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THE EFFECT OF ETHANOL AND KHAT (*CATHA EDULIS* FORSK) ON THE CEREBELLAR CORTEX OF EARLY POSTNATAL RATS

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Summary

Rats of post natal day 6 were treated with ethanol, khat or vehicle for 30 days using blunt needle. At the end of the experiment, animals were scarified, their brains were dissected out and immersion fixed. The brain as a whole and cerebellum, separately were weighed, and cerebellum was processed for routine histology. Samples of serially sectioned tissues of cerebellum were stained with toluidine blue and observed using light microscope. At the end of the experimental period the body weight increment was found to be significantly less in the ethanol and khat treated (ET and KT) rats than their respective controls by 18.05% and 21.75%, respectively ($P < 0.01$). Between the treated rats, body weight increment was less for the ET rats by 4.51% than the KT rats, although it was not statistically significant ($P>0.05$). Similarly, the weight of the brain as a whole and cerebellar weight separately of the treated rats were significantly less than their respective controls ($P<0.01$). These weights were also less for the ET rats than for the KT rats, although not statistically significant. The mean diameter of Purkinje neurons was found to be less in the ethanol treated and khat treated rats than their respective controls by 28.66% and 11.44%, respectively ($P<0.01$). Moreover the attempt to study the effect of combination of ethanol and khat treatment was interrupted because the rats died after two days, and such combined consumption of these substances at this age was found to be fatal. In conclusion, the study depicted that PND 6 is an extremely vulnerable period to the exposure of high concentration of ethanol and khat, which result in morphological change of cerebellar cortex and reduction in diameter of Purkinje neurons of cerebellum.

Key words: *Cerebellar cortex, Purkinje neurons, Khat, Ethanol, Postnatal day*

Introduction

Maternal ethanol consumption during pregnancy can result in fetal alcohol syndrome (FAS) of offsprings (1). Diagnostic criteria for FAS include growth retardation, facial abnormalities as well as central nervous system (CNS) disturbance (2). The cerebellum is most consistently affected by prenatal ethanol exposure and is associated with delay in motor development, problems with fine motor tasks, tremors, ataxia and gait disturbance (3).

In laboratory animals such as rats, the brain growth occurs primarily at about postnatal days (PND) 6-8 (4). Thus, ethanol administration during this period has been shown to result in behavioral anomalies and various cerebellar deficits (2).

Khat (Catha edulis Forsk, Celestrasae) is an evergreen flowering tree or shrub, first identified by the Danish botanist named Forska in 1762 in Yemen (5). It is known by a variety of names such as “chat” in Ethiopia, “Qat” in Yemen, “Mirra” in Kenya, “Jaad” in Somalia and Khat in English. It is widely cultivated in East Africa and Arabian Peninsula, both for domestic usage and for commercial purposes (6). Fresh khat leaves are chewed daily by over 20 million people in Yemen and East African countries (7).

Khat contains psychoactive components, namely, cathine, cathidine, norephedrine and cathinone (8). Cathinone, the major component of khat, stimulates CNS, increases locomotor activity and results in sympathomimetic effect (5) which are analogous to the effects of amphetamine. The sympathomimetic effects include elevated blood pressure, anorexia, insomnia, alertness, elevated mood and loquacity (7). Furthermore, chronic khat chewing for many years results in unpleasant effect of cognitive defects and psychosis associated with severe neurological illness. It also causes abnormalities deep in the white matter of cerebral hemispheres and marked cortical atrophy (9). These clinical complications might be exacerbated by intermittent use of khat and ethanol.

The prevalence of concomitant use of khat chewing and drinking alcohol is sharply increasing particularly in the young generation of Ethiopia and even in pregnant women. It appears that these individuals are attracted by the antagonistic effect of ethanol on the stimulant and insomnial effect of khat (6). However, drinking ethanol after khat chewing may exacerbate the risk factors of clinical complications of both ethanol and khat. The complications could even be fatal for those who are chronically addicted with khat and alcohol (10). There is, however, no much research that has been done on the combined effect of khat and ethanol on the cerebellum.

The aim of the present study is therefore, to address this issue with particular attention on the structural changes of cerebellar cortex as well as the size of Purkinje neurons of the cerebellum.

MATERIALS AND METHODS

PLANT MATERIAL PREPARATION

Khat leaves grown in Gelemso (Ethiopia) were purchased from a local market in Addis Ababa. Methods developed by Connor et al. (11) and Makonnen (12) were employed for the extraction process. The leaves were finely chopped with knife, weighed by electronic digital balance and placed in an Erlenmeyer flask containing organic solvents diethyl ether (Whitehouse Industrial Estate, Reagent Chemical Services Ltd., Cheshire) and chloroform (BDH Chemicals Ltd) in a 3:1 ratio. Enough volume of volatile solvent was added in such a way that it covered the minced plant material in the flask. The flask was closed by flask stopper and the contents were continuously stirred using magnetic stirrer for 24 hours. The extractant was decanted, filtered by Whatman No.1 filter paper, and was concentrated using a Rota-vapor under low pressure. The concentrated extractant was then poured on a petridish and subjected to a vacuum until the organic solvents were completely evaporated. The dry residue was weighed to calculate the total yield, which was found to be 0.73 %. The resulting residue was kept covered and refrigerated until use. On the day of experimentation, khat extract was reconstituted with 2% Tween 80 in distilled water to dissolve cathinone. The dose was expressed in terms of dry weight of extract per body weight.

