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Journal of Medicinal Materials, 2023, Vol. 28, No. 2 (pp. 109 - 116)

AMELIORATIVE EFFECTS OF *BACOPA MONNIERI* (LINN.) WETTST *n*-BUTANOL EXTRACT ON NEUROBEHAVIORAL DEFICITS IN TRANSGENIC DROSOPHILA EXPRESSING HUMAN AMYLOID PRECURSOR PROTEIN

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(Received December 12th, 2022)

Summary

Ameliorative Effects of *Bacopa monnieri* (Linn.) Wettst *n*-Butanol Extract on Neurobehavioral Deficits in Transgenic Drosophila Expressing Human Amyloid Precursor Protein

Alzheimer's disease (AD) is a neurodegenerative disorder and the most common form of dementia in the group aged above 60 years. It is accompanied by neuronal disorders along with learning and memory defects, as well as locomotor impairment. Because 70% of human disease-related genes are conserved in *Drosophila melanogaster* (*D. melanogaster*), especially those associated with types of risk factors for AD, this is a powerful model to study AD and develop the therapeutic AD drugs. In this study, we evaluate the effect of n-butanol extract from *Bacopa monnieri* (BM) on behaviors in transgenic flies expressing human amyloid precursor protein (hAPP) as a model organism for AD. Behavioral experiments were conducted to evaluate the crawling ability and short-term memory of third-instar larvae as well as climbing ability of adult flies. Furthermore, the expression of hAPP was evaluated in the head of adult flies which were exposed by 4 and 6 mg/mL BM extract using Western blot method. The results showed that the n-butanol extract from BM was effective in improving the ability of third-instar larvae to crawl and short-term memory, the ability of adult flies to climb, and at the same time suppressed the hAPP expression in adult brain. The results suggest that BM extract is a candidate for the treatment of behavioral deficits in transgenic *Drosophila* expressing hAPP protein.

Keywords: *Alzheimer's disease, Drosophila model, Bacopa monnieri, Behavioral alterations, hAPP expression.*

1. Introduction

Alzheimer's disease (AD) is a disease characterized by symptoms of impaired memory and cognitive function and is the most severe form of the group of dementia diseases today. According to statistics from the World Alzheimer's Association 2015, the incidence of AD increases gradually with age, from about 5% of people under 75 years old to 40-50% of people after 85 years of age [1]. AD always comes with a huge treatment budget and a physical and emotional burden on patients and their loved ones [2, 3, 4]. Drugs for the treatment of AD include cholinesterase inhibitors, N-methyl D-aspartate (NMDA) receptor antagonists, and drugs acting on pathological amyloid. However, these drugs have many side effects [5]. Therefore, the need to research and develop new drugs derived from medicinal herbs to support and treat Alzheimer's disease is very necessary.

Bacopa monnieri (Linn) Wettst (BM) is a medicinal plant used in traditional Indian medicine as a nerve tonic, memory booster, and epilepsy treatment. In Vietnam, BM grows wild in many places and is used as a daily vegetable and to treat a number of diseases such as neurasthenia, epilepsy, loss of muscle tone, according to folk experience [6]. There are much research works in the world that have proven that BM has the effect of improving memory and cognitive impairment by models of memory impairment in mice with ethylcholine aziridinium ion, colchicine, $AlCl_3$ and in mice carrying AD gene (PSAPP mice) [7],[8],[9]. In Vietnam, BM has also been studied very carefully for its effect on preventing/improving memory impairment and its mechanism of action on a mice model of trimethyltin (TMT)-induced memory impairment and olfactory bulbectomized (OBX) model of AD [10],[11]. In addition, Nguyen Thi Thu

Huong et al. reported that BM ameliorates learning deficits and cognitive dysfunction in scopolamine-induced memory impairment in mice [12],[13].

In humans, it is proposed that AD is initiated by the aggregation of peptides called Aβ from amyloid precursor protein (APP). When expressed in the fly brain, human APP (hAPP) is cleaved by endogenous *Drosophila* γ-secretase, resulting in the generation of the Aβ peptide. Degree of intracellular Aβ accumulation correlates with early phenotypes of AD such as locomotor dysfunction and memory impairment [14]. In addition, recent advancement of genome-wide association study analysis revealed that many types of risk factors for AD are identified and *Drosophila* homologue of those risk factors showed genetic interaction with fly model of AD [15]. Therefore, trangenic *Drosophila* expressing hAPP is a useful model developed to study the pathogenesis of AD as well as used for studying medicinal products from plants/ plant-derived compounds [16].

