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Psychobiological Assessment of Smoke of Agarwood (*Aquilaria spp.*) in Male Rats

Sayyed Shahram MIRAGHAEE[1] Isaac KARIMI[2*] Lora A. BECKER[3]

[1]Department of Pharmacognosy and Pharmaceutical Biotechnology, School of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, IRAN

[2]Department of Biochemistry, Physiology and Pharmacology, School of Veterinary Medicine, Razi University, Kermanshah, IRAN

[3]Department of Psychology, University of Evansville, IN 47722, USA

[*]Corresponding Author
e-mail: isaac_karimi2000@yahoo.com

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Abstract

Burning of agarwood as incense has been part of oriental religious and cultural ceremonies through the millennia. To evaluate effects of agarwood smoke, ten adult male rats were divided into two equal groups. Male rats were either exposed to compressed air or compressed agarwood smoke for 1 hour, once a day, for four days per week throughout 28 days. Standard behavioral rat models for the evaluation of anxiety, depression, memory and stress and steroid hormone profile were examined. Smoke of agarwood significantly depressed swim time in forced swimming test. No differences were found between two groups for object recognition in object recognition test, spatial memory in Morris water maze, anxiety in elevated plus-maze, or stress in the tail pinch stressor paradigm. Correspondingly, plasma level of cortisol did not differ due to exposure. However, plasma testosterone level was significantly decreased following exposure to agarwood smoke. This result suggests that agarwood smoke may function as an endocrine disruptor.

Keywords: Aquilaria spp.; Pharmacological assays; Testosterone

INTRODUCTION

Throughout history, mankind has searched herbs for enlightenment and esoteric allusions. Herbs that fulfill these purposes are called entheogenic; literally, 'God inside us'. In the strict sense, an entheogen is a psychotropic substance used in a psychotherapeutic, religious, shamanic, or spiritual context. Recently, research on plants eliciting psychoactive effects, such as Cannabis sativa L., Papaver somnifera, Boswellia carterii, and Nicotiana tabacum, has opened new horizons into the mechanisms that are beyond the behavioral changes that occurred following intake of these psychoactive herbs [1,2].

Agarwood is the resinous heartwood from Aquilaria spp. trees (family Thymelaeaceae) native to oriental regions. The trees become decayed, injured or infected with mold and begin to produce an aromatic resin in response to this attack [3]. The incense of this endangered centuries-old plant has been used in religious ceremonies in Asian religions like Zen, Buddhism, Taoism, Sanskrit, Christ, Jew and Islam (Sufism and Mysticism). For example, in Sufism, agarwood is used as medium for decisive phases of development, in the Bible, agarwood is described as scented wood or as balsam, in Buddhism only joss sticks of agarwood are allowed for certain sacrificial ceremonies and in the Islamic mysticism, agarwood is used for advanced stages of spiritual growth. This valuable incense (Ood, Oud and Oudh in Persian and Arabic literatures) also known with different names like agarwood, aloewood, eaglewood (in the United States), jinkoh (in Japanese), agar (in Sanskrit) and aguru and aghil (in Tamil).

Agarwood is held in high regard as incense along with other uses as a sedative, analgesic and digestive in Kampo medicine [4]. Okugawa et al. [5] reported that six sesquiterpenoids, namely, jinkoh-eremol, agarospirol, α- and β-santalols, dehydrocostus lactone, and costunolide, isolated from agarwood, inhibited acetic acid-induced writhing in mouse model of pain. Some of the isolated sesquiterpenes of agarwood have sedative and analgesic effects [6,7]. Aquilaria sinensis (Lour.) Gilg. leaf extract found to be a strong analgesic and anti-inflammatory in animal models, which support its folkloric use for some diseases associated with painful and inflammatory conditions such as trauma etc [8]. In the Middle East and Europe, distilled oil of agarwood is more commonly used as a blending material for perfume and balm [9].

