

Neuro-Histo-Morphological Changes Seen Associated with Learning & Memory in Hippocampal Neurons of Rats Administrated with *Catha edulis*

Manoj Kollukkad Mani¹

(Department of Anatomy, Faculty of Medicine & Health Science, Hawassa University, Hawassa, Ethiopia)
Corresponding Author: Manoj Kollukkad Mani

Abstract: The tender *Catha edulis* leaves (*Khat*) have stimulatory properties due to the presence of alkaloid called cathinone, which is known to have a stimulating effect at lower dosages and can act as depressant if consumed at higher quantities. This study was aimed at assessing the effect of *khat* leaf extract on learning and memory of the rats. The study was conducted in 40 adult male Wistar rats. The rats were randomly assigned to eight groups, four groups each for RA Maze and T MAZE trails respectively. The calculated dosages were 100mg/kg Bwt (T1), 200 mg/kg Bwt (T2), 300mg/kg Bwt (T3) and control group were provided with normal saline. At the end of the experiment the rats were sacrificed and the hippocampus CA3 region of brain was identified and thereafter processed for rapid Golgi staining. The data obtained were analyzed using descriptive statistics and the means compared using Duncan's Multiple Range Test ($P < 0.05$). The results show that the rats in both the experimental groups showed better ($P < 0.05$) learning abilities at T2 diet, where as those reared on T3 diet showed learning disorders with however no differences were recorded among those in T1 and control diets. The results of the dendritic branching counts supplement the above findings with lowest counts recorded among the rats receiving T3 dosages and highest among those reared on T2 dosages. In conclusion, at higher dosages, *Khat* extract leads to deterioration of learning and memory while at medium dosages it has a stimulating effect on the learning and memory.

Keywords: Wistar rats, *Khat* extract, learning, memory, Hippocampus

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I. Introduction

The present study was aimed at estimating the morphological and histological changes seen in neurons of hippocampus in rats which are fed with *Khat*. Apart from neuronal count estimation, other morphological changes like increase or decrease in dendritic arborization are aimed to be estimated.

Golgi' staining was famously used by Spanish neuroanatomist Santiago Ramón y Cajal (1852–1934) and this method was employed in this study also for analyzing the neuro morphological changes¹. The Golgi stained neurons were traced using camera lucida or Simple Neurite Tracer², and the traced image was subjected to Sholl analysis³. Sholl analysis creates a series of concentric circles around the soma of the neuron. Within each sphere various metrics was obtained such as the number of spines that intersect it, or the total length of intersecting neurites etc.

Previous studies on sub chronic oral administration of *khat* displayed enhanced locomotor activity but its effect on memory is not studied⁴. From this study it is expected to understand the morphological and histological details of learning and memory changes seen associated with *Khat* use. In this research, the researchers analyzed the effect of *khat* on brain especially the hippocampus, which is associated with learning & memory process. The *khat* contains cathinone & many other alkaloids, most of them having amphetamine like substances^{5,6}. The extracts from *khat* may be useful for developing pharmaceutical drugs for several neurological disorders (such as Attention Deficit Hyperactivity Disorder) related to hippocampus & brain as a whole. It can also be used as an antidepressant.

II. Materials and Methods

The current study was conducted in Hawassa University College of medicine, Hawassa, Ethiopia.

Experimental animals: The present research experiments were carried out in 40 adult male Wistar albino rats weighing in the range of 180 ± 30 g, which was 60 days of age when considered for the experiments. The rats were maintained at the animal research facility (Rodent house) at the college of medicine & health sciences, Hawassa University, Hawassa, Ethiopia & were acclimatized for two weeks at room temperature. Rats were housed in polypropylene cages (30 x 22 x 14 cm). Three to four rats were housed in each cage. Standard chip

wood was used as bedding material, which was changed on alternate days. The colony was maintained in a well aerated room with at 12 hrs light-dark cycle. The room temperature was maintained at 21 ± 4 °C. Animals were provided with pelletized feed and tap water ad libitum until they aged 60 days at the beginning of the experiment. All animals were handled according to internationally accepted guidelines.