In this study, we evaluated the effect of n-butanol extract of BM on behaviors in a transgenic fly expressing hAPP, as an AD model with the aim of providing more scientific evidence on the therapeutic effects of this plant for AD.

2. Materials and Methods

2.1. Plant extraction

BM was collected in the central coast of Vietnam (April, 2018), andauthenticated by Prof. Pham Thanh Huyen, National Institute of Medicinal Materials (NIMM). The voucher specimens of BM were deposited at the Herbarium of Medicinal Material Resources Center, NIMM (vourcher specimen 9967. The extraction process is briefly described as follows: The aerial part of BM was dried in a hot-air oven at 50°C, cut into small pieces and then crushed. The herbal powder was extracted with 50% ethanol (1:8 w/v) at 85°C for 2 hr. This extraction was repeated 2 times. After filtration, the aqueous alcohol extract was concentrated under reduced pressure at 50°C and then further extracted with n-butanol three times. The combined organic phase was evaporated under reduced pressure and dried in a vacuum oven at 50°C until obtain the dry BM extract (4.5% moisture). The extract was analyzed by UPLC (with diode detection)-TOF-MS plus MS/MS (Fig. 1).

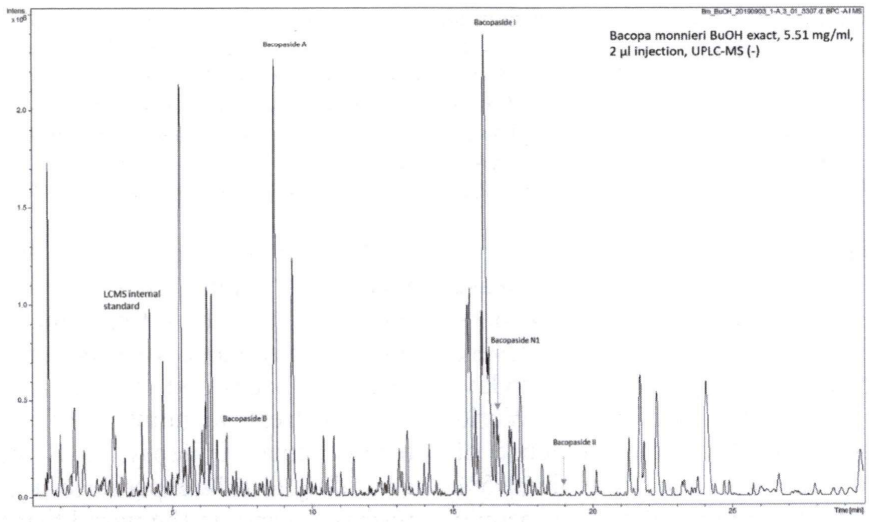

Fig. 1. Chemical profile of BME using UPLC (with diode detection) -TOF-MS plus MS/MS

2.2. Drosophila culturing and BM extract treatment

This study used two *Drosophila* strains provided by Prof. Masamitsu Yamaguchi, Kyoto Institute of Technology, Kyoto University, Japan: The first strain, Elav-GFP (also known as Elav) is a wild-type flies which carrying the Elav-GFP gene complex, were used as physiological controls. The second strain, UAS-Gal4>hAPP (also known as hAPP or hAPP64385) is a hAPP transgenic Drosophila with the code number 64385 carrying Alzheimer's disease. Flies were cultured on media containing 1% agar, 5% sucrose, 5% dry yeast, and 3% powdered milk at 25°C with a relative humidity of 50%-60% in a day/night period 12hr/12hr (7:00 to 19:00). Fly stocks were cultured in standard media and moved onto fresh food every 2 days.

Male flies of hAPP were crossed with virgin female flies of Elav in the standard media with or without BM extract with a concentration of 4 and 6 mg/mL. This results in first-generation (F1) offspring expressing hAPP, which served as our *D. melanogaster* model for AD in the assays of odor-taste learning, crawling, and climbing.

2.3. Behavior tests

Behavioral experiments were carried out between 9:00 and 17:00. Flies included in the study consisted of 4 groups:

- Physiological group (Elav strain): The Elav flies were fed with a standard media.

- Pathological group (hAPP): The first-generation (F1) offspring expressing hAPP were fed with a standard media.