The phytochemical analysis of agarwood has been subject of several studies [3, 10-18]. According to these reports on the fragrant compounds of agarwood, sesquiterpenoids and phenylethyl chromone derivatives are the principal compounds in the resin of agarwood derived from different Aquilaria spp. The agarwood contains numerous components: p-methoxycinnamic-acid, agarotetrol, agarol, agarospirol, alpha- and beta-agarofuran, dihydroagarofuran, 4-hydroxydihydroagarofuran, oxo-nor-agarofuran and others [4]. The burning of incense may provide some spiritual or physical comfort [19]. However, if not properly vented, the accumulated high levels of polycyclic aromatic hydrocarbons (PAHs) inside a temple or house may become a potential threat to humans [20].

To evaluate the activity profile of smoke of agarwood, we used behavioral, pharmacological, and physiological assays. They included standard behavioral rat models for the evaluation of anxiety, depression, memory and stress (elevated plus maze, forced swimming test, object recognition test, Morris water maze and tail pinch stressor, respectively) and steroid hormone profile.

MATERIALS AND METHODS

Animal subjects

This study reviewed and approved by the Laboratory Animal Care Committee of School of Veterinary Medicine, Razi University, Kermanshah, Iran. Adult weight- and age-matched healthy male Wistar rats (n=10), were maintained on normal chow diet in an air-conditioned room (23° ± 1°C) and were divided into two groups of 5 each. Control group animals (C) were handled and exposed to compressed air for 1 hour, once a day, four times per week throughout 28 days. Agarwood smoke exposed animals (S) were handled the same and then exposed to agarwood smoke from inflamed agarwood (Agarbatti in Indian; Vasuagarwoodes brand, India) incense stick in compressed air flow for 1 hour, once a day, for four days per week throughout 28 days.

Agarwood exposure

The smoking apparatus consisted of two glass chambers separated by a perforated wall. In the first chamber (combustion), the sticks were burnt, and the animals were placed in the second chamber (inhalation). During exposure compressed air fed the combustion and directed the smoke flow into the inhalation chamber and from there to an exit. The smoke from agarwood sticks were completely burnt and ventilated by compressed air flow. The animals in group C were put in second chamber that was reserved exclusively for that group, with a similar compressed air flow, at the same frequency and duration and at the same times of day as the animals in group S.

Behavioral tests

All behavioral tests and their corresponding sessions were conducted 30 min after exposing to smoke of agarwood in the light phase between 09:00 and 17:00.

Object recognition test: ORT

The apparatus used for ORT was the same smoking apparatus because rats were habituated to it throughout study. The experimental session comprised two trials with three time arrangements to test the effects of agarwood inhalation on acquisition, consolidation and restitution phases of learning during ORT. In the first trial, two copies of the same object like colored sands were presented. In the second trial, one of the familiar object and a new object were presented. In the first trial (T1), one object-stimulus, the sample (A), was placed near the central area of the box in a location equidistant from the walls of the box and the animals explored objects during 10 min. During the second trial (T2), a new object (B) was added. Here, each object was placed in a front corner. The object (A') presented during T2 was a duplicate of the sample presented in T1 (A) in order to avoid olfactory traits. The animals explored objects for 3 min. Objects and their positions during T2 were counterbalanced and randomly changed. These precautions were taken to reduce object and place preference effects. At the beginning of each trial, rats were placed near the center of the back wall of the box, with their heads oriented in the opposite direction to the object.

To test the acquisition phase of ORT, rats were treated for 1h with smoke of agarwood before T1 and then tested throughout T2. The consolidation phase of ORT was evaluated by smoking rats just after the T1 and tested them throughout T2. Finally to test the restitution phase of ORT, rats were smoked 0.5 h before T2. In all experiments, the gap between two trials was considered 24 h. The duration of T1 and T2 was 3 min. The basic measure was the total time spent by rats in exploring objects during T1 and T2 trials. Exploration of an object was defined as follows: directing the nose at a distance 2 cm to the object and/or touching it with the nose. Turning around or sitting on the object was not considered exploratory behavior. In this sense, the following variables were defined: A = the time spent exploring the sample during T1, B + A' = the time spent exploring a duplicate of the familiar object A (A') and a new object (B) during T2. Object recognition was measured by the variable B – A'. Since B – A' may be biased by differences in overall levels of exploration, the variable B – A'/ B + A' was considered as the discrimination ratio [21,22].

