# Sandalwood Oil Treatment During Growth Spurt Period Improves Learning And Enhances Memory

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## Summary

To evaluate the effect of sandalwood oil treatment during growth spurt period on learning and memory in Wistar rats. The effect of sandalwood oil on learning and memory was assessed using various methods viz., open field, passive avoidance, elevated plus maze and T-maze. The rats were administered sandalwood oil, with dose of 125mg/kg and 250mg/kg for two groups (SW1) and (SW2) respectively. From postnatal day 7, rat pups were administered sandalwood oil for 30 days. On $38^{th}$postnatal (early test) and $68^{th}$ postnatal day (delayed test), learning and memory development in rats were evaluated using all the models mentioned above. After all the tests the rats were sacrificed, the brain was taken out and the Acetylcholinesterase (AchE) levels were estimated using Ellman *et al* procedure. Both test groups showed significant improvement in learning and memory. Compared to control group in all the models, both the test groups showed improved learning and memory in early test ($p<0.01$) as well as delayed test ($p<0.05$) and showed significant reduction in AchE ($p<0.01$). However, both the test groups do not have any effect on general behaviour. The results signify that sandalwood oil treatment during growth spurt period improves learning and memory.

**Keywords:** Sandalwood oil, acetylcholinesterase, 'T' maze, open field test, passive avoidance.

## Introduction

The ability to learn something new and then to store the information in long term memories is part of normal development. [1] Memory is the recollection of the past experiences. [2] Brain is able to store new information, and recall after sometime. The field that deals with learning memory and cognitive disorders is called cognitive neuroscience. It has originated in two disciplines. In psychology the development of rigorous methods for analyzing behaviour and cognition, and in neurobiology, the effort to understand the structure and function of neuronal circuits of the sensory and motor systems of the brain. [3] In Ayurvedic system of medicine, "Medhya drugs" – a group of herbal medicines are known for their actions on nervous system. Some of the herbal drugs reported to act on nervous system include *Centella asiatica*[4], *Clitoria ternatea*[4], and *Withania somnifera*[4], etc

Sandalwood (*Santalum album*) oil is derived from heartwood of medium sized and evergreen parasitic tree is reported to have effect on central nervous system and also reported to improve memory.[5] It is also reported to be used as blood purifier and it has got its own importance in rituals of Hindu religion. Several texts including *Charaka Samhita, Shrungadhara Samhita*, mention a complex polyherbal medicated oil that contain chandana (Sandal wood oil) as chief constituent, called *chandanadya taila* is applied topically and taken internally to treat spiritual and mental disorders.[6] Though mentioned in Ayurvedic texts the effect of sandal wood oil on learning and memory, is not scientifically documented. Therefore, the present study aims at exploring the details of sandalwood oil on learning and memory in animal models.

## Materials and Methods

### *Drugs, reagents and chemicals*

The Sandalwood oil was procured from Karnataka Soaps and Detergents Limited, Bangalore, Karnataka. Acetylthiocholine iodide (Hi Media,India ), Dithiobisnitrobenzoic acid (Sigma St. Louis, U.S.A.),Trichloroacetic acid (S.D. Fine Chemicals, Mumbai), sodium hydroxide, potassium dihydrogen orthophosphate, sodium bicarbonate, buffer tablets pH 4.0, pH 9.2, pH 7.0 (Nice Chemicals Co., India) were purchased for the study .

### *Preparation of drugs and reagents:*

The sandalwood oil emulsion was prepared by using span 20 as an emulsifying agent. Sandalwood oil being an essential oil the standard ratio of 4:2:1 (parts of oil/emulsifying agent/water respectively) was used for preparing the o/w emulsion of the oil. The milky white emulsion so obtained was diluted with distilled water to obtain a concentration 27.82mg/ml of sandalwood oil. Ellman's reagent [7] was prepared by using phosphate buffer of pH 7.