ANIMAL PREPARATION

Pregnant white Wistar rats were obtained from the Animal House of the Faculty of Medicine, Addis Ababa University and were housed in a standard plastic cage on straw bedding in a temperature controlled room (21 10C) maintained at 12/12 hrs light/ dark cycle. They were fed on pellets and were given drinking water ad libitum. The rats were checked everyday to determine whether they had given birth or not. The day of birth for any group of pups was assigned as postnatal day (PND) 0.

ANIMAL TREATMENT

In this study pups of 6 PND were used. These animals were categorized randomly into control, ethanol treated, khat treated and combination of ethanol and khat treated groups, where each category contained 5 pups.

On each day of the experiment, all the animals were taken from their cage. They were weighed using Swiss Quality electronic digital balance with 0.01 precision, since weight of animals was necessary to determine the dose of drugs (khat and ethanol) and vehicle. Test substances as well as the vehicle were administered into the stomach through a blunt feeding needle daily for one month. The ethanol treated group received 3ml/100 gm body weight of 20% ethanol and their control group received the same amount of vehicle (distilled water) according to their body weight. The khat treated group received 20mg/100 gm of body weight khat suspension and their control received vehicle (2% Tween 80 in distilled water) corresponding to their body weight. The combination group received 20mg/100 gm body weight of khat first followed by 20% of ethanol (3ml/100 gm of body weight) after an hour, and their control received only vehicle (2% Tween 80 in distilled water).

ANIMAL PERFUSION, BRAIN DISSECTION AND FIXATION

After one month of treatment, the rats were deeply anaesthetized with diethyl ether and transcardially perfused with 4% formaldehyde in 0.1M phosphate buffered saline at PH of 7.3. Perfusion was carried out with about 14% of total body weight of perfusate solution for 10-15 minutes until the fluid that comes out of the rat became clear and free of blood. The rat was placed in a prone position and the skin on the head was incised and reflected posterolaterally, and held with pins to expose the skull. The skull was cut coronally at the level of lamina cribrosa and then sagittally to the level of the foramen magnum with a small bone cutter. The dissected portion of the skull was reflected laterally to expose the brain as a whole. The brain was separated from spinal cord at the level of foramen magnum using a pair of scissors and was removed. The entire brain was immersed in fixative and kept in the refrigerator for 18 hours (13).

TISSUE SAMPLING AND PROCESSING

After fixation, the brain as a whole and cerebellum were separately weighed. Cerebellum was then cut according to stereological, multistage fractionator rules. This process incorporates four stages. In the first stage, cerebellum was cut sequentially into 2mm thickness parasagittally. This gave six slices. These slices were arranged in sequence and a random sampling, which was accomplished by a lottery system, was used to select any three slices. The chance of each slice for being selected was ½ (f1=2). In the second step, the selected slices of tissues were further sectioned in an approximate area of 4 mm2 (2mm x 2mm) and nine small squared stripes were obtained, three from each slice. A random sampling procedure with a lottery system was also implemented here and three stripes were selected out of nine with a probability of 1⁄3 (f2= 3). The chosen tissues in the latter step were processed for routine paraffin procedure. By taking such 3 blocks of tissues from each animal, a total of 15 blocks per groups were therefore collected. Tissues were dehydrated in increasing concentration of alcohol (Ethyl alcohol absolute 99.7 %, El Nasr Pharmaceutical Chemicals, Egypt), cleared with xylene (BDH Laboratory supplies Poole BH15 1TD, England), impregnated and embedded in paraffin wax (Paraffin wax m.pt. 58-600C, Dongnam petrochemical MFG. Co. Ltd, Korea).

Each tissue blocks were sectioned on Zeiss Microtome (Carl Zeiss Zunch AG, West Germany) at 6 m thickness and collected on to egg albumin coated microscopic slide. About 400 sections were obtained from each block. For the purpose of stereological analysis, every 20th section of the tissues was collected in random fashion. The probability of any tissue to be selected is 1 /20(f1=20). Subsequently, sections were deparraffinized, cleared and hydrated as stained with toluidine blue for 20 minutes. Stained tissues were then dehydrated and cleared in a reverse direction and mounted in pertex (medite GmbH, Wollenweberstrasse12, D-31303 Burgdorf, Germany) and cover slipped.

MICROSCOPIC EXAMINATION

Slides were examined with a Zeiss binocular microscope (Carl Zeiss, Axiostar, Germany) fitted with x10 and x 40 magnification objective lens. All the changes observed in the examined tissue sections were recorded and photographed using a Leitz Dialux 20 wild photoautomat MPS 51(Wild Heerbrgg Ltd., Heerburgg, Switzerland).