- Group BM4 and BM6: The first-generation (F1) offspring expressing hAPP were fed with a media containing a concentration of 4 mg/mL and 6 mg/mL BM extract, respectively.

2.3.1. Evaluation of short-term memory of fly larvae:

The larval short-term memory assay based on odor-taste [17] was conducted with third-instar larvae that were collected randomly and washed with phosphate-buffered saline (PBSX1) to remove food traces.

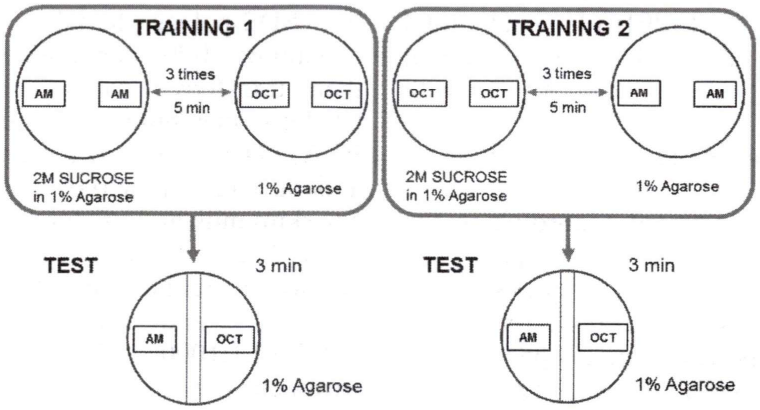

Fig. 2. Scheme of the experimental procedure of short-term memory in fly larvae

The odor-taste learning assay was performed three times for each group, each time with two rounds; each round has two trials: the training trial and the test trial. 10 µL of each odorant (n-amyl acetate and 1-octanol) were added in 0.2 mL Eppendorf tubes with a perforated lid, so that the smell can diffuse in the petri dish. For the training trial, larvae were placed onto the middle of a dish with a thin layer of 1% agarose containing 2M sucrose-labeled X and exposed to n-amyl acetate (AM). After free wandering on the X dish for 5 min, the larvae were transferred onto another dish with a thin layer of 1% agarose only-labeled Y and then exposed to 1-octanol (OCT) and allowed to move freely for 5 min. This trial was defined as AM+/OCT, indicating "+" as the reward. These training trials were repeated three times, then larvae were subjected to the test trial: twenty-four larvae were divided into three groups, each with 8 larvae. After training, groups of 8 larvae were immediately transferred onto the test dish with a thin layer of 1% agarose, and an AM-filled odor container on one side, and an OCT-filled odor container on the opposite side. At 3 min, the number of larvae on the AM side and on the OCT side of the dish was counted. The preferences (PREF) of the larvae in each test dish are calculated as:

PREF = (#AM - #OCT)/(#Total)

#AM: the number of larvae observed on the AM side;

#OCT: the number of larvae observed on the AM side;

 # Total: the number of larvae observed on both sides;

For the next round of experiments, the new larvae were used for reciprocal training and a fresh set of petri dishes. That was combined AM with the Y-labeled and OCT with the X-labeled petri dishes. After that, the learning index (LI) was calculated as:

$LI = (PREF_{AM+} - PREF_{OCT+})/2$.

$PREF_{AM+}$: The preferences of the larvae in test combining AM with sucrose dishes;

$PREF_{OCT+}$: The preferences of the larvae in test combining OCT with sucrose dishes;

The total number of flies in each study group was about 150 (Each experiment was conducted 6 times and 25 larvae each time).

2.3.2. Evaluation of locomotor activity of flies:

2.3.2.1. Evaluation of crawling ability of fly larvae:

Male third-instar larvae (F1 generation larvae) were collected and raised on standard food containing BM extracts (4 and 6 mg/mL). Locomotor activity was analyzed by the crawling assay: After washing with PBSX1 to remove food traces, 4 larvae were transferred to 100 mm petri dish containing 1.5% agarose and the movements of the larvae in 60 sec were recorded using a digital camera (Nikon D3500, Tokyo, Japan). The average movement speed of each larva was analyzed using Image J software according to Brooks et al (2016) [17]. The total number of larvae in each study group was about 32 (each experiment was conducted 8 times with 4 larvae per time).