Plant material: *Catha edulis* leaves (2000 g) were purchased fresh at a local market in Wondognet, 40 km away from Hawassa, Ethiopia. The fresh bundles were packed in plastic bag and transported in an icebox to the laboratory. The plant was identified by a taxonomist.

Khat extraction: The fresh Khat leaves were separated from the shoots. The fresh leaves were washed thoroughly with distilled water, blotted neatly, then chopped on glass plates and finally crushed. Crushed material was immersed in conical flask containing sufficient amount of methanol and kept on rotary shaker for 18 hours. Filtration of the previous mixture was carried in two steps; firstly using the gauze roll to separate the larger particles and secondly with Whatman No. 1 filter paper. The non-filtered plant material was re-extracted in fresh methanol⁵. The filtrate was admixed with the initial filtrate. The resultant filtrate was collected in pre-weighed conical flasks and exposed at 60-65 °C to evaporate the methanol completely⁷, thus leaving semi-solid material which was dried and collected to be kept as a powder in the refrigerator. The fresh solution of Khat extract was prepared by dissolving the powder of Khat extract in distilled water, just prior to its oral administration to the rats daily throughout the experiment for 30 days.

Grouping of experimental animals & dosing: All animals were randomly divided in to 8 groups consisting of 5 rats each. Four groups for T maze behavioral tasks & four groups for Radial arm maze behavioral tasks. Within T maze behavioral task groups, Group 1 consisted of Healthy controls normal rats for acquisition and retention test for T maze fed on normal saline. Group 2 consisted of Test group for acquisition and retention test for T maze in which the animals received khat in low dose @ 100mg/kg weight for all the days of experiment. Group 3 consisted of Test group for acquisition and retention test for T maze in which the animals received khat in medium dose @ 200mg/kg weight for all the days of experiment and finally group 4 consisted of Test group for acquisition and retention test for T maze in which in which the animals received khat in high dose @ 300mg/kg weight for all the days of experiment.

Within Radial arm maze behavioral task groups, Group 1 consisted of Healthy controls normal rats for acquisition and retention test for Radial arm maze fed on normal saline. Group 2 consisted of Test group for acquisition and retention test for Radial arm maze in which the animals received khat in low dose @ 100mg/kg weight for all the days of experiment. Group 3 consisted of Test group for acquisition and retention test for Radial arm maze in which the animals received khat in medium dose @ 200mg/kg weight for all the days of experiment and finally group 4 consisted of Test group for acquisition and retention test for Radial arm maze in which in which the animals received khat in high dose @ 300mg/kg weight for all the days of experiment..

Design of behavioral experiments

Apparatus

T-maze: The T-maze was made of wood with smooth polished surface. It consists of a stem (35×12 cm), a choice area (12×12 cm) and two arms (35×12 cm); the end of each arm contain a food well. The sidewalls were of 40 cm high. The choice area was separated from the arms by a sliding door.

Radial arm maze (RAM): Radial arm maze was made of Plexiglas, consisting of eight equally spaced arms radiating from an octagonal central platform. Each arm had a length of 56.2 cm, width of 7.9 cm and height of 10 cm (Fig 1). The entire maze was elevated 80 cm above the floor for easy locating of spatial cues by rats.

Figure 1: Rat within a Radial Arm Maze



Behavioral tasks: All the behavioral experiments were carried out in three phases viz; orientation and training session, learning performance test (acquisition test) and memory performance test (retention test). The rats were semi starved for 48 hrs before the start of behavioural experiments. The body weight was maintained at 85% of the original body weight, through out one session of behavioural experiment. Behavioral experiments were conducted in the same room, with the same allocentric cues, such as doors, windows, posters and the experimenter. Experimenter always maintained same position throughout the whole of the experiment. The following behavioral tasks were included.

T-maze (TM) task: This is analogous to non-matching to sample task, where the rat was rewarded only if the current choice doesn't match the previous one. As reward was used, it can also be considered as a learned alternation procedure.

In the orientation phase, the starved rats were allowed to spend 10 minutes / day for three days in the T-maze and were trained to collect food pellet from the food wells.