ESTIMATION OF DIAMETER OF PURKINJE NEURONS

Cerebellar cortex has abundant neurons with variable size. Among these, Purkinje neurons are the largest in size. The size of neurons can be estimated from their diameter. To determine the diameter, randomly selected Purkinje neurons with clear and visible nuclear profile was measured by aligning up an eyepiece calibration bar using x25 objective. It was carried out by measuring the major (a) and minor (b) axis of each neuron, and then the mean diameter was calculated by using the formula:

$$D = \sqrt{a.b} \quad (14)$$

Where D: Mean diameter of neuron
a: Long axis of neuron
b: Short axis of neuron

STATISTICAL ANALYSIS

The data obtained from body weight, weight of brain as a whole and cerebellum independently, and diameter of Purkinje neurons of cerebellum were analyzed using version 11.5 statistical packages for social sciences (SPSS). The significant differences among different categories were tested by one way analysis of variance (ANOVA). All the data were presented as mean ± S.E.M. $P<0.01$ was considered statistically significant.

RESULTS

QUALITATIVE OBSERVATIONS ON RATS

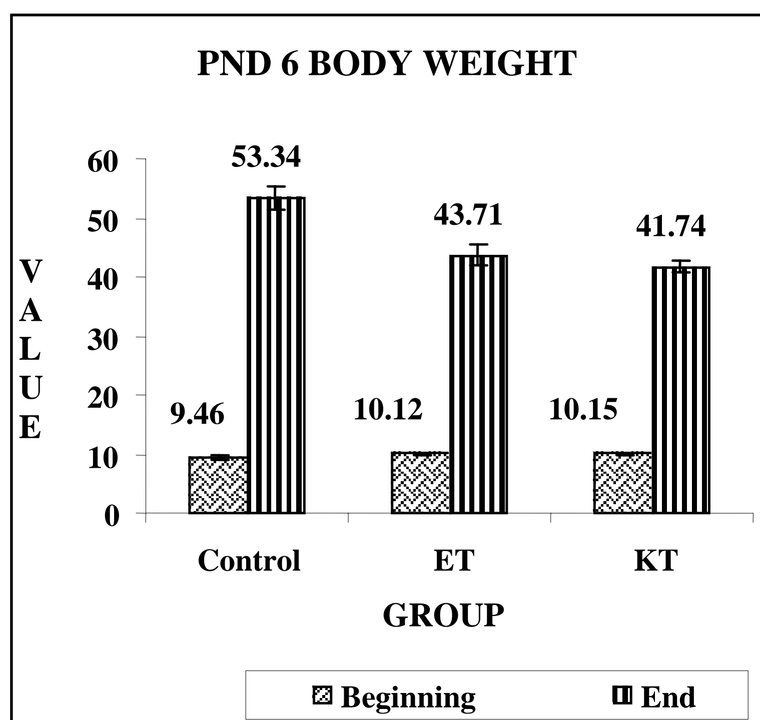
At the end of each day of ethanol treatment, the animals showed obvious signs of intoxications. Many of the intoxicated animals appeared to be asleep and depressed. All the animals, however, recovered from the symptoms of intoxication within a few hours after treatment. On the other hand, khat extract treated animals showed an increased motor activity and were restless. After about an hour of khat treatment, however, increased motor activity was followed by depression, then, they returned to normal. In the combined treated animals, khat extract enhanced motor activity when ethanol was administered after one hour. The animals showed signs of depression and the motor activity was reduced. However, these groups of pups that received the combination of khat and ethanol died after two days of treatment. Therefore, further investigations on the combination of khat and ethanol treated category were interrupted. Their autopsy showed extensive hemorrhage in the posterior region of the brain, however no gross pathological change was observed in the liver and lung.

BODY WEIGHT MEASUREMENT

The body weight of the animals of PND 6 was recorded just at the beginning of treatment and at the end of the experiment after 30 days, after which the animals were sacrificed for histological examination, as indicated in Figure 1. At the beginning of the treatment there was no statistically significant difference in the body weight among different categories. At the end of the experiment, the body weight of the rats increased. However, the increment was different for each category.

The body weight increment was 82.26%, 76.85% and 75.68% for control, ET and KT rats, respectively. This indicated that the body weight increment of the control groups was more by 18.05% and 21.75% over those treated with ethanol and khat, respectively. This result was statistically significant at the level of P< 0.01. In addition, the body weight of ET was greater than KT by 4.51%, although this was not statistically significant (P>0.05). Post hoc test also showed significant differences between the control and different experimental groups. However, the weight difference between ET and KT groups was not statistically significant (P>0.05).

Figure 1: Average body weight (g) of rats treated with ethanol and khat and their age matched control at the beginning of the experiment and after 30 days of treatment.



BRAIN WEIGHTS

At the end of the experiment the average brain weight of the animals of ET and KT were 1.55 gm and 1.7 gm, respectively whereas their respective controls was 2.1 gm., (see table 1). This depicted that the brain weight of ET and KT animals was significantly less than that of the controls by 26.19% and 19.05%, respectively (P<0.01). In addition, the brain weight of ET was significantly less than those of KT by 8.8% (P<0.01). Furthermore, the weight of cerebellum of the ET (0.15 ± 0.01 gm) was less than the control (0.27 ± 0.01 gm) and KT (0.17± 0.01 gm) animals. It was significantly decreased by 44.44% and 11.76% from their age matched control and KT categories, respectively (P<0.01). Moreover, the cerebellar weight of KT rats decreased by 37.04% from those of controls. This was statistically significant (P<0.01).

