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The Effect of *Vespa simillima* Extracts on Long-Term Memory and MK-801-Induced Learning Disability in Mice

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Extracts of adult worker bodies of *Vespa simillima* in 2 % NaCl or acidified methanol were administered orally to mice for 70 days. Following this period, memory at one-day and one-month periods, and the effects on scopolamine-induced amnesia were examined using a step-through passive avoidance task. Changes in MK-801-induced disability after 8 days of training, and in memory one month after the trial were also assessed. Mice treated with the 2% NaCl extract showed significant improvement in memory in the behavioral tests one month after the trial, whereas mice receiving the extract in acidified methanol, did not differ from the controls in any trial. The results inidicate that *Vespa simillima* contains substances acting favorably on the cerebral functions of mammals.

Key words: *Vespa simmillima*, Body extract, Memory, Learning, Improvemnet.

Introduction

Pharmacological studies on entomoresouce are largely based on the traditional medicine in Asia and the Central and South America (Wahrendorf and Wink, 2006; Gan *et al*., 2006). The wasps of the genus *Vespa* (Hymenoptera: Vespidae) are used as a souce of remedies throughout East Asia where the genus probably originated and evolved into a number of species (Vecht, 1959). Seven species (*Vespa mandarinia, ducalis, analis, crabro, dybowskii, simillima* and *affinis*) have been classified in Japan (Matsuura, 1971). The wasps are social insects that live on colonies composed of immature individuals, workers, queens, and drones. The immature larvae and pupae are eaten as a part of various recipes in the Yunnan province of China, the northern part of Thailand, and the mountainous areas of Japan. Adults (primarily workers) are utilized as a traditional nutritious tonic in shochu (a kind of sake) and honey. The nest, which is constructed with the aid of worker's saliva, is used in Chinese herbal medicine to accelerate blood coagulation, stimulate heart beat, exert a hypotensive and diuretic effect, and provide relief of oedema and inflammation (Yoshimura, 1946; Hong, 1960).

It is known that long-term potentiation of synaptic transmission in the hippocampus of mammals is induced by a venom peptide from a wasp relative, the honeybee, *Apis mellifera* (Cherubini *et al* 1987). It may be expected that the wasps of *Vespa* genus also contain biologically active compounds, but no pharmacological studies have been conducted to evaluate possible effects of this entomoresource on the cerebral or limbic functions. We examined such effects in mice with two kinds of extracts prepared from the bodies of adult workers of *Vespa simillima*: a 2% NaCl extract (NE) and an acidified methanol extract (AE). A step-through passive avoidance task and a Morris water maze task were to evaluate whether these extracts may improve learning and memory and be considered as a potential source of useful pharmacological materials. To our knowledge, this is the first report demonstrating that an insect extract prevents decrease in the learning and memory capacities.

Materials and Methods

Mice

ICR mice (females aged 5 weeks) were purchased from

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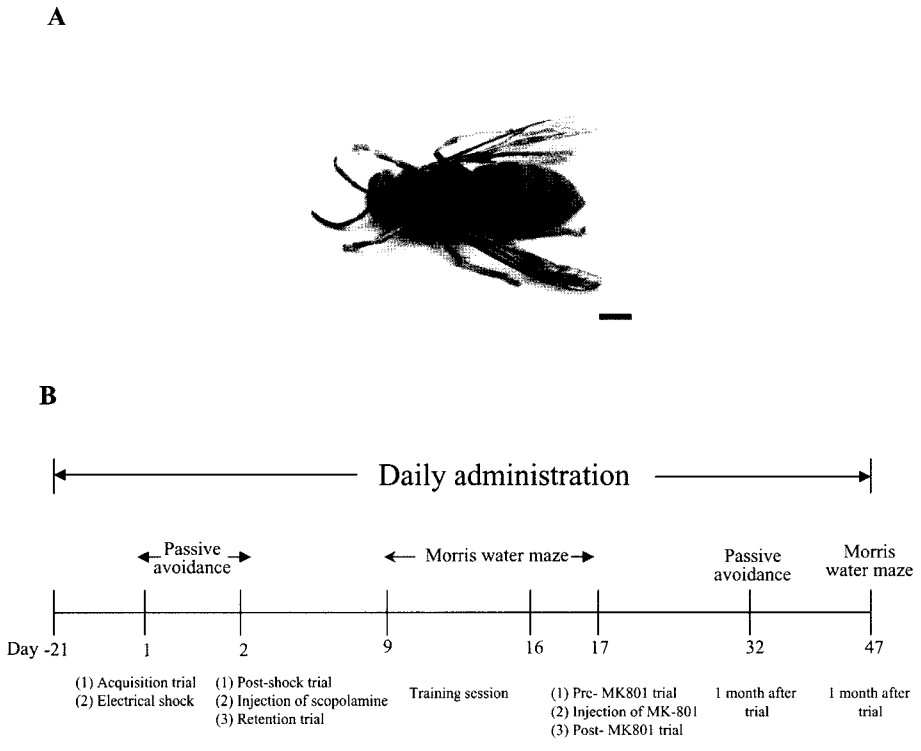