### *Animals and treatment*

The albino Wister rat pups of either sex were procured along with parent rats (since they are in growth spurt period) from Venkateshwara Enterprises, Bangalore. They were housed as a set of parent rat and pups in each cage and maintained under natural day and night cycle at 25± 2°C ambient temperatures, 45-55 % relative humidity. The rats were allowed with free access of standard pellet and water *ad libitum*. The experiments were conducted under these ambient conditions between 9 to13 h. Before conducting the experiment, ethical clearance was obtained from Institutional Animal Ethical Committee, K.L.E.S' College of Pharmacy, JNMC, Belgaum. The literature review suggest that the sandalwood is safe upto oil is 5g/kg. [8] Since the rat pups were 7 days old and the dosing should be done upto 30 days from post-natal day 7, $1/20^{th}$ and $1/40^{th}$ of dose was selected.

Rat pups were divided into 4 groups each consisting of 6 rats of either sex.

Group 1 – Control (Received water)

Group 2 – Saline control (Received normal saline) and the two test groups 3 and 4 received sandalwood oil 125mg/kg and 250mg/kg respectively.

All animals received sandalwood oil for 30 days of $7^{th}$ to $37^{th}$ postnatal day. The dose 125mg/kg and 250mg/kg were administered orally using plastic oral pipe of infant saline drip.

**Screening for learning and memory [4]**

All four groups of rats were subjected to the following tests –

a) Open field behavioural test (General behaviour model) (b) Passive avoidance test (Exteroceptive behaviour model) (c) Elevated plus maze test (Exteroceptive behaviour model) and (d) T-maze (spatial learning), commencing from postnatal day 38 (i.e. a day after the last dose, early test). These rats were later again subjected to the same tests on postnatal day 68 (i.e. 30 days after the last dose, delayed test) in order to test the behavioural changes on learning and memory if any, is transient or permanent.

***a) Open field behaviour test***

This test was carried out to assess general behaviour. Open field behavioural test apparatus consisted of a large wooden box 100x100cm with 40 cm high walls. The floor consisted of a black painted wire mesh grid dividing the field into 25 equal squares 5 x 5 cm with 16 peripheral squares (PS) close to the wall and a central squares (CS). A 100W bulb was placed centrally, 5 feet above, for bright illumination.

The rat was placed in a corner of the apparatus and was allowed to explore the apparatus for 5 min. During this period the number of peripheral and central squares entered by the rat and the number of boli of excreta excreted were counted. Incidences of expression of rearing grooming/preening behaviours were also noted. Increase in the number of peripheral squares entered and more time spent in the peripheral area were considered as an increase in motor activity. Increased number of central squares entered and more time spent in the centre of the field indicates decreased fear and anxiety and emotional disturbance. Increased number of grooming, number of boli of excreta and decreased rearing are the expressions of emotionality, which are measures of autonomic function in the animals.

***b) Passive avoidance test***

The passive avoidance paradigm based on negative reinforcement was used to examine the long-term memory. The passive avoidance apparatus consists of a box/compartment with 50 cm x 50 cm grid floor and a wooden wall of 35cm height and illuminated with 15W bulb during experiment .A small wooden platform of 15x15 cm is placed in the centre of the platform.

On day one of the test (early test), rats were allowed to explore for 5 min and gently placed on the platform at the centre of the grid floor. When the animal stepped down the grid floor (placing all their paw). Inescapable electric foot shock (50 Hz, 1.5mA.for 1sec) was delivered and step down latency (SDL). SDL was defined as the time taken by the animal to step down from the wooden platform to grid floor with all their paws on the grid floor. Retention performance for each rat was again repeated after 48 h and 30 days (delayed test). Increase in SDL after an inescapable foot shock was interpreted as good retention performance. Test was repeated on day 68.

***c) Elevated plus maze***

Elevated plus Maze served as the exteroceptive behavioural model to evaluate learning and memory in laboratory animals .The procedure, technique, end point and dimension of EPZ was followed in accordance with suggestions / reports of several investigators working in area of neuropsychopharamcology.