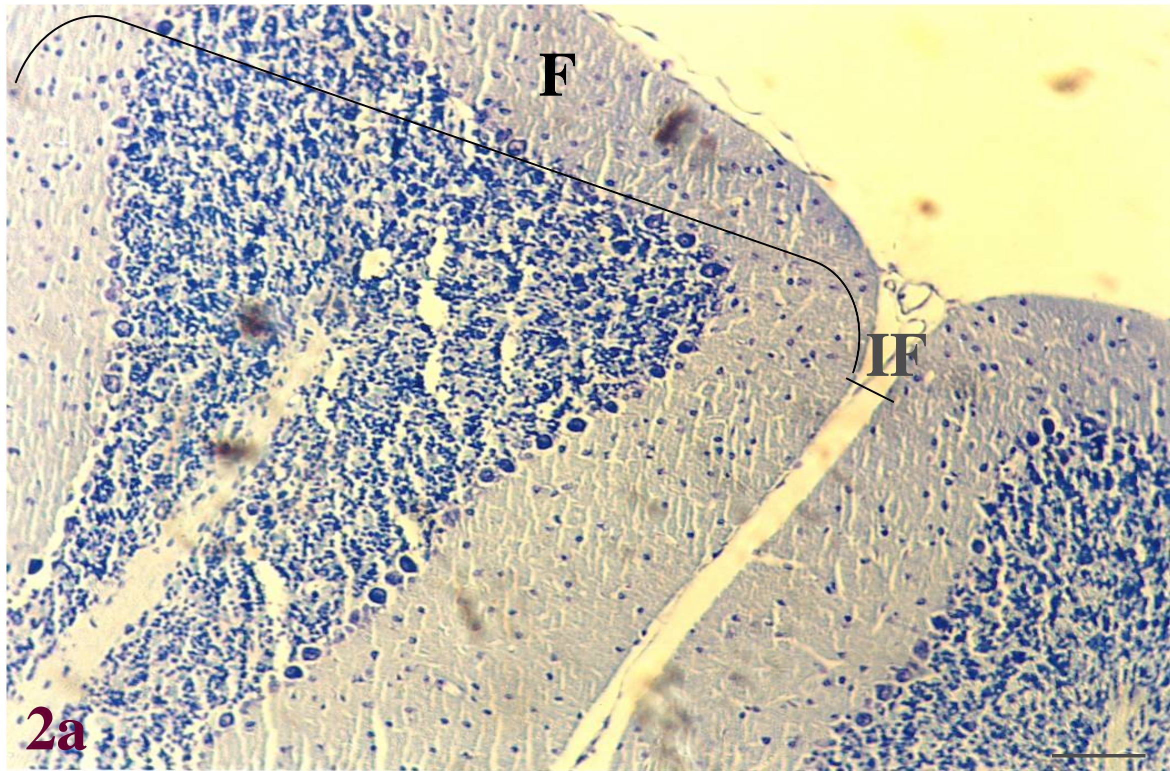
Table 1: Mean + S.E.M. of the whole brain and cerebellar weights (g) of rats treated with ethanol and khat and their age matched controls after 30 days of treatment.

Group	Brain	Cerebellum
PND 6		
Control	2.10 ± 0.01	0.27 ± 0.01
ET	1.55 ± 0.03	0.15 ± 0.01
KT	1.70 ± 0.02	0.17 ± 0.01

MICROSCOPIC OBSERVATION OF CEREBELLAR CORTEX

In the molecular layer of the cerebellum nerve fibers, a few dispersed neurons and lightly stained neuroglial cells were distinctly observed (figure 2a –c). In the inner layer (granular layer) tightly packed small rounded granule cells were identified. In the Purkinje layer, on the other hand, very large flask shaped cells with dendrite were observed lying separately at intervals. In most neurons of the three layers of cerebellar cortex, nuclei, which were surrounded by Nissl substance, were clearly observed. In most cases the nuclei were located centrally. In the control animals, the Purkinje neurons contained one or two nucleoli whereas in other layers most neurons contained one nucleolus. In addition to neurons and neuroglial cells, blood vessels were observed. The different morphological changes observed in the cerebellar cortex for each investigated categories as studied by light microscope were as described below.

