ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

EFFECT OF ETHANOL AND KHAT (*Catha edulis* Forsk) ON CEREBELLAR CORTEX OF THE RAT

By : Abebe Muche

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THESIS

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"This thesis is my original work, has not been presented as a thesis work for a degree in this or any other University and that all sources of material used for the thesis have been duly acknowledged".

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ABBREVIATIONS

µm	–	Micrometer
ANOVA	–	Analysis of variance
APA	–	American Psychiatric Association
BAC	–	Blood alcohol concentration
BBB	–	Blood brain barrier
cm^2	–	Centimeter square
CNS	–	Central nervous system
CSF	–	Cerebrospinal fluid
CT	–	Combination of khat and ethanol treated
CT	–	Computer tomography
D	–	Mean diameter
ET	–	Ethanol treated
GABA	–	Gamma aminobutric acid
GIT	–	Gastrointestinal tracts
gm	–	gram
H_2O_2	–	Hydrogen peroxide
KT	–	Khat treated
mg	–	milligram
mg/ dL	–	Milligram per deciliter
mg/gm	–	milligram per gram
ml	–	milliliter
ml/gm	–	milliliter per gram
ml/hr	–	Milliliter per hour
mm	–	millimeter
mm^2	–	millimeter square
mm^3	–	Millimeter cube
MRI	–	Magnetic resonance imaging
NAD+	–	Nicotinamide adenine dinucleotide
NMDA	–	N- methyl- D- aspartate
PND	–	Postnatal day
S.E.M.	–	Standard error of the mean

SPSS	–	Statistical package for social sciences
US	–	United States
WHO	–	World health organization

ABSTRACT

This experimental study included three age groups of rats: post natal day (PND) 6, 13 and 30. Each group contained control, ethanol treated (ET), khat treated (KT) and combination of khat and ethanol treated (CT) categories. They were treated with vehicle, ethanol and khat, respectively for 30 days using blunt needle. At the end of experiment, all the animals were scarified, their brain was dissected out and immersion fixed. The brain and cerebellum were separately weighed, and cerebellum was processed for routine histology and sectioned. The serially sectioned tissues of cerebellum was stained with toluidine blue and observed using light microscope.

In the rats of all age groups, the body weight increment at the end of experimental period was significantly less in the treated ones than their respective controls at $P< 0.01$. Between the treated rats, this was less for the ET rats than the KT rats, although not statistically significant ($P>0.05$). Similarly, the weight of the brain as a whole and cerebellar weight, part of brain, 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, though not statistically significant.

In the rats of PND 6 group, the following results were found: The volume of cerebellar cortex as well as the total number of Purkinje neurons of the ET rats were significantly less than the controls and KT rats at $P<0.01$ and $P<0.05$, respectively. However, no statistically significant difference was observed between the controls and KT rats. The numerical density and volume fraction of Purkinje neurons of ET rats was found to be significantly greater than those of control or KT rats ($P<0.05$). In addition, the numerical density and volume fraction of Purkinje neurons were greater in the KT rats than their corresponding controls, but no

statistically significant difference was observed. The mean diameter of Purkinje neurons was significantly less in the ET rats than in KT rats which in turn was significantly less than the control rats ($P<0.01$).

In the rats of PND 13 and 30, the patterns of the results of all the different parameters investigated consistently followed those of the rats of PND 6 as summarized above, However, the values were found to be statistically non- significant. In addition, the results of all the parameters for the CT rats of PND 30 rats showed values in between KT and ET rats, though these were also statistically non- significant. However, CT rats of PND 6 and 13 died after two days of treatment.

In conclusion, the study depicted that PND 6 is an extremely vulnerable period during which the rat cerebellar Purkinje neurons are particularly susceptible to the effect of high dose of ethanol. However, a similar level and duration of ethanol exposure commencing during PND 13 and 30 has no significant effect on the volume of cerebellar cortex, numerical density of Purkinje neurons, total number of Purkinje neurons and volume fraction of Purkinje neurons. Treatment of khat and combination of khat and ethanol is lethal at an early age, however it does not significantly change the above mentioned parameters at the latter ages (PND 30).