Morris water maze: MWM

The MWM was used to test the effects of agarwood inhalation on spatial memory [23]. The water-maze consists of a plastic child swimming pool 150 cm in diameter with 60 cm high walls. The maze was divided into four quadrants with string or wire stretched across the top. A height transparent circular platform was located in one quadrant. The pool was filled in drinking yoghurt to about 1 cm above the platform. The maze itself was featureless and the only obvious cues were photos on the surrounding walls. Each session was comprised of four trials with an inter-trial interval of 30 s. A trial consisted of randomly releasing the animal from one out of four compass locations around the pool and allowing the animal to swim until it either came upon the hidden platform (North quadrant) or until 60 seconds had elapsed. The latency to the platform was recorded. If an animal failed to find the platform within 60 seconds, it was taken back there for 15 s. The final test (probe trial) was performed on the afternoon of the 6th training day, during which the platform was removed from the maze. During the probe trial the animal was released into the quadrant opposite to the one that had previously contained the platform (the ''platform'' quadrant), and allowed to swim in the maze for 1 min. The video/computer system automatically recorded the latency for finding the platform.

Elevated Plus-Maze: EPM

The EPM was used to test the effects of agarwood inhalation on anxiety [24]. The wooden, plus-shaped apparatus was elevated to a height of 50 cm, and consisted of two 50×10

cm open arms, and two 50×10×50 cm enclosed arms, each with an open roof. The maze was in the center of a quiet and dimly lit room. Thirty minutes following exposure to the smoking apparatus, rats were placed individually in the center of the plus-maze, facing one of the open arms. The rats' behavior was recorded by a camera that was suspended at an angle above the maze, then films were evaluated for following parameters: (1) time spent in the open arms, (2) time spent in the closed arms, (3) number of entries into the open arms, and (4) number of entries into the closed arms during the 5 min test period. An entry was defined as all four paws in the arm. The maze was cleaned with distilled water after each rat was tested. For the purpose of analysis [24], open-arm activity was quantified as the amount of time that the rat spent in the open arms relative to the total amount of time spent in any arm (open/total×100), and the number of entries into the open arms was quantified relative to the total number of entries into any arm (open/total×100). The time spent in open arms, number of entries into the open arms and the ratio of open arm entries were evaluated as an index of anxiety. The number of total entries was also evaluated as an index of locomotor activity [25].

Forced swimming test: FST

The FST was used to test the effects of agarwood inhalation on depression [26]. Rats were exposed to the swimming apparatus (individual color plastic cylinders 20 cm diameter, 46 cm height, 30 cm water depth and 23–25°C water temperature) for 15 min pre-exposure 24 h prior to their test session [26]. Swimming sessions were conducted 20 days after treatment. Each rat was forced to swim for 5 min and then removed from the cylinders, dried with paper towels and placed in heated cages for 30 min, before returning to their home cages. Test was run between 19:00 and 22:00 h. All behaviors were recorded by camera that located above the swimming apparatus. A time sampling technique was employed to score three different behaviors. During the session, following behaviors have been rated 2 min after beginning of test (1) immobility-floating in the water without struggling, and doing only necessary movements to keep the head above the water and (2) swimming-showing moderate active motions around in the cylinder, more than necessary to merely keep the head above water.

Tail pinch stressor: TPS

The TPS has been used to stimulate stress-related behavioral activities in rats [27]. The tail pinch cages were cleaned and dried before each trial. Animals were placed in the one side of a cage, the length of the tail was guided through the hole in the cage's wall, and a sponge-padded paper clip (2.3 cm x 0.5 cm) was applied for 10 min to a position 2 cm from the tip of the tail. Animals were observed for a period of five minutes. After observation, the paper clips were removed and the animals were returned immediately to their home cages. The cumulative times of gnawing or biting toward a wooden chip was recorded as non-functional masticatory activity (NFMA). This stereotypic behavior was considered as a coping response to the acute stress.

Blood collection

On the last day of the experiment (28th day), after 14-16 h of fasting, blood samples were collected into tubes containing heparin by cardiac puncture following deep anesthesia with an i.p. ketamine/xylazine injection. All plasma samples were separated by centrifugation at 1400 × g at 4 °C for 15 min, and stored at –20 °C until analysis.