During the acquisition test, all the rats were given six trials / day with an inter trial interval of one hour. Each trial consisted of four sample and choice run. In the sample run, the rat was placed at the start end of the T-maze stem. Was allowed to move towards one arm and collect the food pellet, while keeping the sliding door of other arm closed. In the choice run, the rats were placed at the start end of stem and both arms were kept open. If the rat visits the same arm as that of sample run, it was recorded as error and the rat was not rewarded with food.

Instead, if the rat visits the alternate arm, it was recorded as correct score and the rat was allowed to eat food pellet (reward) in the food well. There was an interval of 30s between each run.

Score was given for alternate selection of arm during choice run and a maximum score of '4' can be obtained per trial. The acquisition test was continued until the rats attain the learning criteria of obtaining '4' correct score without any error for three consecutive trials.

Ten days after the last day of acquisition of the task, the rats were subjected to retention test. The test was conducted similar to acquisition test and was continued until the rats attain the learning criteria. A memory score was also calculated by taking the difference between number of trials required for acquisition test and number of trials for retention test.

Radial arm maze (RAM) task: Orientation phase was for three days, where the starved rats were allowed to familiarize themselves with the radial maze for 10 minutes / day. Prior to each acquisition trial, all the eight arms were baited with food pellets. The rat was placed in the center of the maze and allowed to freely explore the maze. The rats were required to take the food pellet from each arm without making a reentry into the arm already visited. The trial was terminated when the animal takes the food reward from all the eight arms or after 10 minutes if all the eight arms were not visited. A correct score was given when the rat visits an arm and collect the food reward, and a maximum score of '8' can be attained per trial. When a rat reenters an already visited arm or doesn't enter an arm, it was taken as error. The acquisition test was continued until the rats attained learning criteria of obtaining a correct score ≥ 7 , and an error ≤ 1 , for three consecutive trials. Six trials / day was given with an inter trial interval of one hour.

Ten days after the last day of acquisition of the task, the rats were subjected to retention test. It was continued until the learning criteria were attained.

Neuromorphological Analysis of Pyramidal Neurons for Dendritic Quantification: After the completion of the behavioral experiments, all the rats were sacrificed, and processed for the neuromorphological analysis of the pyramidal neurons of CA1-CA3 regions of dorsal hippocampus.

Details of the procedures are mentioned below:

Perfusion method: The animal was weighed and the amount of fixative required was 2-3 times the weight of the animal. The animal was deeply anaesthetized with chloroform. The thorax was cut open and heart was exposed; a needle connected to the tubing from the fixative bottle, was inserted into left ventricle. The right atrium was cut open to drain out the blood and fixative. First, 20-30 ml of saline was passed trans-cardially to flush out the blood & then perfused with formalin. After perfusion the animal was decapitated, the brain was shelled out and kept in 10% formalin for minimum 1-2 days for primary fixation.

Processing of the tissue: After 1-2 days, the brain tissue was processed by using Rapid Golgi staining method adopted by Hawassa University College of medicine histology laboratory. Slides were coded prior to the quantitative analysis and the code was broken only after the analysis is complete.

Camera Lucida Tracing: Camera Lucida is a very useful accessory for making accurate drawings of microscopic materials under observations. In the present study, mirror type Camera Lucida was used for tracing the pyramidal neurons in CA1-CA3 regions of dorsal hippocampus for dendritic quantifications of branching points and intersections.

The soma, apical dendrites, basal dendrites and axons were carefully drawn with the help of Camera Lucida and then the minute details were filled in free hand. Identification of hippocampal sub regions was based on “Rat brain in stereotaxic co-ordinates” atlas by Paxinos and Watson⁸.