Fig. 1. *Vespa simillima* worker and experimental schedule.
A, Adult wasp worker harvested from a nest in October 2006. Scale bar=3 mm. B, Timing of the passive avoidance task and the Morris water maze in mice at the start of experimentations, in which mice were supplied orally with four preparations for 21 days and during the experimental period, as shown in the legends to Figs. 2 to 5.

SLC Japan and kept in groups of 6 or 7 per cage. The ambient temperature was maintained at 23 to 25°C, and the light-dark cycle was 12 : 12 hr. During the experiment, all mice were fed standard diet but different drinking water and confirmed to have irregular sexual cycle and no reproductive ability. The control group of 6 to 33 mice was given plain water, while the experimental groups received water with 0.028% NE, 0.043% AE, and 0.5% honey, respectively. Both food and water were provided *ad libidum* and their consumptions, as well as mice body, were measured every 2 days. These same mice were used for the 70-day study period (Fig. 1). This study was performed in accordance with the Guidelines for Animal Welfare Act and with the Guide for the Care and Use of Laboratory Animals approved by the Animal Experiment Committee in Iwate University, Japan.

Insects

Vespa simillima adult workers (Fig. 1A) were harvested from nests in the botanical garden of the Faculty of Agriculture in Iwate University, and in the Sugo area in Morioka, Japan in 2001 and 2002. After freezing the nests at −20°C, the outer shell was removed, and the worker wasps were quickly transferred to −80°C.

Acidified methanol extract (AE)

This extract was prepared by the method of a simple acidified-methanol extraction providing a large yield of peptides (Tanaka *et al.*, 2003). Samples of 50 g of adult workers (without wings) were homogenized in a mortar in 500 ml cold acidified methanol (methanol : water : acetic acid, 90 : 9 : 1). The homogenates were centrifuged at 10,000 × g for 30 min at 0°C, and the precipitates extracted 3 times with this same methanol solution. Combined supernatants were concentrated to pellets using a centrifugal evaporator, heated on boiling water bath for 10 min, and centrifuged. The supernatant was stirred overnight with 80 ml cold acetone. The solution mixture was then centrifuged, and the precipitate dissolved in appropriate volume of distilled water and lyophilized. The resulting acidified methanol extract (AE) of 1,646 mg was suspended in an appropriate volume of distilled water. The mice were given free access to the water solution, but the consumption was measured every 2 days and the AE concentration was adjusted to ensure consumption of 3 mg AE per mouse and day. The dose was established in preliminary experiments (data not shown).

2% NaCl extract (NE) and Chemical analysis

The extraction was accomplished with a method previ-

ously used for the isolation of peptidic hormones (Naya *et al.*, 1994). Extraction began with 70 g adult workers homogenized in a mortar with 200 ml cold acetone. The homogenate was stirred for 30 min and then centrifuged at 10,000 × g for 20 min at 0°C. The precipitate was dried in air to obtain acetone powder that was re-extracted with 200 ml of cold 80% ethanol. Ethanol was evaporated and the remaining powder was suspended in 100 ml 2% NaCl solution. The suspension was stirred overnight, centrifuged, and filtered through standard filter paper. The filtrate was dialysed overnight with the membrane (CE, MWCO : 500, Spectrum) and lyophilized. The resulting extract (NE) of 1,546 mg was suspended in appropriate volume of distilled water. Based on preliminary experiments (data not shown), NE concentration in drinking water was adjusted to provide a consumption of 2 mg per mouse and day. Measurement of the sodium content in NE was performed according to the method of Wright and Stuczyneski (1996).