Biochemical analysis

Cortisol was measured by a commercially available ELISA (IBL, International GmbH, Hamburg, Germany), with intra- and inter-assay coefficients of variation less than 5.6% and a sensitivity of 2.5 ng/ml. Plasma testosterone level was measured using ELISA kits (IBL International GmbH, Hamburg, Germany) with intra- and inter-assay coefficients of variation less than 6.8% and a sensitivity of 0.083 ng/ml.

Statistical analysis

For the statistical analysis we utilized the software SPSS version 13. The discrimination index for ORT in different phases of memory as well as escape latency in different trials of MWM were analyzed using a repeated-measure analysis of variance (ANOVA), followed by a post-hoc Tukey's HSD test. Pairs' comparisons were analyzed using the Student-t test. Data are expressed as the mean ± SEM; $P < 0.05$ was considered as minimum criterion for assigning statistical significance.

RESULTS

Throughout the experimental period, the mean body weights of rats in both C and S groups were similar (Fig. 1). Food intake did not show any significant differences between two groups (data not shown).

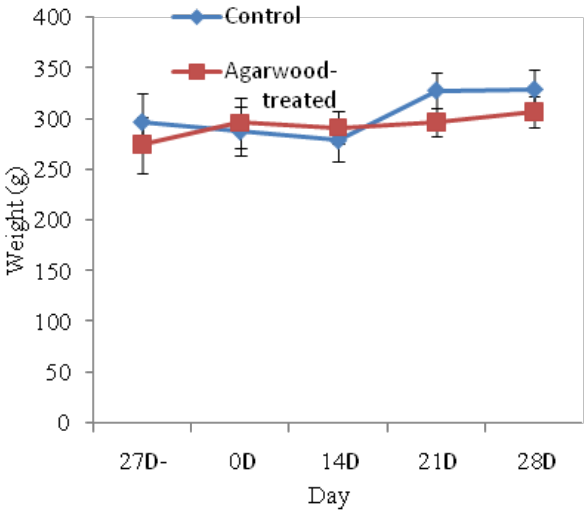

Fig.1. The body weight changes after exposing to the smoke of agarwood in rats. Each bar is mean ± S.E.M.

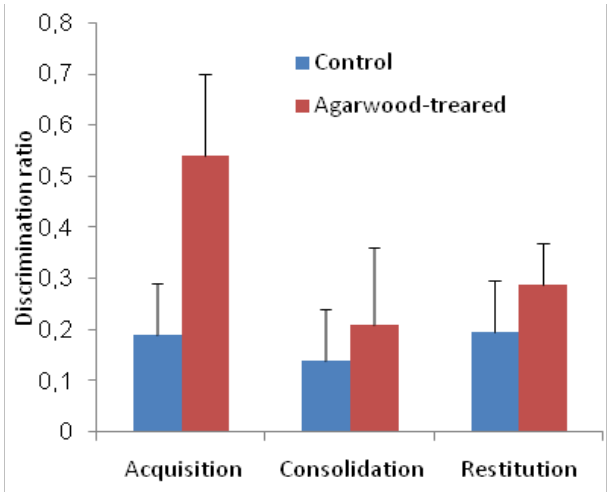

Fig. 2. Effects of smoke of agarwood on discrimination ratio in acquisition, consolidation and restitution phases of learning during ORT [21,22]. Each bar is mean ± S.E.M.

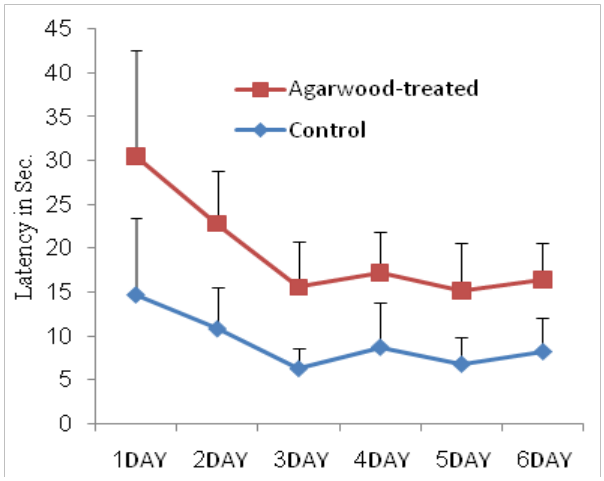

Fig. 3. Time to escape on the platform during acquisition trials of the Morris water maze test. Four trials per day over 5 days were performed for the acquisition test. The task was performed with four daily trials on the sixth day without the platform for the retention test. Vertical lines indicate S.E.M.