10 pyramidal neurons were randomly selected from CA1-CA3 pyramidal cell layer of dorsal hippocampus from each group of animals in such a way that minimum one pyramidal neuron is selected from each rat accounting for 40 neurons in total for dendritic quantification. Their Camera Lucida tracings were made at 45x magnification, using a monocular microscope. Neurons with minimal overlap of dendrites, heavily impregnated with silver nitrate and Neurons without truncate dendrites were considered for the study

Concentric circle method of Sholl, was used for dendritic quantification. Concentric circles drawn on transparent sheet at 20 microns intervals, with the aid of the stage micrometer is then used for dendritic quantification. The sheet was then placed on a pyramidal neuron tracing so that center of the cell body of the neuron coincides with the center of concentric circle. The number of branching points between the two concentric circles i.e. within each 20 micron concentric zone was counted. The points at which the dendrites cross the concentric circles were taken as points of intersection. Both the branching points and intersections were counted up to a radial distance of 100 microns. The dendritic branching points represent the dendritic arborization and dendritic intersections represent the length of dendrites. Dendritic branching points and dendritic intersections give the gross morphology of the pyramidal neuron. The mean dendritic branching points at each concentric zone and mean dendritic intersections at each concentric circle was calculated from each group and these values was used for statistical analysis using SPSS.

Statistical analysis:

Statistical evaluation of the data was done using SPSS v20 for windows software package. Descriptive statistics was used for estimating the means, the means were compared across the treatments using Duncan’s post hoc multiple range test. The values were considered significant at P<0.05.

III. Results

At the end of the treatment period, all animals in each treatment group appeared to be healthy. The behavioral study using T-maze & Radial arm maze was mainly oriented to find out the effect of khat on hippocampal pyramidal neurons & learning and memory; by conducting rewarded alternation test in a T-maze & Radial arm maze. The criteria of assessment of T-maze & Radial arm maze behavioral task for acquisition and retention has already been mentioned in material and method section.

In acquisition trials of T maze, low dose khat administered group took less number of trials compared to control group. Medium dose khat administered group took less number of trials compared to control and low dose administered group. High dose khat administered group took more number of trials compared to other groups (Table 1).

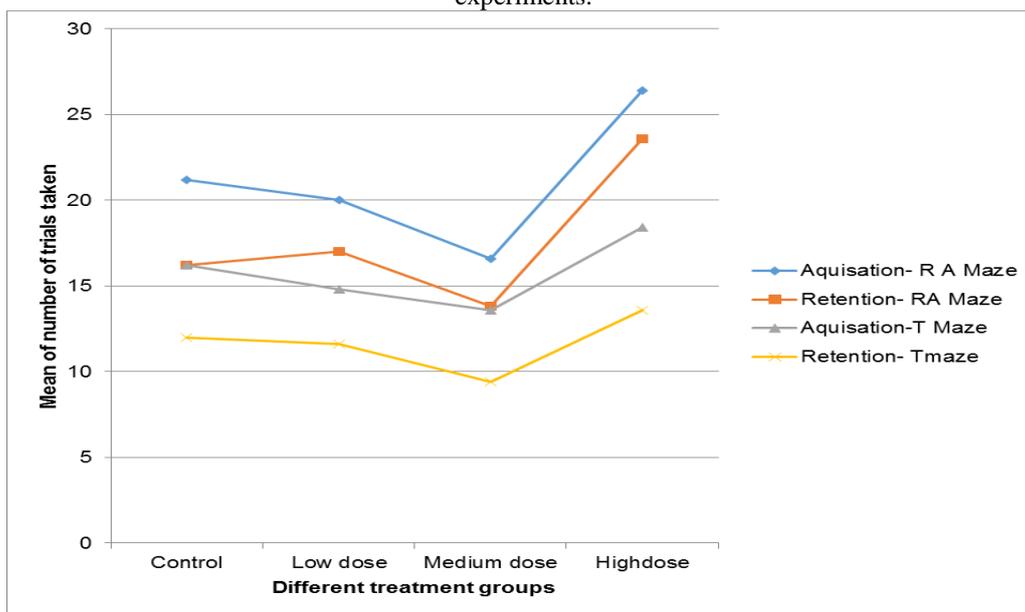
Table no 1: Average values (Mean ± SE) for acquisition and retention (T-maze and RAM) experiments of rats receiving different dosages of Khat leaf extract