Honey from *Robinia pseudo-acacia* (HO)

In respect to the high metabolic rate of glucose in the brain tissue (Donohoe and Benton, 2000), a 0.5% solution of *Robinia pseudo-acacia* honey (supplied by the Fujiwara Apiary in Morioka, Japan) derived from *Apis mellifera* was used as a positive control in our tests. The mice were allowed to consume honey water *ad libidium*, but the concentration was adjusted every 2 days to deliver about 40 mg HO per mouse and day.

Injection of chemicals

Mice used for the step-through passive avoidance task were subcutaneously injected with scopolamine-HCl (Sigma) at a dose 0.5 mg/kg body weight. MK-801 [(+)-5-methyl-10, 11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine] (Tocris Cookson) was injected intraperitoneally 0.25 mg/kg body weight into mice prepared for the Morris water maze task.

Step-through passive avoidance task

We adopted the methods of Zhang *et al.* (1994) to assay learning and memory. Tested sample solutions were administered to mice for 21 days before they were taken for the step-through passive-avoidance task (Fig. 1, referred to as passive-avoidance test hereafter). The apparatus consisted of a bright compartment (19 × 19.5 × 17.5 cm) illuminated by a fluorescent light (15 W) and a dark compartment (the same size as the bright one) with an electrified grid floor. The two compartments are divided by a vertically sliding door, which could be opened and closed freely.

On the first day of the experiment, the mice were placed in the bright compartment with the door closed. The door was opened 30 sec later to measure their reaction latency when they entered the dark compartment (acquisition trial). The door was then closed, and an electric shock (0.5 mA, 4 sec) was given. After 24 hr, the mice were again placed in the bright compartment with the door closed. The door was opened 30 sec later, and their reaction latency was measured for up to 180 sec at the longest (post-shock trial). Before the retention trial, scopolamine (0.5 mg/kg) was injected. Reaction latency to move from the bright to the dark compartment was measured after 15 min. The same trials as those prior to the scopolamine administration were performed again after one month, and the reaction latency was measured.

Morris water maze task

Morris water maze test (Morris, 1981) was performed one week after completion of the retention trial of the passive avoidance test (Fig. 1B). The apparatus consisted of a circular pool (100 cm in diameter, 30 cm in depth) set on a stand 80 cm heigh. A clear acryl resin platform (10 cm in diameter, 19 cm in height) was situated 1 cm below the water surface at the center of a quadrant prepared by dividing the pool conceptually into four equal parts. The room and water temperatures were both maintained at 25 ± 1°C, and the pool was made opaque by the addition of white poster color (Color acrylique). All objects in the laboratory were kept in their original locations during the experiment. A camera was installed above the center of the pool at a height of 100 cm from the water surface, and images covering all quadrants were recorded at 2 frames/sec. Picture analysis was performed on Macintosh computer using Image WMH 2.08 and Image WM 2.12. (O'Hara), software based on the public domain NIH Image program (developed at the U.S. National Institutes of Health).

A total of 32 training sessions (4 sessions/mouse/day for 8 days) were performed. The time for each mouse to reach the platform was taken as the reaction latency and measured for up to 60 sec. When a mouse reached the platform within the trial time, the animal was detained on the platform for 15 sec before progressing to the next trial. When a mouse did not reach the platform within 60 sec, the reaction latency was assigned to be 60 sec, and the animal was transferred to the platform and detained there for 15 sec before progressing to the next trial. After 8 consecutive days of training, a probe trial was performed on the 9th day. In the probe trial, the platform was removed from the pool, and each mouse was forced to swim for 60 sec. The number of crossings by a given mouse at the position where the platform had been located was determined (pre MK-801 trial). Subsequently, MK-801 (0.25 mg/kg) was

