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***Curcuma comosa* improves learning and memory function on ovariectomized rats in a long-term Morris water maze test**

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Abstract

Aim of the study—*Curcuma comosa* extract and some purified compounds from this plant have been reported to have estrogenic-like effects, and estrogen improves learning in some animals and potentially in postmenopausal women; therefore, this study tested the hypothesis that *Curcuma comosa* and estrogen have similar beneficial effects on spatial learning and memory.

Materials and methods—*Curcuma comosa* hexane extract, containing 0.165 mg of (4*E*,6*E*)-1,7-diphenylhepta-4,6-dien-3-one per mg of the crude extract, was orally administered to ovariectomized Wistar rats at the doses of 250 or 500 mg/kg body weight. 17 β -estradiol (10 μ g/kg body weight, subcutaneously) was used as a positive control. Thirty days after the initiation of treatment, animals were tested in a Morris water maze for spatial learning and memory. They were re-tested every 30 days and a final probe trial was run on day 119.

Results—Compared to control rats, OVX rats displayed significant memory impairment for locating the platform in the water maze from day 67 after the surgery, onward. In contrast, OVX rats treated with either *Curcuma comosa* or estrogen were significantly protected from this decline in cognitive function. Further, the protection of cognitive effects by *Curcuma comosa* was larger at higher dose.

Conclusions—These results suggest that long-term treatment with *Curcuma comosa* has beneficial effects on learning and memory function in rats.

Keywords

Curcuma comosa; Zingiberaceae; Phytoestrogen; Ovariectomized rat; Morris water maze; Spatial memory

1. Introduction

Estrogen replacement therapy (ERT) has been reported to prevent or delay cognitive decline in postmenopausal women and in estrogen-depleted animals. These benefits of estrogen therapy have been supported by numerous longitudinal and cross-sectional clinical studies (including randomized controlled trials) (Anderer et al., 2005; Lethaby et al., 2008; Sherwin, 2003). However, studies into the beneficial effects of estrogen replacement therapy in menopausal women demonstrated potential adverse outcomes, including increased breast

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cancer (Beral et al., 1997; Chlebowski et al., 2003), epithelial ovarian cancer (Barnabei et al., 2005; Bosetti et al., 2001) and hemorrhagic stroke (Anderson et al., 2004). These findings have made most women reluctant to use estrogen replacement therapy for menopausal symptoms. In humans, several phytoestrogens appear to have estrogenic-like effects on reproduction, bone and the cardiovascular systems and to have neuroprotective effects in the central nervous system (Dang and Lowik, 2005; Glazier and Bowman, 2001; Tham et al., 1998). In ovariectomized rats, some dietary phytoestrogens enhance spatial memory and increase spine density in the hippocampus and prefrontal cortex (Luine et al., 2006; Lund et al., 2001).

Ovariectomized rats have often been used as the model of cognitive impairment following ovarian hormone deprivation. Several studies using these models have reported that exogenous estrogen administration improves spatial working (Daniel and Lee, 2004) and reference memory (Sandstrom and Williams, 2004). The Morris water maze has been the classic method to test spatial learning and memory function, and the outcomes of studies using this method have correlated with human studies of cognition (D'Hooze and De Deyn, 2001).