Key words: *Cerebellar cortex, Purkinje neurons, Stereology, Khat, Ethanol, Post natal day*

1. INTRODUCTION

1.1. ANATOMY OF CENTRAL NERVOUS SYSTEM

The central nervous system (CNS) is a component of nervous system that contains brain and spinal cord. It is protected by bony encasement –the cranium and connective tissue called meninges and bathed in the cerebrospinal fluid (CSF) which circulates within the ventricles of the brain, central canal of spinal cord and subarachnoid space surrounding the entire CNS (Kingsley, 1996).

CNS is made up of neurons, which are the structural and functional units of nervous system. Neurons have variable size with their diameter ranging from 4µm for granule cells to 120µm for Betz cells, and shape ranging from circular to pyramidal (Kingsley, 1996). Although they are variable in shape and size, they have three basic components, namely the cell body (perikaryon) consisting of the nucleus and surrounding cytoplasm, the dendrites and the axon.

CNS is also composed of neuroglial cells which outnumber the neurons by 9:1 ratio (Snell, 1997). They are important in supporting the survival of the neurons. Most are highly branched cells which occupy the spaces between neurons. There are four principally recognized neuroglia cells known as astrocytes, oligodendrocytes, microglia and ependymal cells. Astrocytes are the largest and most numerous of all supportive cells in the CNS, and are recognized by their stellate shape and numerous long processes, which extend into the surrounding neuropil (Marieb, 2000). The long processes have expanded pedicles at their ends that attach to the walls of blood capillaries and completely surround and ensheath the vessels contributing to the blood brain barrier formation (Bharihoke, 2000). Astrocytes are of two types: fibrous and protoplasmic astrocytes (Cohen, 1993). Fibrous astrocytes are characterized

by their thin, less branched processes, which radiate from the cell body for considerable distances. They are highly abundant in the white matter and are often interposed between neurons and adjacent blood vessels. On the other hand, protoplasmic astrocytes are more numerous in the grey matter. They have many but short processes, which envelop the surface of nerve cells, synaptic areas and blood vessels. Astrocytes in general, provide mechanical support and mediate the exchange of metabolites between neurons and the vascular system. In addition, they play an important role in the repair of CNS tissue after injury or damage by disease (Snell, 1997). Oligodendrocytes are smaller neuroglial cells with fewer processes than astrocytes (Hollinshead and Rosse, 1985). They are found both in grey and white matter. In the grey matter, they are mainly localized close to perikaryon, where as in the white matter they appear in rows among the myelinated nerve fibres. They are responsible for the production of myelin sheath in the CNS, similar to the Schwann cells that make myelin sheath in the peripheral nerves. Microglial cells have elongated or triangular nuclei and are found both in white and grey matter, but are more abundant in grey matter (Burt, 1993). They have defense and immunological functions in the CNS (Carpenter and Sutin, 1983). Ependymal cells, which are the fourth neuroglia cells with numerous microvilli on their ventricular surface, are specialized epithelial cells, which line the central canal of the spinal cord and the ventricles of the brain. They have absorptive and secretory function in the CSF (Bharihoke, 2000).

Brain is the center of thought and emotions that occupies the entire cranial cavity enclosed by the skull. Brain has similar structure in mammals both microscopically and macroscopically (Carpenter and Sutin, 1983). Macroscopically, it is composed of six major parts, which are delegated to control specific activities of the body. These include cerebrum, diencephalons, cerebellum, midbrain, pons, and medulla oblongata. Very often, the midbrain, pons and

medulla oblongata are collectively referred to as the brain stem. Microscopically, brain is composed of about 100 billion neurons and 900 billion neuroglial (Waxman, 1996).