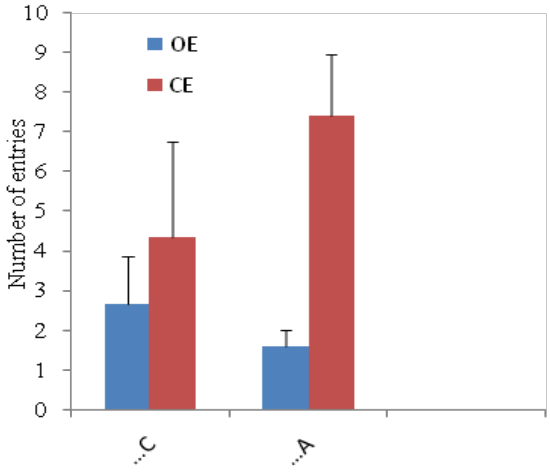

Fig. 4. Effects of smoke of agarwood on rats in the elevated plus-maze. Each bar is mean ± S.E.M., Open arm entries (OE) and closed arm entries (CE), n = 5.

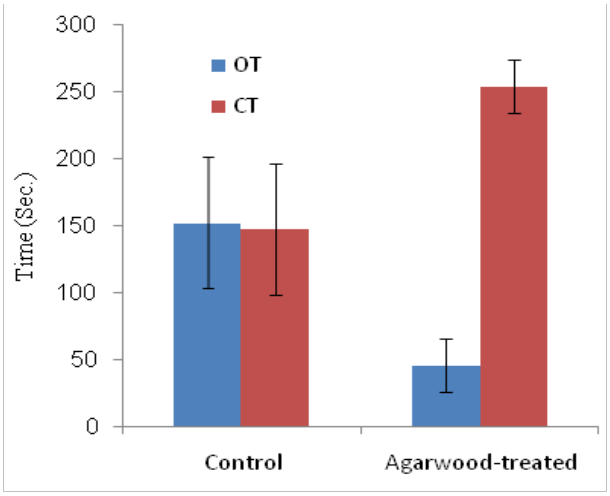

Fig. 5. Effects of smoke of agarwood on rats in the elevated plus-maze. Each bar is S.E.M. Open arm time (OT) and closed arm time (CT), n = 5.

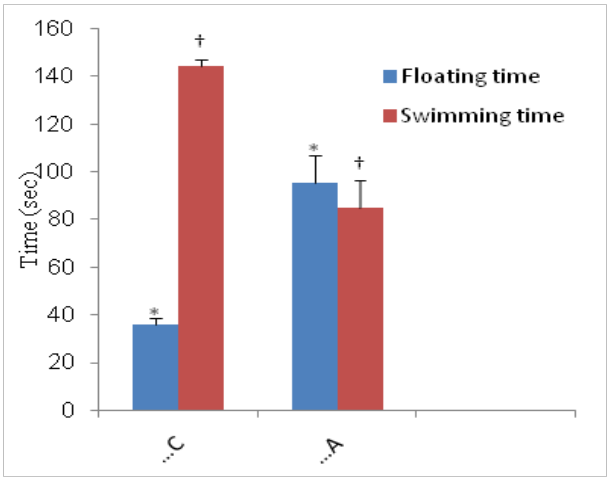

Fig. 6. Effects of smoke of agarwood on immobility (floating) and swimming times in Porslot's forced swimming test in rats. Data are expressed as mean±SEM with 5 rats per group. $^{*,\dagger}p < 0.05$, compared with the control rats.