Transfer latency (TL) was recorded on postnatal day 38, and after 24 h. This was repeated after 30 days (day 68). TL is defined as the time taken by rat to move into covered arm with all its four legs.

***d) Spatial learning test (T-Maze test)***

T-maze was used for assessing spatial learning and memory, when the test animals were subjected to spontaneous alterations and rewarded alterations**.**

The T-Maze consisted of a start box 15 x 12cm, stem 35 x 12 cm, a choice area 15 x 12 cm and two arms 35 x 12cm each, at the end of which were the goal areas 15 x 12 cm each, containing the food pellets. The sidewalls were 40 cm in height. A sliding door separated the stem, from the start box. The T-maze was kept in a sound attenuated room.

***i) Spontaneous alternation test:***

Rats were starved for two days prior to the test in order to motivate them for food reward. Subsequently food was restricted so that the body weight was maintained at 85% of pre-test weight. Rats were placed in the T-maze for 30 minutes daily, for 2 days, to orient them to the T-maze environment. During these sessions 15 pellets of food (10 mg each) were kept in each goal area.

On the following 4 days, six trials were given daily. In each trial, the rat was placed in the start box and the door opened, thus allowing it to enter into the stem and arms of the T-maze. After the rat ate the pellet in the goal area, it was replaced back in the start box. In each trial, the arm chosen by the rat and number of alternations made, were noted. The inter-trial interval was one minute. The rat was deemed to have entered that arm with all its four limbs. Percentage bias was calculated for each rat using the following formula:

$$\text{Percentage bias} = \frac{\text{Total number of choice of more frequently chosen side}}{\text{Total number of trials}} \times 100$$

More number of alternations and less % bias was considered as an index of improved learning ability.

***ii) Rewarded alternation test***

This test was done after completion of spontaneous alternation test. Test consisted of 10 trials/day, for four consecutive days. Each trial had two runs, forced run and choice run. In the forced run, the rat was forced to one of the arms by blocking the other arm and allowing it to consume the pellet. In the choice run, the forced arm was kept empty and pellet was placed in the opposite arm. Both the arms were free for the rat to run. In forced run, the rat had to enter into the arm, opposite, to the forced arm, if it had to be considered as *"correct response"*. The forced arm was predetermined and it was same for all rats on any given day. It was changed on subsequent days. Experiment was repeated for 4 successive days. *"Percentage of correct responses"* was calculated for each rat by using the following formula [9]

$$\text{\% Correct response} = \frac{\text{Total number of choices of correct response}}{\text{Total number of trials}} \times 100$$

Increase in mean percentage correct response was considered as improved learning and memory.

***AchE enzyme estimation[7]***

After early and delayed tests, rats were decapitated and the whole brain was taken out quickly. The cerebral cortex, cerebellum, medulla oblongata and midbrain were dissected as described by Glowinsky and Iverson suspended in phosphate buffer and weighed accurately. Acetylcholinesterase content was estimated following the method suggested by Ellmann *et al*.

***Statistical analysis***

Statistical analysis was carried out by one-way ANOVA followed by Dunnet's 't' test for each group.

## Results

Sandalwood oil is reported to be safe upto 5g/kg. [8] Since the rat pups were 7 days old and upto 30 days dose administration has to be done 125mg/kg (SW1), and 250mg/kg (SW2) were selected for two test groups.

***Open field behaviour test***

In early test there was significant decrease in number of boli excreta ($p<0.05$), rearing ($p<0.01$) and grooming ($p<0.05$) in both test groups compared to control group but in delayed test these parameters showed no significance. Saline control group showed no boli excreta, significant decrease in rearing ($p<0.05$) and grooming ($p<0.05$) in early test but in delayed test boli excreta, rearing and grooming were not significant in value.

There was a significant decrease ($p<0.01$) in number of squares entered and also time spent in central area ($p<0.01$) compared to control in both early and delayed tests. Test group showed significant decrease in number of central squares entered and time spent in central area ($p<0.01$) compared to control, but in delayed test the time spent in central area increased but was found to be not significant. In early test the saline control group showed significant increase in number of peripheral squares entered ($p<0.05$) and also time spent in peripheral area ($p<0.01$) compared to control group. But in delayed test though the group showed increase in number of squares entered and time spent but found to be not significant.