1.1.1. CEREBELLUM

Cerebellum is located in the posterior cranial fossa. This mass of tissue possesses central constriction called vermis and two lateral expanded portions (right and left hemispheres). The surface of cerebellum is thrown into a series of parallel folds (folia). Folia of cerebellum encompass white and grey matter. Within the white matter of cerebellum four nuclei are located namely, fastigial, globose, emboliform and dentate nuclei. The fastigial nucleus receives fibers from the purkinje cells of the vermis which synapse in the dentate nucleus to control balance and posture (Kingsley, 1996). The globose and emboliform nuclei projects to the intermediate or paravermal and paramedian lobes of the cerebellum (King, 1987).

According to Kingsley (1996), based on their morphology the lobes of cerebellum are classified into three. These are paleocerebellum (anterior lobe), neocerebellum (middle lobe) and posterior lobe (archicerebellum). The paleocerebellum is concerned with postural and muscle reflex. It has spinal connection and receives spinocerebellar tract. The neocerebellum, which is concerned in performance of accurate voluntary movements, has cerebropontine connection. Hence, it receives cerebropontocerebellar tract. The third lobe, archicerebellum is related to equilibrium. It has vestibular connection and receives vestibulocerebellar tract.

The grey matter of cerebellum, the cerebellar cortex, has a complex but uniform cellular structure throughout its extent. Within the cerebellar cortex, three layers are differentiated by their cellular composition. From outer to inner, they are named as molecular layer, Purkinje

cell layer, and the granular layer. Within these three layers of cerebellum six different types of neurons are to be found (Cohen, 1993).

Stellate cells, located in the outer two-thirds of molecular layer, have small cell bodies, short thin dendrites, and fine unmyelinated axons. They send axons to the folia and establish inhibitory contact with Purkinje cell dendrites (Burt, 1993).

Basket cells are situated near the Purkinje cell bodies in the deeper part of molecular layer. They provide numerous branching dendrites that extend into the superficial regions of molecular layer. Like stellate cells, basket cells send their axons to the folia at the junction of molecular and Purkinje cell layers. As their axons move to the border, they envelop the soma of the Purkinje neurons like a basket. They empress a powerful inhibitory synapses and receive excitatory input from parallel fibers (Shepherd, 1993).

Purkinje cells are large pear shaped neurons with a diameter of 25 µm and their number in rats ranges from 2×10^5 to 3×10^5 (Miki et al., 1999). They have large dendritic trees in the molecular layer, which are highly branched and fan – shaped and have dendritic spines at their sites of synapses (Burt, 1993). The axons of Purkinje cells which are the only fibers that leave cerebellar cortex form the efferent pathway from cerebellum, and send collaterals in the granular layer. The axons project ipsilaterally to the deep cerebellar nuclei especially the dentate nuclei and form inhibitory synapses (Waxman, 1996).

Granule cells are the most numerous neurons in the brain, ranging from 3-7 million/ mm^3 (Bedi, 1987). They are very small and closely packed neurons having heterochromatic nuclei, scanty cytoplasm, and small dendritic tree in granule layer. Granule cells are the only excitatory neurons in the cerebellar cortex (Cohen, 1993) with unmyelinated axon, which is

projected to the molecular layer and splits in T- shaped manner to form parallel fibers. The parallel fibers run longitudinally along folia and cross dendrites of many Purkinje cells.

As King (1987) described, Golgi cells are mostly situated in the outer part of the granular layer. Their dendrites form excitatory contacts with the parallel fibers of granule cells as they extend to the molecular layer (Snell, 1997). They have short axon with numerous arborizations in the granular layer which in turn inhibit the granule cells.

According to Braak and Braak (1993); Mugnaini and Floris (1994), Monodendritic or brush cells have been described only recently. These cells are located in the granular layer. The soma of the neuron contains a faintly stained nucleus and issues a single short dendrite terminating in a tuft or brush. Mossy fibers make large synaptic contacts with the brush- like process. The cells are strongly immunoreactive with antibodies against calretinin. Brush cells are especially numerous in the nodulus and the flocculus. They give rise to a thin axon, whose termination is presently unknown.