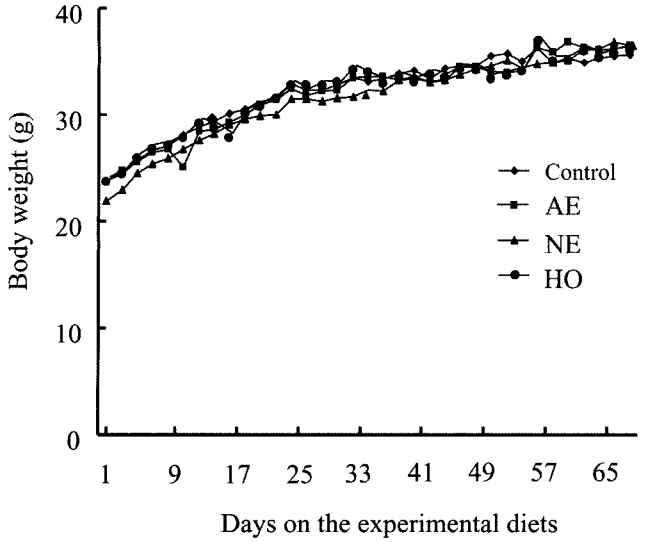

Fig. 2. Body weights of mice supplied with pure drinking water (Control, ◆, n = 6) or drinking water containing acidified methanol extract (AE, ■, n = 6), 2 % NaCl extract (NE, ▲, n = 6), and 0.5% honey solution (HO, ●, n = 6). Data are expressed as the mean.

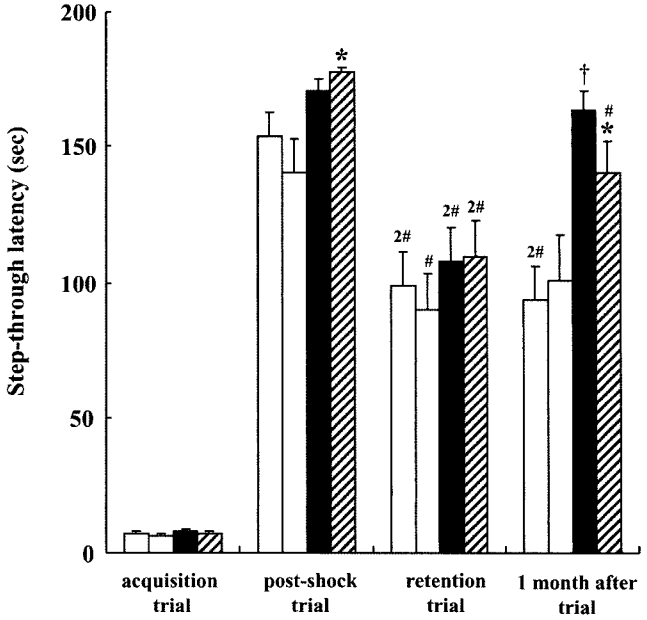

Fig. 3. Effects of *Vespa* extracts on mouse memory in a passive avoidance task. Control mice (Control, ▩, n = 33) received pure drinking water and the experimental mice water supplemented with acidified methanol extract (□, n = 23), 2% NaCl extract (■, n = 32), and 0.5% honey solution (▨, n = 32), respectively. The latency time of the post-shock trial and retention trial was measured and analyzed 1 month after the first trial. Data are expressed as mean ± S.E.M. *, $P < 0.05$, †, $P < 0.01$ versus the control group and #, $P < 0.05$, 2# $P < 0.01$ versus the post-shock trial in each group (ANOVA followed by Dunnett test).

injected intraperitoneally, and the probe test was repeated 15 min later (post MK-801 trial). One month later, each mouse was made to swim in 4 sessions similar to the training trial, and the reaction latency was measured.

Statistical analysis

The statistical significance of the results was evaluated using one-way ANOVA or partially followed by the Dunnett test in comparison with the control group, or the Student's *t*-test. All results are expressed as mean ± S.E.M.

Results

Weight and general condition

The consumptions of food and drinking water did not differ significantly among the 4-groups receiving different supplements in the water (none in the control, acidified methanol extract, 2% NaCl extract, and 0.5% honey in the experimental mice). All animals steadily gained weight during the 70-day study period and there was no significant weight difference between the groups (Fig. 2). The 2 % NaCl extract (NE) contained 60 μg of sodium/mg, but this amount was small compared to about 12 mg sodium received daily with about 5 g of ingested food. Mice receiving AE, NE, and HO, respectively, did not show any physiological abnormality compared with the control group.