In all the three categories of the pups, Purkinje neurons with nucleus were seen clearly. In the control animals the nuclei were located centrally (fig. 3a). On the other hand, in some neurons of ET animals the nuclei were found eccentrically and a few neurons lacked nucleolus in their nucleus (fig. 3b). In addition, in some neurons the Nissl substance was dissoluted. Similarly, in some Purkinje neurons of KT animals the Nissl substance was dissoluted and the nuclei were pushed peripherally (fig. 3c). Moreover, unlike the control and KT rats, the folia in the ET ones were smaller and interfolia were wider (fig. 2 a-c).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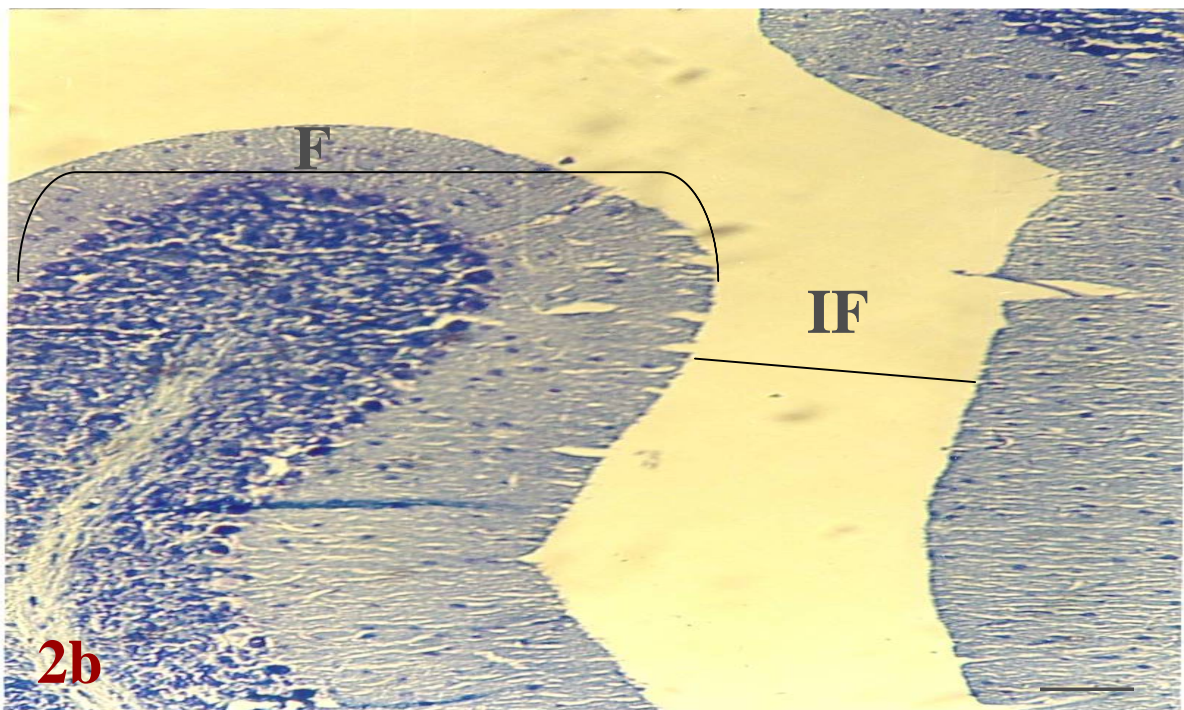
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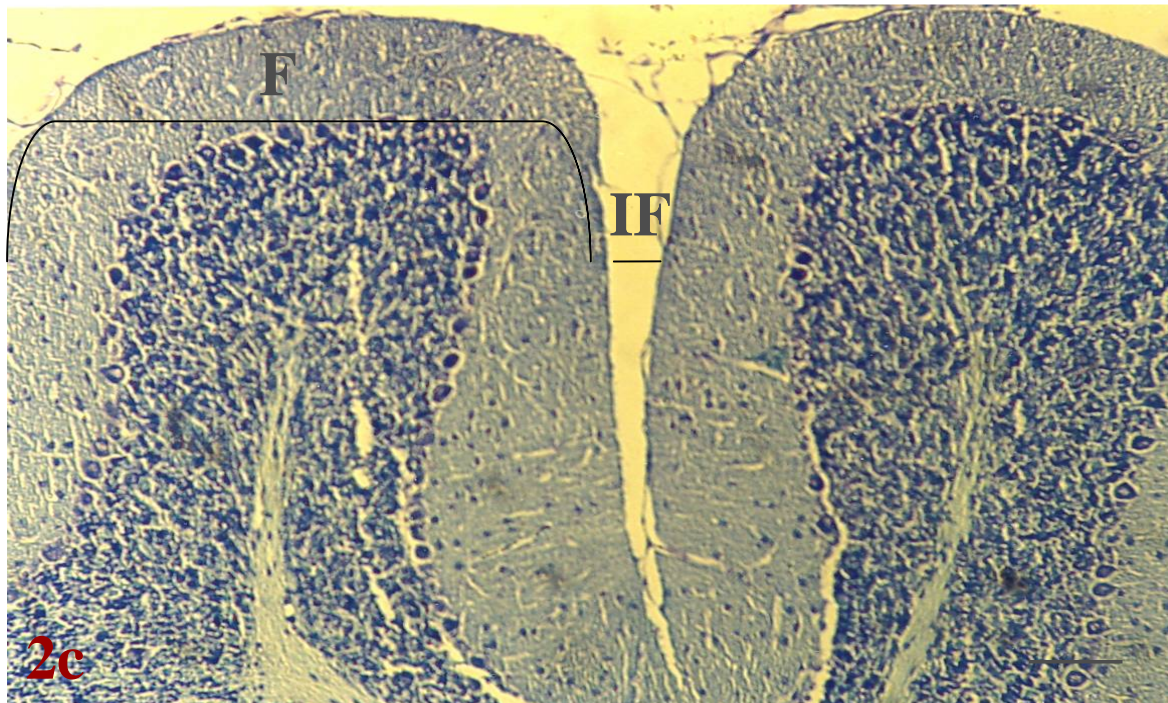
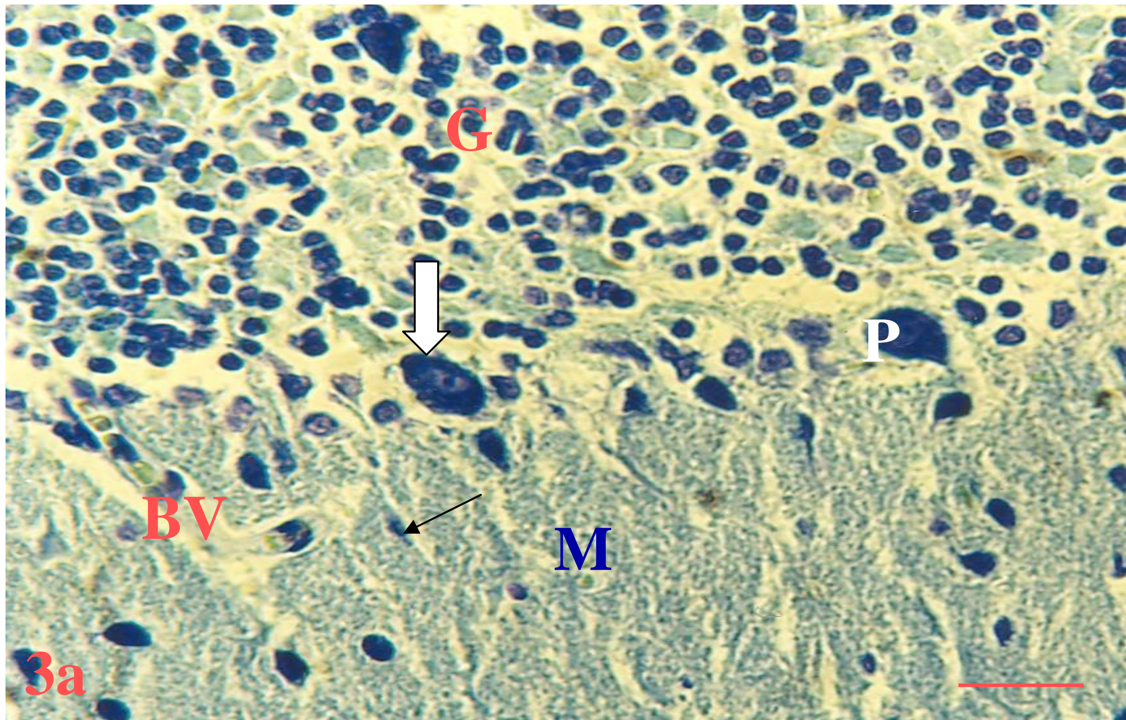