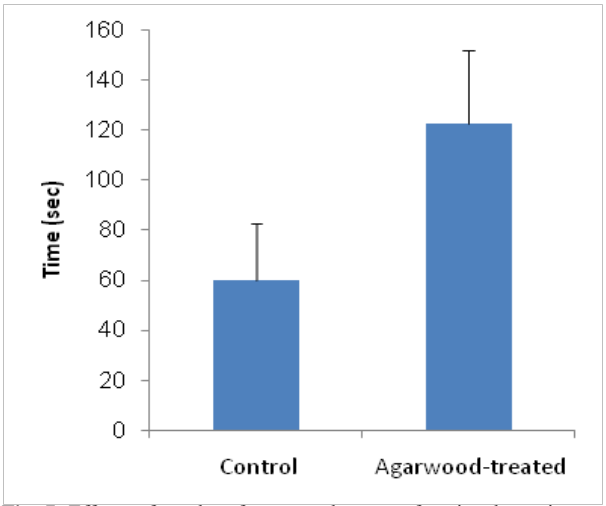

Fig. 7. Effects of smoke of agarwood on non-functional masticatory activity (NFMA) in tail pinch test in rats. Data are expressed as mean±SEM with 5 rats per group.

Object recognition test: ORT

Smoke of agarwood did not alter memory of object recognition in S group compared to C group (see Fig. 2). The discrimination ratio in the acquisition phase of ORT increased in S group (0.54±0.16) compared to C group (0.19±0.120; p>0.05). The discrimination ratio in the consolidation phase of ORT increased in S group (0.21±0.150) compared to C group (0.14±0.025; p>0.05). The discrimination ratio in the restitution phase of ORT increased in S group (0.28±0.081) compared to C group (0.19±0.003; p>0.05).

Morris water maze: MWM

All latencies for finding the platform for the different rat groups are shown in Fig. 3. Latencies during water maze acquisition showed a significant decrease throughout the trials in both group, which indicated that all rats improved performance during the trials. But data analysis did not show significant difference between groups. However, the total escape latency throughout 6 days (24 trials) had a marginal increase in S group (10.38±7.280 s) compared to C group (9.27±5.679 s; p>0.05), however it was not statistically significant. The total escape latency of four trials in the water maze task was similar in agarwood-treated group (8.20±4.443 s) compared to control group (8.25±3.840 s; p>0.05) on the day 6 (probe trial; see Fig. 3)

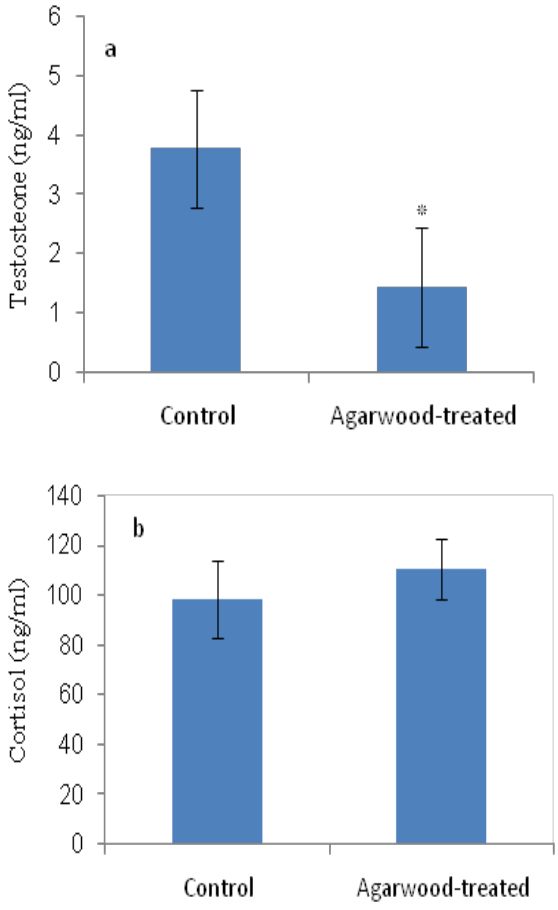

Fig. 8. Changes in steroid hormone concentration of testosterone (a) and cortisol (b) following exposing to the smoke of agarwood. Data are expressed as mean±SEM with 5 rats per group.

Elevated Plus-Maze: EPM

Figure 4 and 5 show the effect of smoke of agarwood in the EPM in rats. Rats exposed to the smoke of agarwood spent a lesser time (45.8±19.66) in open arm of EPM than control rats (152.33±49.09; p>0.05). However, agarwood-treated group spent a greater time (254.2±19.66) in closed arm of EPM than control rats (147.66±49.09; p>0.05). The number of entries into the open arms in agarwood-treated rats (1.60±0.40) was non-significantly lesser than control rats (2.6±1.18; p>0.05) while the number of entries into the closed arms in agarwood-treated rats (7.4±1.53) was non-significantly greater than control rats (4.3±2.40; p>0.05). No change in the locomotor activity was observed between two groups.