Curcuma comosa Roxb. belongs to the Zingiberaceae family and is distributed throughout tropical and subtropical regions of Asia, especially in Thailand, Indonesia and Malaysia. *Curcuma comosa* is widely used in traditional medicine as an anti-inflammatory, and in treatment of postpartum uterine bleeding, pre-menopausal bleeding and uterine inflammation (Piyachaturawat et al., 1995a). The effectiveness of *Curcuma comosa* in the treatment of reproductive system disorders triggered studies into its potential estrogenic-like functions. Piyachaturawat et al. (1995a) demonstrated that oral administration of *Curcuma comosa* hexane extract increased uterine weight and improved glycogen content in ovariectomized rats. These effects were similar to the effects of estradiol in this model. Moreover, the *Curcuma comosa* extract treatment effectively induces and increases specific estradiol binding in uterine nuclei (Piyachaturawat et al., 1995b). These results suggest that *Curcuma comosa* may have estrogenic-like effects by acting at specific estradiol binding sites. In these studies, the hexane extract was the most active fraction of the botanical, and it significantly and dose dependently suppressed the growth of the organs, including testes, epididymis, ventral prostate, seminal vesicle and levator ani muscles (Piyachaturawat et al., 1998). Recently, several pure compounds had been isolated from the rhizomes of *Curcuma comosa* and two groups of structures, sesquiterpenes and diarylheptanoids, were isolated and identified from the purified compounds (Suksamrarn et al., 2008; Xu et al., 2008). Some of the isolated diarylheptanoids and their modified analogues exhibit estrogenic activity comparable to that of estradiol and genistein (Suksamrarn et al., 2008).

The present study investigated whether *Curcuma comosa* extract could protect against spatial learning and memory decline in ovariectomized rats.

2. Materials and methods

2.1. Plant material

Curcuma comosa rhizome was harvested in January, 2008 from Nakon Pathom Province, Thailand, (identified and provided by Professor Piyachaturawat Pawinee; Mahidol University, Bangkok). A voucher specimen was filed and kept in our laboratory (BS-C-03).

2.2. Chemical preparation and plant extract standardization

Dry *Curcuma comosa* rhizome was crushed to crude powder and extracted in n-hexane Soxhlet apparatus until the outlet hexane was colorless. Then the hexane fraction was evaporated to a brown-yellow oily extract, which was kept at 4 °C until use. HPLC was used to confirm the stability of the extract within the experimental period; a pure compound (98% pure), (4E,

6E)-1,7-diphenylhepta-4,6-dien-3-one (kindly provided by Prof. Apichart Suksamrarn from Ramkhamhaeng University, Thailand), which was purified from the *Curcuma comosa* hexane extract and proved to have the estrogenic-like effects was used as the standard to standardize the crude extract. In the present study, 1 mg crude extract was equal to 0.165 mg (4E,6E)-1,7-diphenylhepta-4,6-dien-3-one. The HPLC chromatograms and the structure are shown in Fig. 1.

The *Curcuma comosa* hexane extract was diluted in olive oil (Sabroso[®], Spain) for administration. As a positive control, 17 β -estradiol (Sigma, USA) was also diluted in olive oil at a concentration of 10 μ g/ml. Control rats were treated with the vehicle (olive oil; 1 ml/kg body weight).

2.3. Behavioral testing apparatus

The Morris water maze consisted of a large circular plastic pool (diameter 200 cm, height 40 cm) with a blue painted bottom, located in a room which was separated by black curtain from outside stimuli. It was filled with room-temperature (25 ± 2 °C) water to the depth of 20 cm and a clear plastic circular platform (diameter: 10 cm) was located 2 cm below the surface of the water, and white talcum powder was sprinkled on the water surface to make it opaque. Various objects or geometric images such as circles, squares, triangles with different colors were hung on the wall in the testing room as visual spatial cues. Swimming activity of each rat was tracked via a camera linked to a computer monitoring system from which latency to find the platform, total distance traveled, time and distance spent in each quadrant were calculated.