Figure 2a-c : Photomicrographs of toluidine blue-stained paraffin sections of cerebellar cortex of control (a), ET (b) and KT (c) illustrating the width of folia (F) and interfolia (IF). Note that: in the control (2a) and khat treated (2c) rats the respective width of folia and interfolia are almost equal, where as in the ethanol treated (2b) rats the interfolia (IF) is wider and thickness of folia (F) is smaller. Bar = 21 μ m: x 140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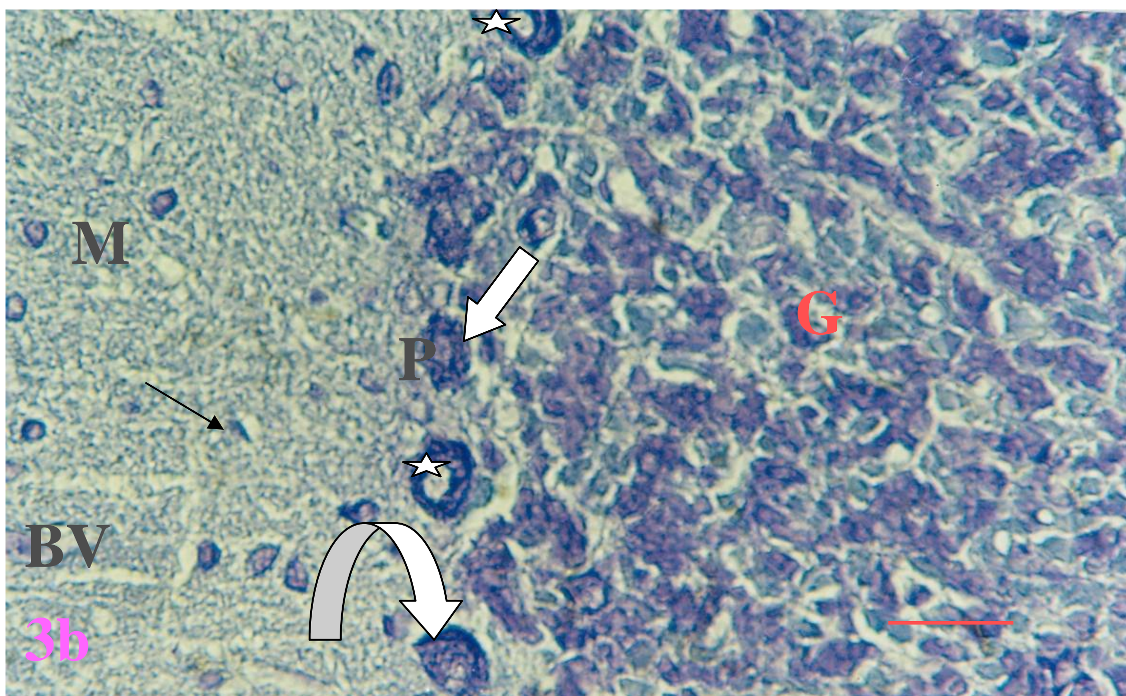
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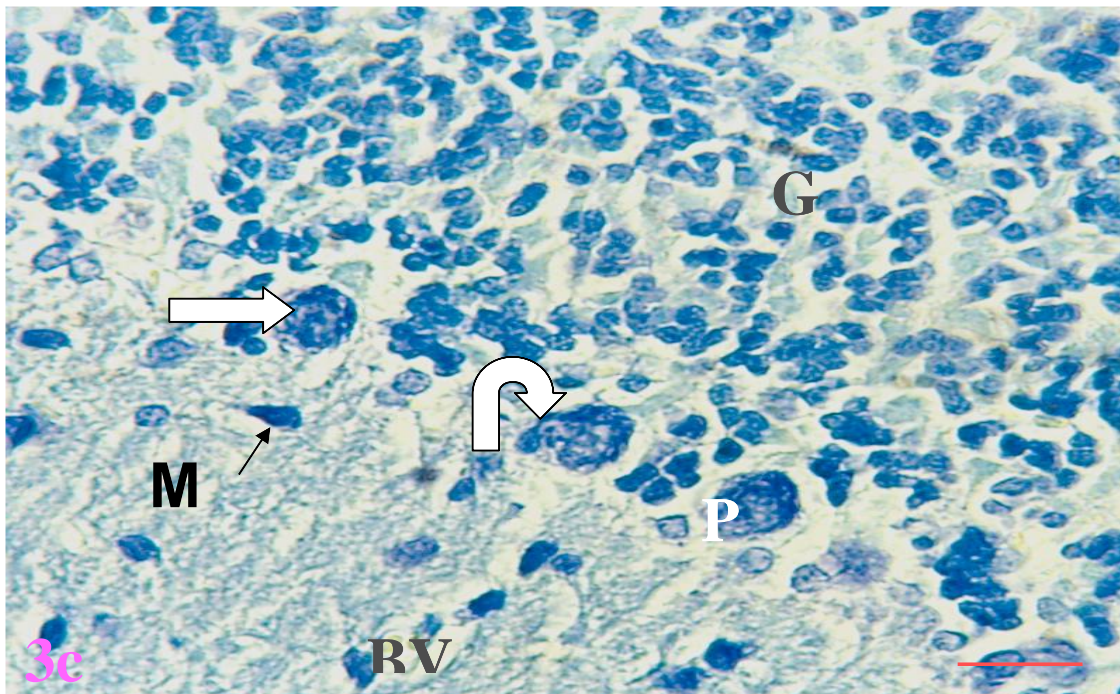
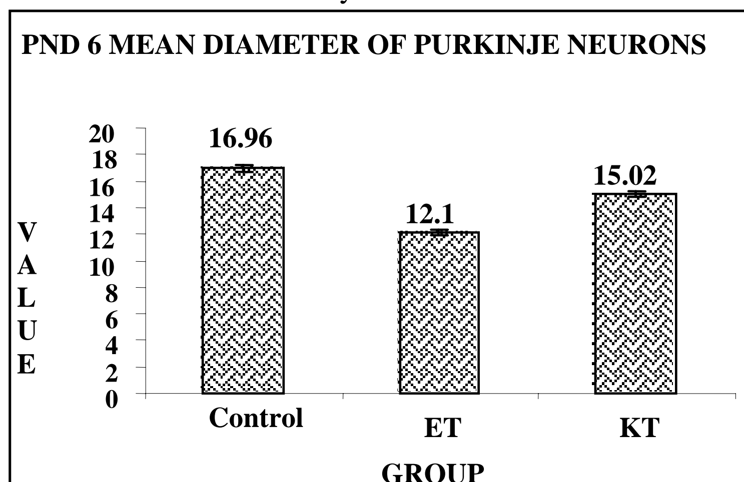