Forced swimming test: FST

By exposing to smoke of agarwood, the mean immobility time in forced swimming test was significantly ($p < 0.05$) prolonged (35.6±2.9 s) for control rats versus (95.2±11.5 s) for agarwood-treated rats as shown in Fig. 6. The swimming times for the agarwood-treated rats (84.8±11.594 s) were significantly shorter than control rats (144.33±2.962 s; $p < 0.05$).

Tail pinch stressor: TPS

Total time of stress-induced NFMA non-significantly ($p > 0.05$) increased in agarwood-treated group (122.6±29.43 s) compared to control group (59.7±22.8 s; Fig. 7).

Steroid hormone profile

The plasma level of cortisol was non-significantly increased in S group (110.56 ± 12.131) compared to C group (98.36 ± 15.662; p>0.05). However, plasma testosterone level was significantly decreased in S group (1.44 ± 0.291) in comparison to C group (3.77 ± 0.375; p<0.05); see Fig. 8a, b).

DISCUSSION

The effects of smoke of agarwood were evaluated in adult male rats. Several behavioral tests such as TPS, EPM, FST, MWM, and ORT were conducted to evaluate the effects of smoke of agarwood on stress, anxiety, depression, spatial memory, and visual recognition memory, respectively and plasma cortisol and testosterone levels were considered as endpoints.

Tail pinch has been used to stimulate eating and other stress-related behavioral activities in rats [27]. Application of an unavoidable peripheral stressor (like tail pinch) produces a profile of behaviors including eating, gnawing, and licking. This response may have interesting parallels with stress-induced eating in humans [27,28]. Total time of stress-induced NFMA showed no significant increase in agarwood-treated group compared to control group. This finding is in contrast to the traditional usages of agarwood as an adaptogenic remedy. Agarwood is one of the major components that used in preparation of Chyawanprash as an adaptogen and anti-aging remedy in Ayurvedic medicine [29]. However, in the present study, the adoptogenic or anti-stress activities of the smoke of agarwood was not observed. It seems the route of

administration may be involved here. Agarwood is consumed as an oral ingredient in Chyawanprash formulation and its combustion would change its properties. Higher level of cortisol in agarwood-treated group with respect to the control led us to conclude that agarwood or smoking process may be caused one type of emotional stress. Also, it was previously reported that rats were exposed to chronic restraint stress for 4 hours a day for 2 months showed marked suppression of spermatogenesis, increase of cytoplasmic vacoulation in Leyding cells that indicates the suppressed activity of these cells and finally remarkable decrease of blood testosterone concentration [30]. A number of studies have found that smoke emitted by incense burning contains PAHs [19,31,32,33]. It appears that different types of incense produce various amounts of PAHs [34]. As previously reported, testosterone decreased following exposing to PAHs in males [35]. In another investigation, Kim et al. [36] have suggest that the lower the testosterone levels in young male Koreans that use "PC Game Room" might be mediated by heavy PAH exposure. The decline of testosterone in agarwood-treated group may be related to the PAHs content of smoke of agarwood that possibly disrupt the processes of synthesis and/or elimination of testosterone. Naphthalene alone accounts for 38 to 68% of total PAH mass emitted from combustion of various incenses [33]. Other most important PAHs include phenanthrene, acenaphthylene, flouranthene, benzo(a) pyrene and anthracene [33]. Naphthalene is recognized as an endocrine disruptor because acute (1 to 6 h) and prolonged (1 to 5 days) exposure of rainbow trout to naphthalene resulted in decreased plasmatic cortisol and 17-β-estradiol levels [37]. The cortisol level did not decrease following exposure to smoke of agarwood in the present investigation and agarwood-treated rats responded to stress by increasing blood cortisol level. This disagreement between this study and Gesto et al's study [37] may be related to the different studied species, content and duration of exposure to naphthalene or possibly other PAHs. Further studies could be directed at identifying the enzymes involved in this response, as well as the amount and types of PAHs in the smoke of agarwood.