2.3.2.2. Evaluation of the climbing ability of adult flies:

The model was conducted according to the description of Nichols et. al (2012) [18]. The flies selected for the experiment were anesthetized and then transferred into graduated glass tubes, each line is spaced 2 cm apart (15 - 20 flies/tube). After stabilizing for 10 minutes, the flies were brought to the bottom of the glass tube (to the same starting line) by hitting hard enough 5 times continuously. Then wait 30 seconds for the fly to crawl up, repeat the same movement for the first time- hit hard enough continuously 5 times to bring the fly to the bottom of the glass tube, wait 30 seconds. A total of 5 repetitions of the step "smash with enough force continuously 5 times to bring the flies to the bottom of the tube; wait 30 seconds. After the experiment was completed, the flies were transferred back to the feeding tube. Video data recorded at the first 5 seconds after the end of 5 consecutive hits were used to analyze and compare the climbing ability of flies.

Evaluation criteria: The height of the tube corresponds to the following scores: <2 cm: 0 points; 2-3,9 cm: 1 point; 4-5.9 cm: 2 points; 6-7.9 cm: 3 points; 8-9.9 cm: 4 points; >10 cm: 5 points. Calculate the score of each fly after the first 4 seconds of each experiment repetition. The average score of the test tube is calculated by the formula:

$$S = \frac{\sum_{i=1}^{5} \quad \sum_{k=1}^{x} \quad (n_{ik} * s_{ik})}{5}$$

S: Average score of a test tube; x: Number of animals in a test tube; n_{ik}: Number of flies in a point level; s_{ik}: The corresponding score of flies in each level. The data were processed by Image-J software and statistically by SPSS.

2.3.3. Evaluation of hAPP protein expression level in the head of flies using Western blotting:

Total proteins from adult heads of flies carrying Elav-GFP and UAS-hAPP-HA treated with vehicle (water), 4 and 6 mg/mL of BM extract were extracted by using 2x SDS sample buffer (2% SDS, 10% glycerol, 0.002% BPB, and 0.063 M Tris-HCl), heated at 95°C for 2 min, followed by centrifugation at 14000×g at 4°C for 20 min. Protein extracts were further separated by SDS-polyacrylamide gel electrophoresis containing 10% acrylamide (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Merck, Millipore, MA, USA). The blotted membranes were blocked by Tris-buffered saline/0.05% Tween 20 containing 5% skim milk at 25°C for 1 hr and then incubated at 4°C for 16 hr with the proper primary antibodies: rabbit anti-HA IgG (1:1000, Sigma-Aldrich) and mouse anti-Actin IgG (ACTA2,1:1000, DSHB #JLA20). After the treatment with the primary antibodies, membranes were incubated with HRP-conjugated secondary antibodies: anti-rabbit and anti-mouse IgG (1:5000, Thermo Scientific, IL, USA), at 25°C for 1 hr. Antibody binding was detected using ECL Western blotting detection reagents (Thermo Scientific, IL, USA) and analyzed by AE-9300H Ez-Capture MG (ATTO).

2.4. Statistical analysis

Statistical analyses were performed using SPSS software (version 23.0). The data obtained in the odor-taste learning assays were expressed as the median with 25 and 75 percentile and analyzed using Mann-Whitney U test for two-group comparisons or Kruskal-Wallis test followed by post hoc Dunn's test for multiple comparisons. The data obtained from crawling assay and climbing assay were expressed as the mean ± S.E.M. and analyzed by One-Way ANOVA followed by post hoc Tukey test. Differences of $p < 0.05$ were considered significant.

3. Results

3.1. Evaluation of short-term memory of fly larvae

We first evaluated the effect of BM extract on learning and memory of the transgenic flies expressing hAPP using the odor-taste learning

assay. The learning index (LI) was used to test olfactory learning, with assays that measured short term memory for either AM-sucrose or OCT-sucrose. Results indicated that hAPP larvae exhibit a significant reduction of learning index compared with physiological group (Elav Gal4 (III)) ($p < 0.05$) (Fig. 3), and that hAPP larvae fed with two different concentrations of BM extract (4 and 6 mg/ml) showed significantly improved learning indices in a dose-dependent manner ($p < 0.01$) compared with untreated hAPP larvae.