2.4. Animals and treatment

All experiments were conducted under the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and approved by the Ethics Committee (Animal Care and Use Committee) of Khon Kaen University (Reference No. 0514.1.12.2/30). All efforts were made to minimize pain and suffering of the animals. Fifty 6-month-old female Wistar rats (National Animal Center of Mahidol University, Bangkok) were housed at constant temperature (25 ± 2 °C), humidity (30–50%) and light-dark cycle (12/12). Rats had access to food (C.P. Ltd. Code: 082) and water *ad libitum*. The rats were randomly separated into five groups ($n = 10$) as follows: Group 1 (OVX + E), ovariectomized and receiving 17- β estradiol daily via subcutaneous injections (10 μ g/kg body weight); Group 2 (OVX + C1), ovariectomized and daily gavaged with *Curcuma comosa* extract at a dose of 250 mg/kg body weight; Group 3 (OVX + C2), ovariectomized and daily gavaged with *Curcuma comosa* extract at a dose of 500 mg/kg body weight; Group 4 (OVX + V), ovariectomized and receiving daily gavage of the vehicle; and Group 5 (Sham + V), sham operated rat receiving daily gavage of vehicle. From 1 day after ovariectomy, all the animals received the designed treatment every day at 8.00 to 10.00 AM, except on behavioral testing days, when the treatments were performed at 3 h before the testing. Thirty days after the ovariectomy, the animals were given the first Morris water maze testing for 5 days, four trials per day. The platform was located at the NE quadrant for all trials. The release point for the four trials was (1) SW, (2) NW, (3) SE and (4) SW quadrant. Thirty days later, animals were again tested in the Morris water maze for 2 days of probe testing. For each trial the platform was removed to test the reference memory after long-term intervals. Thereafter the animals were trained with the platform moved to the NW quadrant, and then they were trained for 5 days. Twenty days later, the animals were tested for 2 days in a probe test. Thereafter, the platform was moved to SW quadrant and the animal were trained for 5 days, and a probe test was conducted 20 days later (Fig. 2).

2.5. Ovariectomy

Rats were anesthetized and ovariectomized as described previously (Puertas et al., 1996). Briefly, the animal was anesthetized by exposure to a few drops of ether. Then the ovaries were removed via a dorsolateral incision, and ovarian blood vessels were tied off with sterile suture then skin was secured with wound clips. In general, the animals recovered from the anesthesia within 10 min after the surgery.

2.6. Morris water maze testing procedures

2.6.1. Hidden platform test—Four trials were given to the rats each day. The rats were allowed to swim 60 s to find the hidden platform at each trial. When successful, the rat was allowed 15 s to stay on the platform. If unsuccessful within 60 s, the rat was given a score of 60 s and then physically placed on the platform for 15 s. A 3–5 min interval was allowed between each trial. The time to find the platform and the distance to the platform were recorded to compare the spatial memory and learning ability between groups.

2.6.2. Probe test—For the probe test, three trials (60 s each; 5 min apart) were given each day with no platform present. The time that the animal spent in the currently “Correct” quadrant was recorded as an index of reference memory.

2.6.3. Video tracking and analysis systems—All swimming behavior was recorded by the camera set above the apparatus. Then the video document was analyzed by the Wintrack software (version 2.4.50923) which was developed by Dr. David P. Wolfer (Institute of Anatomy, University of Zurich).

2.7. OVX and estradiol efficiency inspection

The body weight of each animal was recorded every 3rd day throughout the experiment and the uterus weight was measured postmortem as indices of the efficiency of the ovariectomy and the estradiol and botanical treatments.

2.8. Statistics

Statistical analysis of the experimental data was carried out using SPSS (version 11.0). The parameters that were used to evaluate the performance were analyzed for significant differences by one-way and two-way ANOVA and repeated-measures ANOVA (Bonferroni confidence interval adjustment, Greenhouse–Geisser correction for non-sphericity). The criteria for statistical significance was at $P < 0.05$.

3. Results

The repeated-measures ANOVA result showed that the body weight of the OVX + V group significantly increased up to 240–260 g over the course of the study ($F_{1,62}, 16.22 = 36.36, P < 0.001$), and the body weights of all other groups stayed consistent during the course of the study. Postmortem analysis by one-way ANOVA demonstrated that ovariectomy significantly decreased uterine weight in the OVX + V group (0.2 g) compared to Sham + V group (0.5 g) and *Curcuma comosa* treated (0.4, 0.5 g, 250/500 mg/kg dose) or estradiol treated rats (0.7 g) ($F_{4,50} = 13.87, P < 0.001$). Therefore these data suggest that the ovariectomy reduced endogenous circulating estrogen.