Figure 3a-c: Photomicrographs of toluidine blue-stained paraffin sections of cerebellar cortex of control (a), ET (b) and KT (c) illustrating molecular layer (M), Purkinje cell layer (P) and granular layer (G). Neurons with clear profile of nuclei typical of those used for size determination are marked by thick with arrows. Neuroglial cells can be seen in the molecular layer (thin arrow). Besides, blood vessels (BV) were evident. In the control rat (3a), normal histological structure was observed. In ethanol treated rat (3b), the Nissle substance of Purkinje neuron is distorted and the nucleus with nucleoli is pushed peripherally (white curved arrow) or nucleoli completely absent (white star). In the khat treated rat (3c), the Nissle substance of Purkinje neuron is distorted and nucleus with nucleoi is pushed peripherally (white curved arrow). Bar 17 µm : x 590

ESTIMATION OF DIAMETER OF PURKINJE NEURONS

The mean diameter of cell bodies of Purkinje neurons of each category of pups at the end of the experiment are as presented in figure 4.
The average diameter of Purkinje neurons in the ET and KT categories was 12.10 micrometer (µm) and 15.02µm, respectively where as it was 16.96 µm in the control animals. This indicated that the mean diameters of the neurons of the ET and KT animals were significantly less by 28.66% and 11.44%, respectively from their controls (P< 0.01). Furthermore, the size of Purkinje neurons was significantly greater by 19.44% in the KT than ET groups. Thus, the mean size of Purkinje neurons of rats exposed to ethanol was smaller than those rats exposed to khat extract and the control groups. These differences were further reflected by Post-hoc analysis using Scheffe test, which showed significant decrement in mean diameter of Purkinje neurons of cerebellum of ET and KT animals.