Both test groups showed significant increase in time spent in peripheral area ($p<0.01$), but there was significant decrease in number of peripheral squares entered in both early ($p<0.05$) and delayed test ($p<0.01$). (Table I and II)

***Passive avoidance***

In early test, both test groups showed significant increase in step down latency compared to control ($p<0.01$). In delayed test the higher dose (SW2) group showed significant increase in transfer latency ($p<0.01$) compared to lower dose group (SW1) ($p<0.05$). (Table III )

***Elevated Pus Maze***

Higher dose group (SW2) showed significant ($p<0.01$) decrease in transfer latency compared to control in both early test and delayed test, the lower dose (SW1) group showed significant decrease ($p<0.05$) in transfer latency to enter dark chambers.(Table IV)

***T-maze***

***Spontaneous alternation test***

In early test both test groups showed significant increase in number of alternations ($p<0.01$), and decrease in percentage bias. In delayed test also, both the groups showed increase in number of alternations and decreased percentage bias ($p<0.05$). .(TableV and VI)

***Rewarded alternation test***

In early test the higher dose (SW2) group showed significant increase in percentage correct response ($p<0.01$) compared to control. But SW1 group showed less significant increase in % correct response ($p<0.05$) compared to control, but in delayed test the percentage was reduced and was significant in both groups ($p<0.05$). (Table VII and VIII)

***Anticholinesterase activity***

The *in-vitro* analysis for anticholinesterase activity showed that there was no effect or change in enzyme activity in medulla portion. But there was significant decrease in enzyme activity ($p<0.01$) in cortex, mid brain and cerebellum in both test groups as shown in (Table IX)

**Table. I Effect of sandalwood oil treatment on various parameters in open field test (Early test)**

| Groups | **No. of central squares entered** | **Time spent in central area (in seconds)** | **No. of peripheral squares entered** | **Time spent in peripheral area (in seconds)** | **No. of rearing** | **No. of grooming** | **Boli excreta** |
|---|---|---|---|---|---|---|---|
| Control | 24.00±3.44 | 30.05±3.54 | 67.00±3.80 | 270.0±3.54 | 32.25±4.27 | 5.75±3.54 | 3.50±1.19 |
| Vehicle control | 7.50±2.36** | 12.32±1.87** | 75.75±11.88* | 287.7±1.87** | 18.25±4.32* | 4.25±0.85* | 0.00±0.00 |
| SW1 | 3.00±2.12** | 03.35±1.98** | 33.75±13.34* | 296.6±2.00** | 5.25±1.49** | 3.00±1.08* | 1.25±1.12* |
| SW2 | 4.00±0.40** | 03.95±1.33** | 46.50±2.10* | 296.1±1.33** | 4.50±1.19** | 3.00±1.00* | 3.75±1.10* |

**Table. II Effect of sandalwood oil treatment on various parameters in open field test (Delayed test)**

| Groups | **No. of central squares entered** | **Time spent in central area (In seconds)** | **No. of peripheral squares entered** | **Time spent in peripheral area (In seconds)** | **No of rearing** | **No of grooming** | **No. of boli excreta** |
|---|---|---|---|---|---|---|---|
| Control | 23.25± 2.81 | 29.72± 3.22 | 63.25± 3.96 | 270.8± 3.44 | 38.50± 4.66 | 3.50± 1.44 | 4.00± 0.70 |
| Vehicle control | 12.75± 1.10** | 25.32± 1.45 | 70.50± 4.66 | 274.7± 1.45 | 34.00± 4.56 | 7.25± 1.37 | 0.75± 0.47* |
| SW1 | 5.50± 1.32** | 7.73± 1.78** | 36.00± 3.48** | 292.3± 1.78** | 5.50± 1.44** | 3.75± 1.03 | 1.75± 0.85 |
| SW2 | 4.25± 0.85** | 3.11± 1.05** | 24.00± 4.26** | 296.9± 1.05** | 4.25± 1.10** | 3.50± 1.19 | 3.25± 1.10 |