Two-way ANOVA was applied to analyze the difference between groups and across the 5 days of the hidden platform tests. Within each of the three periods of hidden platform testing, the latency to the platform of all groups significantly decreased across the testing days (Period 1: $F_{4,244} = 45.18, P < 0.001$; Period 2: $F_{4,234} = 7.32, P < 0.001$; Period 3: $F_{3,187} = 18.57,$

$P < 0.001$), indicating that the animals were learning from the day by day practice (Fig. 3). However, differences between groups in each period were found. During the 1st hidden platform test, the OVX + C2 groups showed shorter latencies than the OVX + C1 and OVX + V groups ($F_{4, 244} = 2.83$, $P = 0.025$) (Fig. 3A). In the 2nd hidden platform test, the latencies of OVX + C2, OVX + C1 and OVX + E groups were similar, which were significantly better than that of the OVX + V group ($F_{4, 234} = 16.32$, $P < 0.001$) (Fig. 3B). The OVX + V group had the slowest latencies in the 2nd period trials, indicating impairment of the memory already at 67 days after the ovariectomy. During the 3rd hidden platform testing period, only the OVX + V groups had significantly longer latencies than that of the other four groups. However, there were no significant differences of latencies among these four groups ($F_{4, 187} = 8.83$, $P < 0.001$) (Fig. 3C).

In the 2nd hidden platform test, the one-way ANOVA analyzed result showed that in the 1st and 2nd testing days, the OVX + V group used significantly longer time to find the platform when compared to the other four groups (Day 1: $F_{4, 46} = 3.07$, $P = 0.026$; Day 2: $F_{4, 46} = 3.77$, $P = 0.010$) (Fig. 3B).

When comparing the learning ability between the OVX + C2 and OVX + V of the three periods by using two-way ANOVA, the OVX + C2 group showed a significant improvement in latencies from the 1st period to the 2nd period ($F_{2, 130} = 27.20$, $P < 0.001$), and remained at period 2 latencies in period 3 (Fig. 5A). By contrast, OVX + V rats displayed a significant improvement in latencies only from period 1 to period 3, but not from period 1 to period 2 ($F_{2, 139} = 5.26$, $P = 0.006$) (Fig. 5B).

One-way ANOVA was applied to analyze the difference within groups in the probe test of each testing period. In the 1st probe test, OVX + C2 rats showed significantly longer time in the “correct” quadrant at 30 days after the initial training. The other four groups did not show any significant differences in the first period testing ($F_{4, 46} = 3.05$, $P = 0.027$) (Fig. 4A). In the 2nd probe test at 20 days after the 2nd hidden platform test, OVX + V rats remained in the “correct” quadrant for the shortest time. By contrast, OVX + C2 rats spent significantly longer in the “correct” quadrant ($F_{4, 46} = 2.97$, $P = 0.030$) (Fig. 4B). The 3rd probe trial was similar among the groups, except Sham + V rats spent significantly less time in the “correct” quadrant than the OVX + E, OVX + C1 and OVX + C2 groups ($F_{4, 46} = 2.74$, $P = 0.041$) (Fig. 4C).

The swimming speeds of each group during periods 1–3 were also measured (0.24 m/s on average). There were no significant differences of the swimming speeds between groups in all periods, indicating that the swimming speed did not have influence on the latencies.