Treatment	Control	Low Dose(T1)*	Medium dose(T2)**	High dose(T3)***
T-MAZE				
Acquisition	16.2±.58 ^b	14.8±.73 ^{ab}	13.6±0.40 ^a	18.4±.50 ^c
Retention	12±.44 ^b	11.6±.24 ^b	9.4±.50 ^a	13.6±.50 ^c
RAM				
Acquisition	21.2±.58 ^b	20.0±.44 ^b	16.6±.50 ^a	26.4±.50 ^c
Retention	16.2±.37 ^b	17.0±.44 ^b	13.8±.37 ^a	23.6±.40 ^c

^{a,b,c} P<0.05, values across rows are different

* 100 mg/kg weight, ** 200 mg/kg weight, *** 300 mg/kg weight

Figure 2: Mean number of trials taken by different groups for acquisition & retention in T maze & RAM experiments.



In retention trials of T maze, low dose khat administered group took less number of trials compared to control group. Medium dose khat administered group took less number of trials compared to control and low dose administered group. High dose khat administered group took more number of trials compared to other groups (Figure 2).

In acquisition trials of RAM, low dose khat administered group took less number of trials compared to control group. Medium dose khat administered group took less number of trials compared to control and low dose administered group. High dose khat administered group took more number of trials compared to other groups (Figure 2).

In retention trials of RAM, low dose khat administered group took less number of trials compared to control group. Medium dose khat administered group took less number of trials compared to control and low dose administered group. High dose khat administered group took more number of trials compared to other groups (Table 1).

Table No 2: Results (Mean ± SE) of dendritic quantification on T-MAZE and RA Maze experiments of rats receiving different dosages of Chat leaf extract

Treatment	Control (C)	Low Dose (T1) *	Medium dose (T2)**	High dose (T3)***
T MAZE				
0-20 microns	.80±.24 ^{ab}	.90±.23 ^{ab}	1.2±.20 ^b	.40±.16 ^a
20-40 microns	.60±.16 ^{ab}	.50±.16 ^{ab}	1.0±.21 ^b	.30±.15 ^a
40-60 microns	.50±.16 ^{ab}	.50±.22 ^{ab}	1.0±.14 ^b	.20±.13 ^a
60-80 microns	.40±.16 ^{ab}	.40±.16 ^{ab}	.80±.13 ^b	.10±.10 ^a
80-100 microns	.40±.16 ^{ab}	.40±.16 ^{ab}	.80±.13 ^b	.00±.00 ^a
RA-MAZE				
0-20 microns	1.00±.14 ^b	1.10±.10 ^{bc}	1.50±.16 ^c	.40±.16 ^a
20-40 microns	.70±.15 ^{ab}	.70±.15 ^{ab}	1.1±.17 ^b	.30±.15 ^a
40-60 microns	.60±.16 ^{ab}	.70±.21 ^{ab}	1.00±.14 ^b	.20±.13 ^a
60-80 microns	.50±.16 ^b	.50±.16 ^b	1.00±.00 ^c	.00±.00 ^a
80-100 microns	.40±.16 ^{ab}	.40±.16 ^{ab}	.60±.13 ^b	.30±.12 ^a

N= 10^{a,b,c} P<0.05, values across rows are different for a given dose,

* 100 mg/kg weight, ** 200 mg/kg weight, *** 300 mg/kg weight

Statistical comparison of mean dendritic branching points at each concentric zone between each group was analyzed by one-way analysis of variance (ANOVA) and followed by Duncan’s post hoc test. Significance was accepted at P< 0.05.

The results as presented in Table (2) indicate that in the T MAZE behavioral experiment and at every concentric zone the number of branching points of hippocampal pyramidal neurons of the rats administered with khat extract at the rate of 300 mg/kg body weight (T3) was the lowest. While the higher values were recorded (irrespective of the concentric circles) for rats receiving khat extract at the rate of 200 mg/kg body weight (T2). The values for the rats administered with khat at the rate of 100 mg/kg body weight (T1) were

similar to those receiving the control diet (C) (Figure 3). The reports of Kimani and Nyongesa indicated that the rats reared at higher concentrations had better memory formation which differs from the present findings and may be associated with the type of narcotic compounds present in the khat.

Figure 3: Mean dendritic branching points of different groups across concentric zone in T maze experiments.