**Table.III Effect of sandalwood oil treatment on step down latency in passive avoidance task during early and delayed test.**

| *Groups* | **Step Down Latency** | |
|---|---|---|
| | **Early test** | **Delayed test** |
| *Control* | 18.25±4.97 | 22.50 ±1.93 |
| Vehicle control | 16.25±3.37 | 28.50 ±3.30 |
| SW1 | 49.50±2.59** | 42.00 ±5.61* |
| SW2 | 53.25±2.56** | 51.75 ±5.89** |

Table. IV Effect of sandalwood oil treatment on transfer latency on an elevated plus maze during early and delayed test

| *Groups* | **Transfer latency** | |
|---|---|---|
| | **Early test** | **Delayed test** |
| *Control* | 51.88±1.45 | 43.00±2.64 |
| Vehicle control | 46.38±8.43 | 41.00±2.64 |
| SW1 | 26.25±2.11* | 21.00±4.80* |
| SW2 | 20.27±5.55** | 19.25±2.13** |

**Table .V Effect of sandal wood oil treatment on spontaneous alternation in T-Maze spatial orientation (early studies)**

| *Groups* | **Number of alterations** | **Number of entries** | **Percentage Bias** |
|---|---|---|---|
| *Control* | 3.00±1.08 | 6.00±0.0 | 100.00 |
| Vehicle control | 2.75±0.47 | 5.75±0.25 | 95.83 |
| SW1 | 6.75±0.47** | 2.50±0.28** | 41.66 |
| SW2 | 7.25±0.47** | 2.25±0.25** | 37.50 |

**Table .VI Effect of sandal wood oil treatment on spontaneous alternation in T-Maze spatial orientation (Delayed studies)**

| ***Groups*** | **Number of alternations** | **Number of entries** | **Percentage Bias** |
|---|---|---|---|
| *Control* | 4.00±0.40 | 5.00± 0.40 | 83.33 |
| Vehicle control | 3.25± 0.47 | 4.75± 0.47 | 79.16 |
| SW1 | 7.25±0.85* | 2.75±0.47** | 45.83 |
| SW2 | 7.00±0.91* | 2.50±0.28** | 41.66 |

**Table.VII Effect of sandal wood oil treatment on rewarded alternation on T-maze during early test. And Delayed test**

| ***Groups*** | **Number of entries** | **Percentage of correct response** |
|---|---|---|
| *Control* | 4.00±0.40 | 40.00 |
| Vehicle control | 3.50±0.64 | 35.00 |
| SW1 | 6.50±0.28* | 65.00 |
| SW2 | 6.75±0.62** | 67.50 |

**TABLE.VIII EFFECT OF SANDAL WOOD OIL TREATMENT ON REWARDED ALTERNATION ON T-MAZE DURING DELAYED STUDIES**

| ***Groups*** | **Number of entries** | **Percentage of Correct Response** |
|---|---|---|
| *Control* | 4.50±0.64 | 45.00 |
| Vehicle control | 3.75±0.85 | 37.50 |
| SW1 | 7.00±0.40* | 70.00 |
| SW2 | 7.50±0.64* | 75.00 |

**Table. IX Effect of sandal wood oil treatment on concentration of acetylcholine esterase values of different parts of brain at the end of delayed studies**

| **Groups** | **Cortex** | **Medulla** | **Mid brain** | **Cerebellum** |
|---|---|---|---|---|
| Control | 6.08± 0.06 | 4.23±0.10 | 4.06±0.04 | 3.22±0.03 |
| Vehicle control | 6.01± 0.05 | 4.25±0.05 | 4.19±0.06 | 3.51±0.12 |
| SW1 | 5.09±0.05** | 4.43±0.08 | 3.83±0.05* | 2.64±0.05** |
| SW2 | 4.91±0.03** | 3.97±0.07 | 3.73±0.04** | 2.59±0.05** |

Values are Mean ±SEM obtained after applying One-Way ANOVA followed by Dunnett's't' test

*p<0.05 **p<0.01 when compared to vehicle treated group

## Discussion

The observations revealed that sandalwood oil has shown significant improvement in learning and enhancement of memory in rats in all the models tested except open field model. The open field test results suggested that sandalwood oil has no effect on motor and autonomic nervous system of rats similar to Clitoria *ternatea* [4].