The different parts of cerebellum are interconnected with each other and other parts of the brain through short and long tracts (Hollinshead and Rosse, 1985). The short tracts conduct impulses between neurons within the cerebellar cortex and in the white matter of cerebellum. The long tracts enter or leave cerebellum by way of three cerebellar peduncles to conduct impulse. According to Burt (1993), the superior cerebellar peduncles contain fibers which primarily originated from dentate nucleus and pass through the red nucleus to the thalamus and then to the motor area of the cerebral cortex; the middle cerebellar peduncles, containing pontocerebellar tract, convey impulse into the cerebellum from the pons; the inferior cerebellar peduncles, chiefly composed of spinocerebellar, vestibulocerebellar, olivocerebellar

and reticulocerebellar tracts, conduct input into the cerebellum from the medulla and spinal cord.

1.2. GENERAL ASPECTS OF PSYCHOSTIMULANT AND DEPRESSANT DRUGS

According to Lexa (1995), psychotropic drugs are defined as psychoactive or depressant substances that affect the brain to produce alterations in mood, thinking, perception, behavior and motor activity. Among such drugs khat, hashish, tobacco are psychostimulant while alcohol is depressant. In Ethiopia, all these drugs and other solvents, like benzene are found to be the most abused ones by the youth (Mesfin et al., 1999).

Fresh khat leaves (*Catha edulis Celestrasae*) are chewed daily by over 20 million people in Yemen and East African countries (Kassie *et al*., 2001; Al-Motarreb et al., 2002). Khat contains psychoactive components, namely, cathine, cathidine, norephedrine and cathinone (Kalix, 1984; Kalix, 1990). Cathinone, precursor of cathine, stimulates CNS, increases locomotor activity and results in sympathomimetic effect (Kalix, 1992) which are analogous to the effects of amphetamine. The sympathomimetic effects include elevated blood pressure, anorexia, insomnia, alertness, elevated mood and loquacity (Al-Qirim et al., 2002, Al-Mamary et al., 2002). 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 both cerebral hemispheres and marked cortical atrophy (Morrish et al., 1999).

Alcohol is the most widely used drug in the world (Cook, 1998). It profoundly affects the function of several vital organs, particularly the liver, nervous system, gastrointestinal tract, and cardiovascular system (Tabakoff et al., 1996). It is a CNS depressant, which slows down

the activity of the central nervous system so that messages take longer time to travel along nerve fibers (Zimatkin et al., 1998). It has characteristic response of euphoria in small doses, impaired thought and decreased mechanical efficiency (Goldstein, 1983). In addition, alcohol has toxic effects on the CNS (Samson and Harris, 1992). Consequently, it causes shrinkage and neuronal loss in the frontal lobe of cerebrum and cerebellum, especially vermis (Pentney and Dlugos, 2000). Neuronal loss and shrinkage in the cerebrum and cerebellum of chronic alcohol abusers cause impairment of cognition, reasoning, coordination and balance (Lynch, 1996). Nonetheless, the degree of impairment, and neuronal loss, damage of neuroglial cells as well as shrinkage is directly correlated with the amount of alcohol consumed per day, age of an individual and duration of consumption of alcohol (Holdstock and DeWit, 1998).These defects become highly pronounced in individuals who are also addicted with other psychostimulants such as khat (Widler et al., 1994).

1.3. PHARMACOLOGY OF ETHANOL

Ethanol is a clear, volatile, colorless liquid with a pleasant odor, having a general formula of CH_3CH_2OH. The chemical nature of ethanol enables it to become highly soluble in water and consequently changes its taste. In dilute aqueous solution, it has somewhat sweet flavor, but in solutions that are more concentrated it has burning pain.