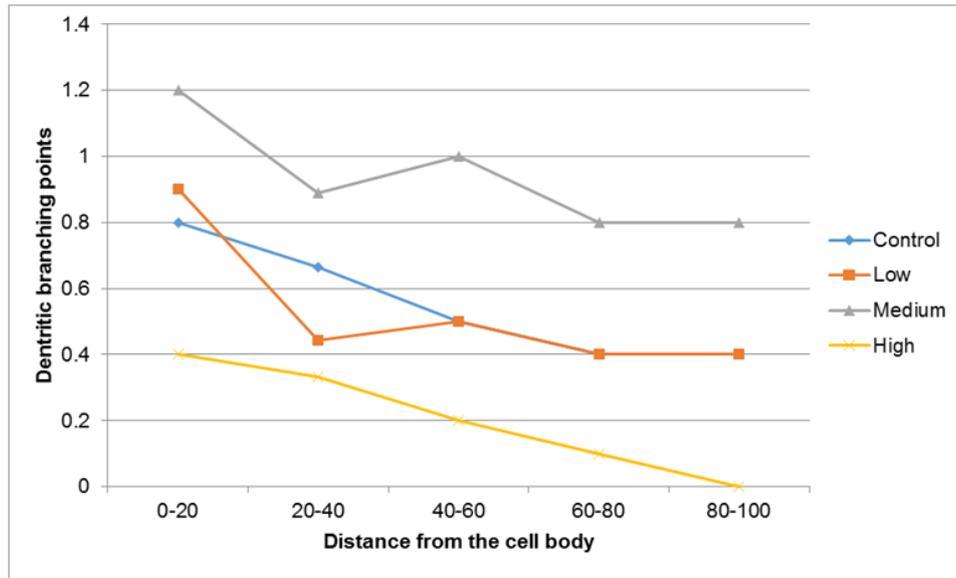


Figure 4: Mean dendritic branching points of different groups across concentric zone in RAM experiments.

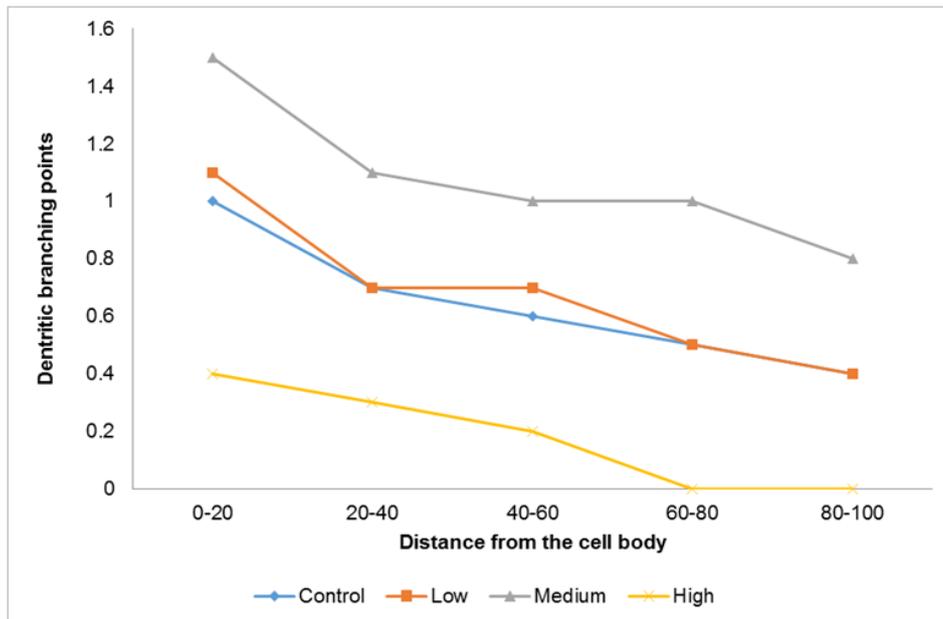
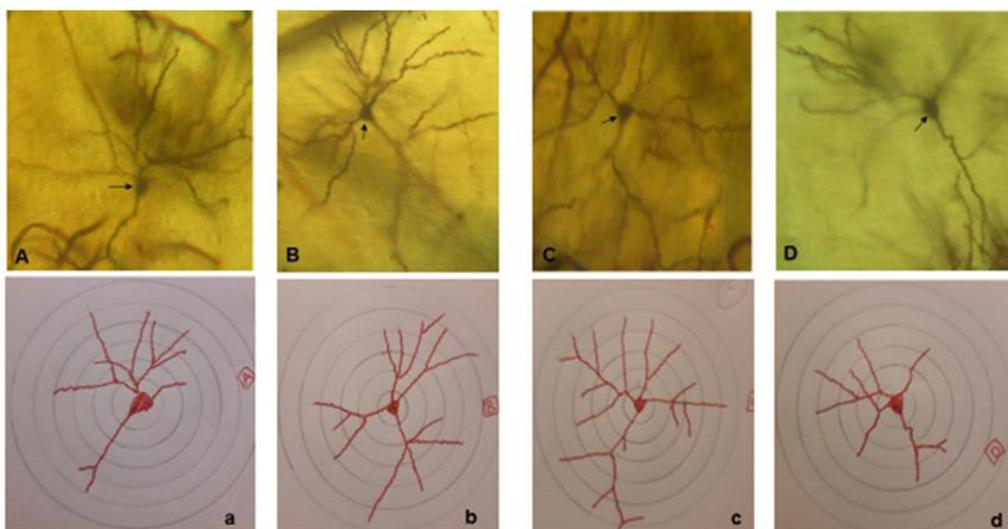