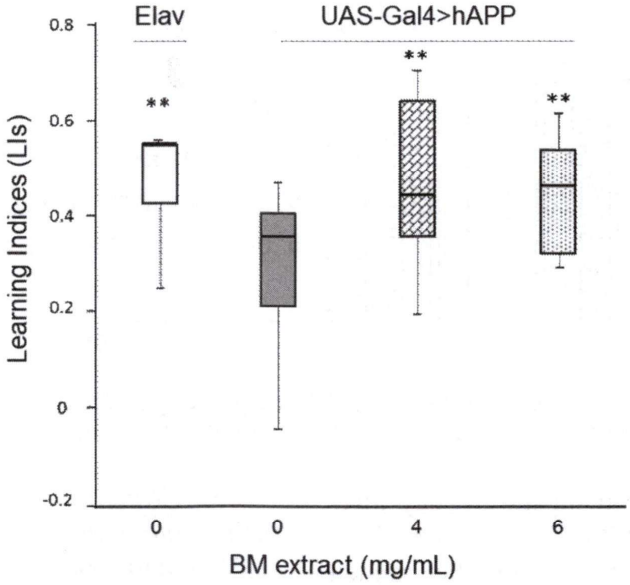

Fig. 3. Effect of BM extract on odor-taste learning in hAPP larvae using Learning Indices (LIs)
(Experiment was conducted 6 times and 25 larvae per each, total sample larvae = 150/group);
***p<0.01 compared with the pathological group*

3.2. Effects of BM extract on locomotor activity

3.2.1. Evaluation of the motility (crawling) of fly larvae:

We next evaluated the effect of BM extract on locomotor activity in hAPP larvae using crawling assay. The results showed that hAPP larvae exhibited significantly decreased crawling speeds relative to Elav larvae ($p < 0.05$) (Fig. 4). hAPP larvae treated with 4 and 6 mg/mL of BM extract were significantly faster than that of untreated larvae ($p < 0.01$), indicating a facilitatory effect on impaired locomotor function (Fig. 4).

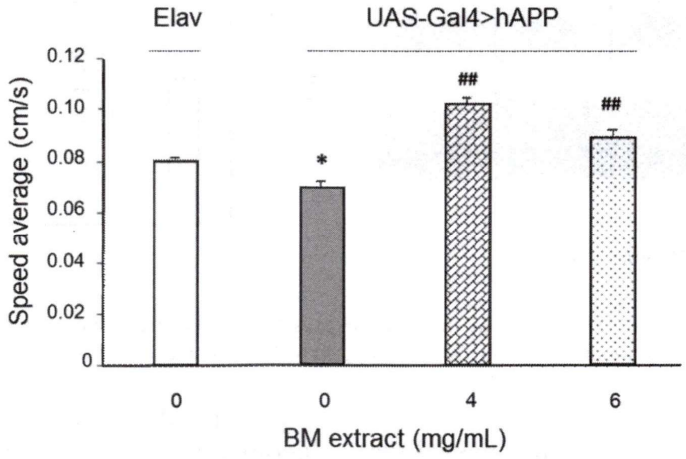

Fig. 4. Effect of BM extract on locomotor deficit assessed by crawling assay in male third-instar larvae of hAPP flies. The total number of larvae in each group was 32 (experiment was conducted 8 times and 4 larvae per each time); *$p < 0.05$ compared with the Elav group; $^{\#\#}p < 0.01$ compared with the hAPP group.

3.2.2. Evaluation of the climbing ability of adult flies:

We also evaluated the effect of BM extract on locomotor activity in hAPP adult flies using climbing assay. The experiment was carried out by bringing all the flies to the bottom of the test tube and then waiting for the flies to crawl up for 30 seconds, based on the climbing ability to calculate the average number of climbing points of each tube.

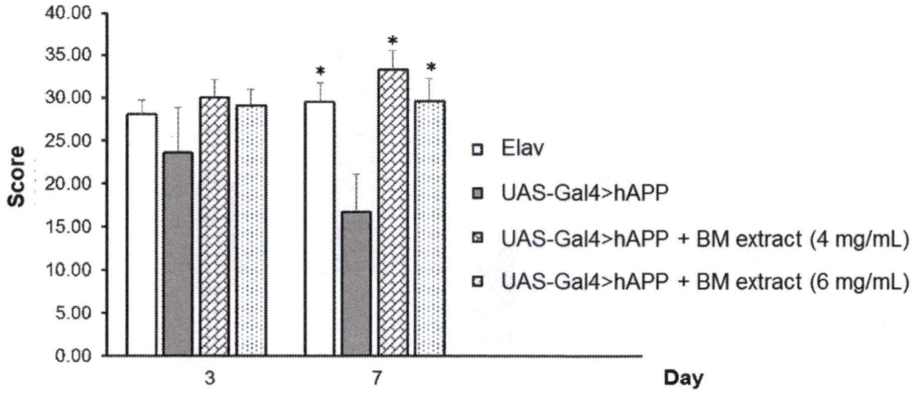

Fig. 5. Effect of BM extract on locomotor deficit assessed by climbing assay in adult hAPP flies after hatching 3 days and 7 days, *p < 0.05 compared with the hAPP group at the same day.