Ethanol, the active ingredient of drinking alcohol, can be easily oxidized in our body with a rate of 10 to 15 mg/hr and then depresses CNS, warms the body (vasodilation) and increases frequency of urination (Dubowski, 1985). In addition, long use of ethanol causes chronic problems in human body as well as in experimental animals. The most prominent pharmacological effect of ethanol is broadly referred to as “intoxication”. Nevertheless, the effect of ethanol depends on the amount consumed (Smith, 1989).

1.3.1. PHARMACOKINETICS OF ETHANOL

Ethanol, highly soluble in water, can be diluted in different proportion with different dilution factors. The dilution factor contributes for variability of absorption and distribution of alcohol in our body. Highly concentrated alcohol can be absorbed and distributed faster than diluted alcohol (Holford, 1987).

1.3.1.1. ABSORPTION OF ETHANOL

Ethanol is absorbed from all parts of the gastrointestinal tracts (GIT) by diffusion into the blood (Holford, 1987). Among the GIT, small intestine is by far the most efficient region for absorption because of its very large surface area. The absorption in the small intestine depends neither on concentration of alcohol nor on the absence of food but on the proportion of intestinal villi (Goldstein, 1983). On the other hand, the rate of absorption of alcohol in the stomach is extremely variable and depends on the amount and concentration of alcohol consumed and availability of food (Dubowski, 1985). Indeed, highly concentrated alcohols are absorbed faster than less concentrated alcohol and its absorption is exacerbated by the absence of food in the stomach (Flack-Yeter and McCullough, 2000). In fasting individuals, alcohol absorption is faster than non- fasting ones, and the blood alcohol concentration peaks within 30 minutes (Pikaar et al., 1988). This is because in non-fasting individuals the presence of food in the stomach dilutes the alcohol and delays absorption by slowing gastric emptying (Flack- Yeter and McCullough, 2000).

1.3.1.2. DISTRIBUTION OF ETHANOL

Ethanol rapidly diffuses throughout the aqueous compartment of the body wherever water goes, with ease (Holford, 1987). The volume of ethanol distribution is largely dependant on the amount of total body water content. For instance, women have lower total body water

content than men; therefore, distribution of alcohol becomes faster and elevates blood alcohol concentration (Hommer et al., 2001; Mumenthaler et al., 1999). In addition, the rate of distribution depends on the capacity of alcohol penetration into biological membrane (membrane having protein and lipid bilayer) and blood supply (Pohorecky and Brick, 1988). In the CNS, ethanol concentration rises quickly because the brain receives a large proportion of blood flow (Pikaar et al., 1988). Furthermore, ethanol is lipid soluble fluid and can dissolve the lipid component of the biological membrane called blood brain barrier (BBB) and consequently increases the proportion of ethanol within the brain components (Samson and Harris, 1992). This leads to impairment of the normal functioning of brain parts and contributes for intoxication (Tabakoff and Hoffman, 1991).

1.3.1.3. BIOTRANSFORMATION OF ETHANOL

Ethanol undergoes two processes of biotransformation. In the first step, after drinking, 90-95% of the ethanol is oxidized to acetaldehyde in the liver with an important enzyme called alcohol dehydrogenase, which requires nicotinamide adenine dinucleotide (NAD^+) as coenzyme (McCarver et al., 1997). Nevertheless, small amount of ethanol may be oxidized to hydrogen peroxide (H_2O_2) in the presence of catalase, particularly in the kidneys (Shepherd, 1993). In the second stage, aldehyde dehydrogenase is an enzyme involved in the biotransformation of alcohol. It utilizes NAD+ like alcohol dehydrogenase and converts the acetaldehyde to free acetate or acetyl coenzyme A in the liver (Holford, 1987). At higher alcohol doses blood acetate concentration thus increases and this contributes to toxicity (Brien and Loomis, 1983).