4. Discussion

In the present study, *Curcuma comosa* hexane extract improved performance of ovariectomized rats in the Morris water maze, indicating that the mild memory impairment induced by ovariectomy is prevented by the botanicals. Further, the effect is very similar to that of estrogen treatment. Especially in the 2nd and 3rd hidden platform tests, the performance of *Curcuma comosa* and estradiol treated groups were always at the same level, but *Curcuma comosa* treated rats seemed to perform better than estradiol treated rats in the following probe test. The estrogenic activity of *Curcuma comosa* also seemed to prevent both body weight changes and increased the uterine weight in OVX rats as seen for the estrogen treated animals. The effects of *Curcuma comosa* and estrogen in both the present and previous studies (Piyachaturawat et al., 1995a,b) showed that the botanical has estrogenic-like effects. The OVX rats that received the 500 mg/kg dose of *Curcuma comosa* extract displayed the lowest latencies of any groups, while the 250 mg/kg dose of *Curcuma comosa* also had a positive effect on

water maze performance. Estradiol treatment also improved performance in ovariectomized rats.

These cognitive enhancing effects were evident in the 1st period on the 3rd and 4th day of learning. The OVX + C2 groups showed the shortest latency and swimming path to the platform. Taken together, these data suggest that *Curcuma comosa* can improve cognitive performance similar to the effects of estrogen and some phytoestrogens (Belcher and Zsarnovszky, 2001; Lephart et al., 2005; Luine et al., 2006). This is the first report of *Curcuma comosa*'s beneficial effects on the central nervous system.

The effects of ovariectomy on impairment of spatial memory in rats are consistent with several previous reports (Daniel and Lee, 2004; Packard and Teather, 1997). In this study, the memory impairment of the OVX rats was evident at 67 days after the surgery, during the hidden platform test of the 2nd period of testing. However at the 1st period (30 days after the OVX), no significant differences were found between ovariectomized and sham operated rats. It is interesting to note that in the 1st probe trial, which was performed just before the 2nd hidden platform training/test period, OVX + V rats did not show any deficit, indicating the poor performance in the maze of ovariectomized rats relates to learning rather than memory.

The amount of time the animal spent in the "correct" quadrant in the probe test was related to the performance in the hidden platform test in the same testing period. For example, the OVX + C2 always used the shortest time in the hidden platform test and then spent the longest time in the "correct" quadrant. The OVX + V group was the slowest one to find the platform and then spent less time in the "correct" quadrant in each period. The OVX + C1, OVX + E and Sham + V groups showed medium performance. However, there was an unexpected result, as observed in the 3rd probe test, in which the sham group spent the shortest time in the "correct" quadrant. Two possible explanations might support to this unexpected performance: (1) The Sham + V group was the only group that not ovariectomized and the might have the best understanding on the rules of this game because they should have no memory loss since the beginning of the experiment. This group may have learnt that there was no platform in the probe trial, and thus they quickly changed their strategy to look for another possible way to escape. (2) Since the sham group was intact, their memory retention or memory recall function should be better than the other groups, so that the animals could still remember the platform location in the 1st and 2nd testing periods. This would result in the animal spending more time in the two previous quadrants when they learned that there was no platform in the current "correct" quadrant. To prove this explanation, further study is required.

Six-month-old rats were used in this study because the current objective was to investigate the effect of estradiol on the memory of a fully developed, mature brain. At the end of the experiment, the rats were in 10 months old, thus it is unlikely that age significantly confounded the interpretation of the hormone effects. At this age, the hormone level in rats is stable. Rats older than 10 months of age show significant fluctuations in circulating plasma estrogen concentrations and estrous cycles (Hof and Mobbs, 2000; Suckow et al., 2006).

The dose of estradiol and the route of administration were referenced from previous researchers (Butcher et al., 1974; Henderson et al., 1977; Hu and Becker, 2008). In those studies, approximately 10 µg/kg body weight per day subcutaneous estradiol injections resulted in circulating maximum levels of 180–300 pmol/l and minimum levels of 20–70 pmol/l in the serum, which was similar to the physiological estrogen level. In the present study, ovariectomy significantly reduced uterus weight whereas *Curcuma comosa* extract reversed this decrease. Since the estrogen receptor (ER) alpha is the dominating subtype in uterus (Buchanan et al., 1999; Wang et al., 1999), it appears that *Curcuma comosa* acts via ER alpha. However, it has

been reported that *Curcuma comosa* hexane extract presented some ER beta effects on HELA cells (Suksamrarn et al., 2008).