The results showed that on the 3rd day after hatching, the climbing ability in groups of Elav as well as BM extract-treated hAPP flies tended to be better than untreated-hAPP flies. However, this difference did not reach statistical significance. On the 7th day after hatching, the climbing ability of these groups were significant improved compared to the untreated- hAPP adult flies (p < 0.05) (Fig. 5).

3.3. Evaluation of the expression level of hAPP protein in adult flies

Aiming to clarify the mechanism underlying the effect of BM extract on improving AD symptoms, we next evaluate the hAPP expression level in head of flies. hAPP flies showed a significant increase in the level of hAPP protein (p < 0.05). Treatment of BM extract at the concentrations of 4 and 6 mg/mL markedly suppressed the hAPP in adult brain flies (p < 0.05 compared to pathological group) (Fig. 6).

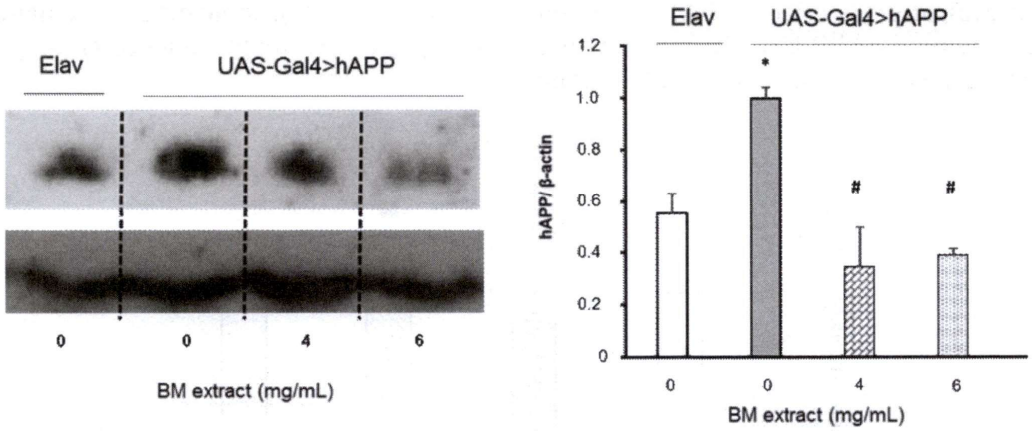

Fig. 6. Effect of BM extract on the hAPP expression level of head flies. (n = 3/ group); *p < 0.05 compared with the Elav group; #p < 0.05 compared with the hAPP group

4. Discussion

The results obtained in our study showed that BM extract is potent in reducing AD symptoms in the transgenic *Drosophila* model of AD. hAPP flies treatment with BM extract not only showed improving the deficit of learning memory and locomotor activities but also suppression of hAPP protein of the fly head.

AD is characterized by memory impairment, difficulty remembering recent events, and an

inability to absorb new information. To assess the memory and learning ability of flies, this study used the odor-taste assay to assess short-term memory of fly larvae. Our results show that BM extract improves memory deficit in AD flies. These results are consistent with our previous studies which indicated that BM has potent to improve memory and cognitive impairment by models of memory impairment in mice models of trimethyltin (TMT)-induced memory impairment and olfactory bulbectomized (OBX) [10],[11].

There are much research works in the world that have proven that BM has the effect of improving memory and cognitive impairment by models of memory impairment in rats caused by aziridinium, colchicine, $AlCl_3$ ions, and mice carrying AD gene (PSAPP mice) [7],[8],[9]. BM extract and its major compounds such as bacoside A, bacoside B act on hippocampus, cerebral cortex, and hypothalamus, leading to increased genes/proteins activity which involve in learning and memory. This extract was also effective in reducing beta-amyloid levels in the brains of PSAPP mice. These evidences suggest that BM improves neurotransmission through synaptic recovery.