In the present study, FST was utilized as a model system for evaluating the effects of smoke of agarwood on an emotional reaction to an inescapable stressor [26,38,39]. The summed immobility time during FST was significantly prolonged in agarwood-treated rats. This depressive state or lowered mood that occurred following exposure to smoke of agarwood may be related to the significant decline of testosterone in agarwood-treated rats in comparison to the control rats. Recently, it was demonstrated that orchiectomized rats showed a prolonged immobility time in FST and this prolongation was shortened by testosterone propionate treatment [40].

The EPM is widely used to test anxiety in animal model [41]. The percentage of open arm entries and time spent in the open arms has been validated as a measure of anxiety [41,42]. Exposure to smoke of agarwood non-significantly modified the rat behavior on the EPM, decreasing the exploration of open arms and increasing the time spent in closed arms. Androstanediol is an endogenous anxiolytic and anti-seizure neurosteroid produced from testosterone and could be a neurosteroid mediator of testosterone actions on neuronal excitability and seizure susceptibility via its activity as a $GABA_A$ receptor modulator and that androstanediol may play a key role in men with epilepsy, especially during the age-related decline in androgen levels [43-46]. Age-related changes in androstanediol levels may profoundly affect anxiety, cognitive function and seizure susceptibility [47,48]. Replacement of androstanediol in aged animals is shown to prevent cognitive deficits and anxiety behavior [49]. Anxiolytic effect of testosterone also requires its conversion to androstanediol. In the present study, decline of testosterone level in agarwood-treated rats may be associated with increased despair state in FST, increased anxious behavior in EPM and lower performance in MWM because of the lower conversion to androstanediol in central nervous system. Further investigations are needed to find the underlying mechanisms. Another study reported male superiority at 30 postnatal days in the spatial version of the MWM task [50]. This difference disappeared when female rats were treated with testosterone propionate at postnatal day 1, which suggests a testosterone-dependant process [50,51]. The MWM is a commonly used paradigm for learning and memory in rodents [52] that relies on distal cues to navigate from start locations around the perimeter of an open swimming arena to locate a submerged escape platform using spatial environmental cues. This ability is believed to require the hippocampal formation [53], and MWM performance has been used to detect cognitive impairment of developmental disorders in rodents [52]. In Western aromatherapy, the inhalation of the scent of agarwood was used for treatment of forgetfulness [54]. Smoke of agarwood had a marginal increase in all phases of acquisition, consolidation and restitution of memory in ORT paradigm. This marginal improvement of memory in ORT paradigm after exposing to the smoke of agarwood may be associated with the induction of nootropic brain-derived neurotrophic factor (BDNF). Ueda et al. [55] have shown that the ethanol extract of Vietnamese agarwood significantly induce BDNF mRNA expression in rat cultured neuronal cells. They also isolated a new spirovetivane-type sesquiterpene, (4R,5R,7R)-1(10)-spirovetiven-11-ol-2-one that induced BDNF mRNA expression. The BDNF is considered as a putative nerve growth factor (NGF) that acts in on certain neurons of the central nervous system and the peripheral nervous system and in the brain, it is active in the hippocampus, cortex, and basal forebrain—areas vital to learning, memory, and higher thinking [56-58] .

The ancient application of the oud chips for relaxation, meditation and physical well being is very common in "Kou-Dou", Japanese incense ceremony. It is also used pharmaceutically as an anti-emetic, sedative and digestive in oriental medical treatments [18]. In the present study, exposure to the smoke of agarwood caused an obvious sedation (data not shown). In this sense, the benzene extract of agarwood showed a prolonged effect on hexobarbital-induced sleeping time, and a reduction in spontaneous motility in mice [7]. The active principles, jinkoh-eremol and agarospirol, were obtained from the benzene extract. They decreased both methamphetamine- and apomorphine-induced spontaneous motility and can be considered to be neuroleptic [7].

CONCLUSION

In summary, the results of the present study demonstrate that smoke of agarwood did not improve spatial and visual recognition memories and has marginal anxious and stressor effects. The major finding of this study is that smoke of agarwood is depressive and decreases the plasma testosterone concentration.

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