In the HPLC chromatogram of the crude extract (Fig. 1), two major peaks with retention times of 8.30 and 14.57 min were observed. However, only the compound of peak with retention time at 8.30 min, which having estrogenic activity was used to standardize the extract in the present study. Moreover, the peak at 14.57 min was identified by NMR method to contain a mixture of two known compounds, (4*E*,6*E*)-1,7-diphenylhepta-4,6-dien-3-ol and (6*E*)-1,7-diphenylhept-6-en-3-ol, which were previously reported by Jurgens et al. (1994) and Suksamrarn et al. (2008).

The animal models (OVX rat) and memory test (Morris water maze task) used in this study are classic methods for testing the role of estrogen in learning and memory. Estradiol improved maze performance in OVX rats, resulting in shorter time to search the platform and longer time to stay in the “correct” quadrant at the probe test. These results were consistent with those previously reported (Iivonen et al., 2006; Luine et al., 1998).

Phytoestrogens are represented by hundreds of different types of molecules that are generally classified as nonsteroidal in configuration with a customary diphenolic structure. Phytoestrogen are reported to affect brain structure, learning, memory and anxiety along with the brain androgen-metabolizing enzyme, aromatase (Lephart et al., 2002). Further, dietary phytoestrogens have been reported to enhance spatial memory and spine density in the hippocampus and prefrontal cortex of ovariectomized rats (Luine et al., 2006). Recently, some of the purified compounds, such as diarylheptanoids, have been isolated from the *Curcuma comosa* hexane extract. Similar to the estradiol and genistein, those compounds display estrogenic-like effect on HELA cells (Suksamrarn et al., 2008).

5. Conclusion

Curcuma comosa hexane extract is beneficial to spatial learning and memory on ovariectomized rats, with the higher dose showing more beneficial effect in the probe test in present study. These effects are similar to that of estradiol.

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Fig. 1.
The HPLC chromatograms of the *Curcuma comosa* hexane extract (0.183 mg/ml) (A) and a purified compound (4E,6E)-1,7-diphenylhepta-4,6-dien-3-one (1.83 mg/ml) (B).

Fig. 2.

The experimental time-line. MWM: Morris water maze, OVX: ovariectomy.

Fig. 3.

The hidden platform test results during three testing periods (A–C) analyzed by two-way ANOVA (a–c) and one-way ANOVA (*). (a) Represents a significant difference of OVX + C2 group when compared to OVX + C1 and OVX + V groups ($P < 0.05$). (b) Represents a significant difference of OVX + V group when compared to OVX + C1, OVX + C2 and OVX + E groups ($P < 0.05$). (c) Represents a significant difference of OVX + V group when compared to other four groups ($P < 0.05$). *Represents a significant difference of OVX + V group when compared to other four groups ($P < 0.05$).

Fig. 4.

Time spent in the “correct” quadrant in the Morris water maze probe trials (A–C). (1) Represents a significant difference of OVX + C2 group when compared to other four groups ($P < 0.05$). (2) Represents a significant difference of OVX + C2 group when compared to OVX + V and Sham + V groups ($P < 0.05$). (3) Represents a significant difference of OVX + C1, OVX + C2 and OVX + E groups when compared to Sham + V groups ($P < 0.05$).

Fig. 5.

The improvement of the water maze performance (time to the platform) of OVX + C2 (A) and OVX + V (B) groups from the 1st period to the 3rd period. (a) OVX + C2 group displayed significant improvement from the 1st period to the 2nd period ($P < 0.05$). (b) A significant difference was not found between 1st and 2nd period in the OVX + V group. The significant improvement was found between 1st and 3rd period in both groups ($P < 0.05$).