Figure 5: Microphotographs (A, B, C & D) and their camera Lucida tracings (a, b, c & d) of Pyramidal neurons used for Neuro-morphological analysis in the present study; **A (a).** Control group **B (b).** Low dose (100 mg/kg) group **C(c).** Medium dose (200mg/kg) group **D (d).** High dose (300mg/kg) group, Arrows indicate the neuronal cell body



The results as presented in Table (2) also indicate in the in the RAM behavioral experiment and at every concentric zone the number of branching points of hippocampal pyramidal neurons of the rats administrated with khat extract at the rate of 300 mg/kg body weight (T3) was the lowest. While the higher values were recorded (irrespective of the concentric circles) for rats receiving khat extract at the rate of 200 mg/kg body weight (T2). The values for the rats administered with khat extract at the rate of 100 mg/kg body weight (T1) were similar to those receiving the control diet (C) (Figure 4 & 5).

IV. Discussion

Hippocampus is one of the important areas of the brain concerned with learning, memory and emotional behavior of the individual⁹. Memory and learning are closely correlated concepts. Many classifications of memory can be seen in literatures. One of the types of memory which has representations in working, long term and short term memory is the spatial memory. Spatial learning and memory is essential for navigation in a space for an individual. Khat extract is said to have selective effects on both learning and memory¹⁰.

Hippocampal pyramidal cells play a significant role in the formation of spatial learning and memory during movement of an individual through the space. Pyramidal neurons in hippocampal dorsal sub regions CA1-CA3 shows place specificity. Pyramidal neurons in these sub regions show a selective increase in activity when the rat is located in a specific region of space. The synaptic activity of pyramidal neurons of CA1-CA3 causes development of the phenomenon of Long Term Potentiation (LTP), when rat is moved through a space repeatedly or engaged in a behavioral task where there is an involvement of working memory. LTP which occurs in pyramidal neurons in a hippocampus is considered to be the physiological basis of spatial learning and memory. This spatial learning and memory helps the animal, or an individual to fulfill the given behavioral tasks or move through environment.

Cathinone, the main component of Khat similar to amphetamine, acts by discharging catecholamines from the presynaptic axon terminals and inhibit their uptake, thus increasing spatial and temporal presence of neurotransmitters like dopamine, serotonin and noradrenaline at the presynaptic receptors sites^{11,12,13}. Neuro-stimulants and related drugs act by inhibiting uptake of a neurotransmitter named serotonin into the presynaptic nerve ends and increase serotonergic neurotransmission by increasing its synaptic concentrations.

