the experiment. Moreover, the melanin concentration of these two groups was not significantly different from that of the sham group (Table 3).

## 4. DISCUSSION

Melanin synthesis pathway consists of the hydroxylation of L-tyrosine to DOPA, followed by the transformation to DOPA-quinone. This process is catalyzed by the reaction of the tyrosinase enzyme; the inhibition of the tyrosinase activity, thereby, will be able to reduce melanin synthesis<sup>12</sup>. The study of Hwang *et al.* of the

screening for the inhibitory activities against tyrosinase and melanin biosynthesis in B16 mouse melanoma cells of 101 extracts showed the promising effect of *Perilla frutescent* and considered as a potential source of novel whitening agents for ultraviolet (UV)-sensitive skin<sup>13</sup>. Our previous study showed the *in vitro* inhibitory effect of the mushroom tyrosinase of EA extract with its  $IC_{50}$  value approximately equal to that of kojic acid<sup>14</sup>. In addition, certain therapeutic effects of the ethanolic total extract of *Perilla* leaves, including the anti-oxidant activity, the anti-inflammatory effect and the tumor suppression, were also elucidated<sup>9,15-16</sup>.

The present study was performed on a skin hyperpigmentation model induced by UVA exposure and progesterone injection. UV radiation enhances the melanocyte activation, followed by the amplification of UV effect by progesterone to increase the number of melanocytes through several sex steroid hormone receptors in the dermis and epidermis, especially in the dermis. The animals selected for the experiment were color rabbits, which responded to the UV radiation by increasing the melanin content<sup>17</sup>. The Color Thresholder application in MATLAB software for morphometric analysis was useful for the observation of the pigment areas. In addition, the quantification of melanin level was performed. The measurement of melanin content produced from melanocytes is essential for the evaluation of the

effectiveness of potential drugs, cosmetics in skin pigmentation disorders treatment and the whitening purpose<sup>18</sup>. The melanin concentration determined in 5% EA-treated group was comparable to that of group treated by HQ, a well-known tyrosinase inhibitor<sup>19</sup>. HQ is an aromatic compound that is naturally present in plants and foods such as coffee, cranberries, and blueberries<sup>20</sup>. HQ-containing cosmetic products are usually compounded 2% to 4% concentrations; the 2% concentrations of HQ available over the counter are not as efficacious as the 4% prescription formulations, as their onset of action is later than with the higher concentrations<sup>21</sup>. Because of the equivalence of the dermal absorption of HQ under a semisolid formulation (oil-in-water creams) and a liquid formulation, HQ is formulated in various topical forms: cream, emulsion, gel, or solution<sup>22</sup>. The efficacy of 4% HQ cream has been elucidated in several clinical trials<sup>23-25</sup>. Therefore, 4% HQ cream has been considered as the gold standard for the treatment of hyperpigmentation for many years<sup>26</sup>.

Generally, the our study has initially shown the promising results of 5% EA extract in the treatment of pigmentation disorders. Further experiments on the pharmaceutical or cosmetic formulations with 5% EA extract should be planned to develop new products containing natural skin lightening agents, especially for the long-term treatment of melasma. Moreover, clinical trials in women with moderate or severe facial melasma as scored by Melasma Area and Severity Index (MASI) scale should be conducted to evaluate the efficacy and safety of these 5% EA extract-containing products<sup>27</sup>.

## 5. CONCLUSION

Ethyl acetate extract of *Perilla* leaves exhibited the *in vivo* anti-melasma effect in pigmented rabbit skin. This opens a new prospect for the application of this extract as a natural skin lightener in the treatment of pigmentation disorders.

## 6. ACKNOWLEDGEMENT

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### Conflict of interest

None to declare.

### Funding

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## Ethics approval

The animal experiments were approved by the scientific committee, speciality of Pharmacology and Clinical pharmacy, Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh city, Vietnam (Number 4380/QĐ-ĐHYD).

## Author contribution

Ngoc Phuc Nguyen and Thanh Hao Do performed experiments and drafted the manuscript. Le Y Nguyen and Thi Thu Hien Pham contributed to the handling process and the analysis of the digital images from dorsal shaved skin of animals. Ngoc Trinh Huynh conceived the idea, the protocol for the experiments and complete the manuscript. All authors critically reviewed and approved the final manuscript for publication.

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