The memory enhancing property was marked in neonatal rats that were in their brain growth spurt period treated with sandalwood oil. Thus it suggests that treatment of sandalwood oil must have produced certain permanent changes in the brain, which were responsible for improved learning and memory. [10, 11]

Results from passive avoidance and spatial learning tests showed improved retention, increased percentage of correct responses and increased number of total alternations and decreased percentage bias in sandalwood oil treated rats. This explains that sandalwood oil may affect the brain structures mainly striatum and amygdale responsible for non-declarative memory. However decreased transfer latency in elevated plus maze reflects that sandalwood oil also has an impact on declarative memory controlled by hippocampus region.

The exposure to new learning experience leads to intracranial self-stimulation and environment within the pyramid model may show alteration in the cyto-architecture of brain regions concerned with learning and memory. Fresh leaf extract of *Centella asiatica* has been shown to improve learning and memory correlated with an increase in dendritic arborization of amygdale and hippocampus whereas cingulate cortex was shown to be affected when the rats were chronically undernourished. The data obtained shows that sandalwood oil may also have improved learning and memory with possible increase in dendritic arborization of amygdale and hippocampus [12,13].

In the present study we also observed that except in medulla there was significant decrease in AchE levels in both SW1 and SW2 groups i.e., in midbrain, cerebellum and cortex where the memory centres are located. This shows more availability of acetylcholine in the brain. In 1983 Hass proposed that the cholinergic input to the hippocampus modulates the hippocampal neuronal excitability [9] and this reveals that the results shown by sandalwood oil has an effect on brain cholinergic system. *Clitoria ternatea* has been reports to affect the brain cholinergic system and enhances memory. [14] Drugs carbamazepine, certain cholinergic and anticholinergics also enhance memory by decreasing AchE activity [9,15].

The connection between memory, acetylcholine and AchE is controversial. Madepalli *et. al.*, reported acetylcholine and AchE have an inverse relationship on memory whereas Agnolli *et. al.*, reports, acetylcholine content and AchE activity were decreased in senile dementia. But many reports suggest that loss of memory is associated with decreased cholinergic activity. Several reports on memory loss or diseases like Alzheimer's are characterized by decreased presynaptic cholinergic markers such as choline acetyltransferase, and degeneration of cholinergic neurons in the nucleus basilis of meynert. The results obtained in present study reflect that sandalwood oil has impact on brain cholinergic system.

In present study it is observed that there is decrease in AchE levels in the parts of brain, which may reveal the factor that the sandalwood oil may have its effect on biosynthesis of neurotransmitters, like acetylcholine, cholinesterase and others. The results show that even after the 30 days gap the memory retention, alternation, percentage bias and percentage correct response, were significant in both doses. This suggests that sandalwood oil treatment during growth spurt period must have been the reason for permanent changes in the brain.[16]

The precise mechanism of action by which sandalwood oil improves learning and enhances memory is unknown. But the mechanism of action can be attributed to a combination of cholinergic modulations by acetylcholine release and muscarinic binding. Enhancement of protein kinase activity in hippocampus, antioxidant and antistress effect may also be the possible mechanism of action.[17]

We conclude that sandalwood oil treatment during growth spurt period improved learning and memory as observed in animal models, further substantiated by decrease in AchE levels in different parts of the brain which is an indication of cholinergic involvement. Results suggest the need to identify and characterise the constituent(s) responsible for nootropic activity.

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