Impaired locomotor behavior is a common diagnosis in many neurological diseases, including AD [19]. The locomotor performance showed an association with the severity of the disease [21]. Therefore, we also evaluate the effect of BM extract on locomotor activities in hAPP flies. hAPP flies showed a decrease in the crawling, climbing activities and BM extract treatment mitigated the reduced locomotor activities at a larvae stage and adult stage of AD flies. The aberrant locomotor function observed in hAPP flies in the present study in line withwith a previous study reported by Beg et al (2018) [22].

It has been reported that expressing human APP in fly nervous system produces amyloid β (Aβ) and shorten the life span as well as memory loss [23]. The aggregation of Aβ peptide in the brain, especially Aβ42 is thought to be a pathological hallmark of AD. Therefore, the inhibition of hAPP/Aβ has been used to develop therapeutic drugs for AD. Our results in this study have demonstrated that n-butanol extract from BM markedly suppressed the hAPP expression in adult brain flies. These results suggest that BM extract has improved the deficit of memory and locomotor activities of AD flies partly via a mechanism involving suppression of hAPP protein.

5. Conclusion

Our results of the present study support the evidence that n-butanol extract from *Bacopa monnieri* has a potential improvement of Alzheimer's disease in a fly model, through criteria including short-term memory, locomotor activities and hAPP expression.

Acknowledgments: *This research was funded by National Institute of Medicinal Materials (Contract No. 21/2021 HĐ-ĐTCS-DLSH issued 03/11/2021). We thanks FIRST Grant (No. 07/FIRST/1.a/NIMM) for supporting some materials in this study.*

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Journal of Medicinal Materials, 2023, Vol. 28, No. 2 (pp. 116 - 122)

ANTI-MICROBIAL AND EXPECTORANT ACTIVITIES OF LOZENGES AND SYRUP FROM *HEDERA NEPALENSIS*

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(Received March 15th, 2023)

Summary

Anti-Microbial and Expectorant Activities of Lozenges and Syrup from *Hedera nepalensis*

Cough is a manifestation of respiratory disease, especially when there is sputum or sore throat caused by the microbes, it is more harmful to health. Hence, it is needed to develop a product which is effective against several respiratory disorders. To avoid the side effects and provide safe medicine, plant products may provide alternative treatments replacing the modern medicine. In this study, we examined the antimicrobial and expectorant activities of two products containing *Hedera nepalensis* extract, a native plant in Vietnam and has been used in traditional medicine for the treatment of cough and inflammation. The syrup and lozenge inhibited the growth of four bacterial strains (*Streptococcus pneumoniae, Streptococcus pyogenes, Haemophilus influenzae, Klebsiella pneumoniae*) with the MIC value of the syrup was 20% (volume/volume) for *Klebsiella pneumoniae,* while it was greater than 20% (volume/volume) for the remaining strains (*Streptococcus pneumoniae, Streptococcus pyogenes, Haemophilus influenzae).* The MIC value of the lozenge was 25 mg/mL for Streptococcus pneumoniae and S. pyogenes, while it was no less than 100 mg/mL for the others (*Haemophilus influenzae, Klebsiella pneumoniae).* Syrup at dose of 16 ml/kg and lozenges at dose of 4 lozenges/kg showed expectorant effects because of a significant decrease (31.15%- 43.56%) on optical absorbance of bronchoalveolar fluid level as compared to normal control. The results of this study could show the potential of this plant and these related products for treating respiratory problems and improving the health of Vietnamese people.

Keywords: *Hedera nepalensis, Anti-Microbial, Expectorant, Syrup, Lozenge.*

1. Introduction

Cough is characterized as a forceful expulsive maneuver, generally against a closed glottis, and is accompanied by a distinct sound [1]. Coughing can be caused by a variety of respiratory tract illnesses, which may need pharmacological therapy for treatment. Most medications are effective for antitussives but there are some side effects and impotence for patients who produce a lot of phlegm. On the contrary, it makes coughing worse. Besides, an upper respiratory infection affects the upper part of your respiratory system, including your sinuses and throat, which can cause more cough and damage your respiratory tract. Therefore, it is necessary to develop a product with contemporary anti-microbial, expectorant, and antitussive activities which are effective against several respiratory disorders.

Because of the limited lack of effective and