Graph 4: Mean ± S.E.M. diameter (μm) of Purkinje neurons of rats treated with ethanol, and khat and their age matched controls after 30 days of treatment.



DISCUSSION

Administration of combination of khat and ethanol resulted in death of the rats within 2 days of treatment. As found from their autopsy, there was sever and extensive hemorrhage in the posterior region of the brain. This may be the main cause of death as there were no other observable gross pathological changes in other tissues. PND 6 in rat is roughly comparable with human fetus of second trimester (4). It is not known, whether such effect is also manifested and adversely affects the pregnancy in human if a pregnant woman takes high doses of ethanol and khat simultaneously. It is a matter that deserves attention and requires future investigations.

The relation between alcohol consumption and total body weight is not clear (15). However, in the present study, ingestion of high amount of alcohol for 30 days caused loss of total body weight of the rats. A similar finding was reported by Miki et al. (16). This might be attributed to the appetite suppressant effect of high alcohol concentration. Similarly, intake of khat extract significantly reduced the body weight of rats and it is in line with the finding of Hassan et al. (17) in humans. This effect might be associated to the anorexigenic effect of cathinone through stimulation of the release of norepinephrine in CNS, which acts centrally to activate the satiety centers and then delays gastric emptying and suppresses appetite (18).

In the present study, both ethanol and khat treatment significantly caused reduction in the weight of the whole brain and cerebellum during early life (PND 6). The functional implications of this reduction in the brain weight as a whole also suggested by (6) in the cerebellar weight are uncertain. Nevertheless, it shows that the brain tissues during PND 6 are vulnerable to dependence producing drugs, such as ethanol and khat. Moreover, Thomas and coworkers (19) have found that rats exposed to high amount of ethanol at PND 4 and 5 or 8 and 9 had a deficit in motor coordination as tested on a parallel bar apparatus.

Although the general morphological arrangement of the three cerebellar layers is found to be comparable in all categories of rats, there were also some differences observed specifically is the structure of the Purkinje neurons. In the ethanol treated animals, the nucleus of some Purkinje neurons was found pushed peripherally and their Nissl substance was dissolute. On top of these, there were neurons without nucleolus in their nucleus. In the khat treated rats, the nucleus of some Purkinje neurons was also pushed peripherally and their Nissl substance was also dissolute. It is known that, displacement of nucleus to the periphery, absence of nucleolus in the nucleus and dissolution of Nissl substance are signs of neuron degeneration (20, 21). The above-mentioned features of the nucleus and Nissl substance observed in this study may also indicate sign of degeneration of neurons as the result of ethanol and khat treatment.

The mean diameter of Purkinje neurons in all categories of the rats was assessed to examine the effect of ethanol and khat ingestion on these particular neurons. The mean diameter of Purkinje neurons of the treated rats were less than their age matched controls. This may be attributed to the selective vulnerability of the bigger sized neurons towards the metabolite of alcohol for the ET rats and towards the active components of khat for the KT animals (22, 23).

In the present study it was found that ET rats have narrower folia and wider interfolia as compared to age matched control and KT rats. This correlates well with one of the main neurological manifestations of cerebellar atrophy that is related to alcohol abuse as described by other investigators (21, 24). It is known that the atrophic changes in cerebellum as a result of alcohol abuse are characterized by a decrease in the volume of molecular and granular layers, gliosis of the granule cells and a decrease in the number of Purkinje cells (20). These lead to shrinkage of the anterior part of the vermis and reduction of the volume of cerebellar cortex resulting in narrowing of folia and widening of interfolia (19) .The mechanism of these neuronal lesions and cerebellar atrophy, however, remains uncertain (25). It is suggested that, it may possibly involve hypoxia due to spasm of cerebral blood vessels and fluidization of membranes (26). It may also be due to focal accumulation of toxic aldehyde resulting from intraneural ethanol metabolism. Acetaldehyde is a toxic substance, which can penetrate the blood brain barrier (BBB) and affect different parts of the brain and neurons (27). The accumulation of acetaldehyde is highly exaggerated in the cerebellum than any other region of the brain (23). In addition, khat consumption has also been implicated in causing cerebellar damage because two of its active components, cathinone and cathine, can pass BBB and possibly affect the white matter, the primary target for dependence producing drugs but the mechanism is uncertain (9).

In conclusion, this study has shown that the exposure of rats to combination of ethanol and khat is fatal to the rats of PND 6. Ethanol and khat ingestion during early postnatal life causes morphological changes of cerebellar cortex. In addition, both ethanol and khat intake significantly reduce the total body weight, brain weight in general and cerebellar weight, independently, and also reduce the diameter of Purkinje neurons of cerebellum.

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