Passive avoidance task

The latency time of the post shock trial, which represented reminiscence of an electric shock after 24 hr, increased significantly in the 0.5% honey solution group, as shown in Fig. 3. The latency time in the retention trial after scopolamine administration did not differ significantly for any of the groups. In comparing the reaction latencies between the post shock trial and the retention trial in each group, there were differences at the 5% level in the acidified methanol extract group and at the 1% level in the other groups. Based on this result, the effect of temporary amnesia induced by scopolamine was not rectified in any of the groups. Latency time measured 1 month after the trial represented memory retrieval of the electric shock given previously. As can be seen in Fig. 3, the result obtained in mice treated with NE was similar to it recorded in the post shock trial, while the responses of the other groups were significantly different. In other words, the wasp extract prevented the decrease in the latency time that occurred after 1 month in mice. The 1-month latency times in the control and acidified methanol extract groups decreased significantly to those at the retention trial level (the significance of the differences was analyzed by ANOVA followed by the Dunnett test).

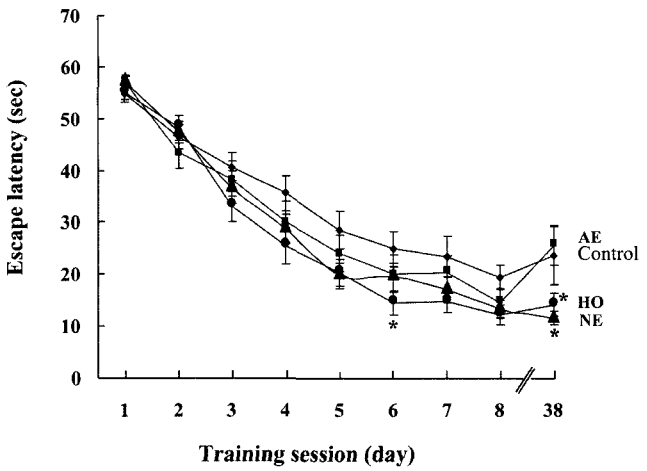

Fig. 4. Effects of *Vespa* extracts on the escape latency in a Morris water maze. Mice were treated orally with one of four preparations {Control, ◆, n=27; acidified methanol extract (AE), ■, n=23; 2% NaCl extract (NE), ▲, n=32; 0.5% honey solution (HO), ●, n=31}. The latency time for escaping onto the platform was measured for 8 consecutive days and then again 1 month after the first trial (in this case, control, n=11; AE, n=11; NE, n=13; HO, n=12). Data are expressed as mean±S.E.M. *, $P<0.05$ versus the control group for each day (ANOVA followed by Dunnett test, and also on day 38, ANOVA followed by Dunnett test).

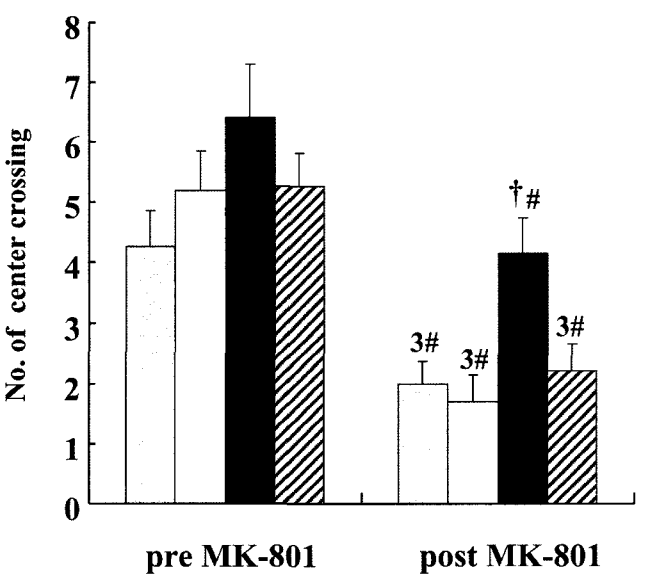

Fig. 5. Effects of *Vespa* extracts on mice performance in a Morris water maze. Mice received pure drinking water (Control, ▒, n=27), acidified methanol extract (□, n=23), 2% NaCl extract (■, n=32), and 0.5% honey solution (▨, n=31), respectively. Number of crossings over the quadrant of former platform location was recorded by a camera. Data are expressed as mean±S.E.M. †, $P<0.01$ versus the control group (ANOVA followed by Dunnett test), and #, $P<0.05$, 3#, $P<0.001$ versus the pre MK-801 trial in each group (ANOVA followed by Student's *t*-test).