According to Toennes et al, the major constituents of khat produces stimulatory effect like amphetamine^{14,15,16}. Amphetamine in turn has more or less same chemical composition as that of methylphenidate which is used to treat attention deficit/hyperactivity syndrome^{17, 18, 19}. Many experimental as well as clinical studies have confirmed the cognitive enhancing property of khat, but only few studies have been done on the effect of khat in learning and memory. In one study, spatial acquisition and reference memory were assessed using modified Morris Water maze & showed shorter escape latency in 120mg/kg khat dose treated rats²⁰. However the impact of extract of khat by evaluating the strength of spatial learning and memory analyzing the neuro morphological changes of pyramidal neurons of dorsal hippocampal CA1- CA3 sub regions have been not yet done.

The neuro morphological changes of pyramidal neurons can be induced by the LTP formed by the synaptic activity caused during the assessment of spatial learning and memory during T-maze & RA Maze behavioral task. This study was an enquiry to find the effect of khat on this spatial learning and memory and thereby finding its influence on synaptic plasticity resulting in dendritic modifications of hippocampal

pyramidal neurons of CA1-CA3 sub regions due to LTP formation. The changes on spatial learning and memory as well as morphology of pyramidal neuronal dendrites of CA1-CA3 are assessed in this study.

In a T-maze & RAM task the animal attains the learning criteria through repeated acquisition trials. Here, each trial can be considered as an experience which leads to learning of the task. The hippocampal pyramidal neurons of CA1-CA3 regions of dorsal hippocampus concerned with the present study, shows morphological modifications as a result of prolonged experience of the animal while learning behavioral tasks in T-maze & RAM. This experience results in the formation of memory.

In the cellular level it can be well thought-out that, a repeated trial in a T-maze & RAM causes stimulation of presynaptic and postsynaptic terminal of a pyramidal neuron of CA1-CA3 region simultaneously. This stimulation causes release of glutamate from presynaptic terminal to post synaptic terminal through NMDA receptor, AMPA receptor, and metabotropic receptors. Release of glutamate causes depolarization of postsynaptic neuron. This depolarization causes release of Ca²⁺ into the postsynaptic neuron.

Ca²⁺ combined with many enzymes present within the postsynaptic neuron produce retrograde messengers which causes further release of glutamate into postsynaptic membranes of CA1-CA3 pyramidal neurons. Ca²⁺ along with dopamine and serotonin activates adenylyl cyclase resulting in production of LTP²¹. To change structure of the neuron, synthesizing of new proteins is required. In the post synaptic neurons, the process of protein synthesis occurs within the dendrites. Every necessary component required for the synthesis of protein are present within the dendrites.

The activation of adenylyl cyclase increases cAMP which results in the formation of PKA. PKA translocate into nucleus causing phosphorylation of CREB (transcription factor). This results in the transcription of CRE leading to the translation of various genes (protein synthesis) which forms growth factors such as brain-derived neurotrophic factor (BDNF) which produce modification in the structure of dendrites and also induce development of new synapses.

Ca²⁺ act to initiate protein synthesis resulting in the establishment of new synapses which is the cellular basis of formation of spatial memory in the pyramidal neurons in CA1-CA3. This process involved in the formation of synapse from a learning experience is the prerequisite factor for the formation of memory.

V. Conclusion

The results of the neuro-morphological studies show increase of dendritic branching points with medium dosages of khat extract. There is an increase in the action of khat in promoting dendritic growth. There is a significant increase in the dendritic branching points at medium dose compared to control & low dosage group. The synapses formed in the newly formed dendrites results effective and rapid conduction of impulse resulting in better performance of the rats in T2 administered group in behavioral task.

Thus, by this study it can be suggested that khat at medium dosages increases the dendritic branching points resulting in a better learning and memory performances where as high dosages deteriorates learning & memory. The point to be noted is that layman consumes larger quantities everyday than the medium dosage that we discussed here, as so it may create adverse effect to the body systems than benefiting. Further studies are required to evaluate effect of khat on specific memory like working memory, short term memory, long term memory, spatial memory etc.

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