Morris water maze task

The results of the Morris water maze task and the 1-month follow-up are shown in Fig. 4. During the training trials, the latency time for escaping onto the platform shortened over the 8 days in all groups. A significant difference was observed in the HO group on the 6th day. A probe test was performed on the 9th day after the removal of the platform. The mean number of crossings in the pre MK-801 trial was 4 to 5 in all groups with the exception of the NE group, which had about 6.5 crossings (Fig. 5). The mean number of crossings in the post MK-801 trial decreased to about 2, again with the exception of the NE group, in which mice crossed the platform about four times. In addition, the differences in the number of crossings between the pre MK-801 trial and post MK-801 trial in each group were significant at 5% level in the NE group and at 0.1% level in the other groups. In the NE group, the latency time on the 38th day (1 month after the trial) was shorter than on the 8th day of training, indicating spatial working memory (Fig. 4).

Discussion

The NE preparation from adult workers of *Vespa simillima* improved long-term memory of passive avoidance and mitigated MK-801-induced learning disability in special cognitive acquisition. These results demonstrate that insects such as wasps may harbor compounds worth of consideration in search for novel treatments of age-related memory impairment.

It has recently been reported that aqueous extracts of the plant *Puerarial flos* and Chinese medicine Oren-gedoku-to, exert antiamnesic effects on the impairment of learning and memory induced by alcohol or surgical cerebral ischemia (Xu *et al.*, 2000; Yamazaki *et al.*, 2005). Another report stated that a honeybee venom peptide (the mast cell degranulating peptide, MCD) composed of 22 amino acids induced a long-lasting increase of synaptic transmission in rat hippocampal slices (Cherubini *et al.*, 1987). The peptides found in honeybee venoms are structurally different from those known from the Vespid venoms, but the sites and modes of their actions are similar (Nakajima, 1986). Fractions extracted with 2% aqueous NaCl from the acetone powder of insect bodies are rich in peptide-like ingredients, as are the extracts prepared with acidified methanol (Naya *et al.*, 1994; Tanaka *et al.*, 2003). Therefore, the 2% NaCl extract prepared from the wasp workers may contain compounds related to MCD-peptide. However, the NE preparation was active orally, indicating that the effects observed in this study might have been be caused by partly digested peptides or by non-peptide

ingredients. Isolation of the effective compounds contained in the extracts is obviously needed for a full appreciation of the findings of this study. Oral administration of an insect-derived compound as a treatment of impaired learning and memory belongs at present to the realm of fantasy but the aging of human population requires exploration of all possibilities.

Certain extracts and compounds (ginsenoside Re, ginseng saponins) originating from plants have an antiamnesic effect on the scopolamine-induced impairment of learning and memory (Espinola *et al.,* 1997; Yamazaki *et al.,* 2005; Yamaguchi *et al.,* 1996; Jin *et al.,* 1999). These functions may be associated with the cholinergic system in the central nervous system (Xu *et al.,* 2000; Das *et al.,* 2002). In this study, scopolamine and MK-801 were used as amnesic agents. Scopolamine is a muscarinic receptor blocker and has been used as a suitable experimental model for Alzheimer's disease (Coyle *et al.,* 1983; Nobili *et al.,* 1997). MK-801 is an inhibitor of the NMDA receptor, and animals treated with MK-801 have been used for modeling of long-term loss of spatial memory (Coan *et al.,* 1987; Ramirez-Amaya *et al.,* 2001; Tsai *et al.,* 2004). Our data show that the NE from adult worker wasps did not have an antiamnesic effect on the scopolamine-induced impairment in the retention trial, but had a significant effect on the memory retrieval in the 1 month latency trial. It is therefore possible that this extract enhances building of the long–term memory (Fig. 3). The results in Figures 4 and 5 provide additional evidence that with the use of the NMDA receptor antagonist MK-801 the 2% NaCl extract may combine to enhance long-term formation of spatial memory.

The wasps and their nests have been used in traditional Chinese medicine for millennia, but until today virtually nothing is known about the mechanism of their action on human body. Our findings suggest that the wasps harbor substances with a potential to be developed for treatments of the age-related memory impairment.

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