1.3.1.4. EXCRETION OF ETHANOL

About 2 to 10% of ethanol is lost in breath, urine, and sweat (Tabakoff et al., 1996). Majority of ethanol is eliminated by metabolism, which accounts for 90-98% (Bosron et al., 1993).

Exercise increases the rate of elimination of ethanol which results in an increase of about 15% in the total loss rate for the period during which it was sustained (Zuckerman, 1994).

1.3.2. PHARMACODYNAMICS OF ETHANOL

According to Cooper and co- workers (1993), CNS is the most severely affected system by ethanol. The severity towards structural and functional impairment of CNS is suggested to be determined by the blood alcohol concentration (BACs) (Pikaar et al., 1988). At low BACs, below 50 mg/dL, there is mood elevation and slight muscle relaxation (Holford, 1987). In addition, as BACs is increased to 50-100mg/dL, the catabolic reaction time in the body increases. At around the limit of intoxication (100-200 mg/dL), impairment of balance, speech, vision, hearing and muscle coordination, accompanied by feelings of euphoria is clearly observed (Pohorecky and Brick, 1988). Above the limit of intoxication, at BACs of 200-300 mg/dL, progressive intoxication, impairment and loss of physical and mental control is observed; and as the concentration rises up to 300 mg/dL and above, ethanol induces coma, respiratory depression, and death (Fadda and Rossetti, 1998). However, on chronic use tolerance develops which as a result the thresholds concentrations of alcohol at which the above effects occur are elevated (Lexa, 1995).

1.3.2.1. EFFECT OF ETHANOL ON BRAIN

According to Smith (1989), long-term ethanol exposure induces neuronal damage in experimental animals. There is, however some doubt whether this is the direct result of alcohol toxicity or acetaldehyde. But researchers believed that acetaldehyde, can pass the BBB and acts as false neurotransmitter, consequently altering structure and function of the brain (Shepherd, 1993). It is found that, the brain of alcohol addicted individuals becomes lighter, smaller, more shrunken than non-alcoholics of the same age and gender (Rosenbloom et al.,

1995). The rate of shrinkage has close association with the amount of alcohol consumption and sex. Some studies indicated that women are more sensitive for alcohol related shrinkage than men of the same age (Hommer et al., 2001). In both male and female alcohol abusers shrinkage of brain is prominent in the cerebellum, frontal lobe and hippocampal gyrus of cerebrum (Pfefferbaum et al., 1997). Shrinkage in these different parts of the brain is thought to be due to neuronal loss and lesions in the grey and white matter and then causes certain pathological changes (Rebecca et al, 2002). This alcohol related shrinkage is accompanied by an increase in the volume of the ventricles because both cerebellum and cerebrum are pushed anteriorly to increase the area of the corresponding cavities (Lynch, 1996). The increment in volume of cavities leads to the increment in CSF volume (Shear et al., 1996).

Cerebral atrophy is also one of the pathological complications of chronic alcohol addiction (Lexa, 1995). It is characterized by brain weight loss, ventricular enlargement as a result of reduction of white matter and cerebral sulci size increment (Rosenbloom et al., 1995). Furthermore, immunohistochemical studies of alcohol abused rat cerebrum indicate neuronal atrophy, pyknotic (shrinked) cells and reduction of neuronal numbers in the cerebrum (Rebacca et al., 2002). These experimental findings from the animals are also supported by the human imaging studies which indicates enlargement of subarachnoid and ventricular spaces in critical alcohol users (Contran et al., 1995).

Cerebellar atrophy is the other major and commonly recognized neuropathological problems due to chronic alcohol addiction (Brumberg et al., 1991). In atrophied cerebellum, narrowing of folia and widening of interfolial sulci are prominent which can be seen by naked eyes. Moreover, in microscopically diagnosed autopsy of cerebellum of alcohol-abusing individuals, there is loss of purkinje cells, loss of granule cells and atrophy of molecular layer (Victor et al., 1997). It is suggested that the clinical manifestations that include instability of trunk, leg

ataxia, and wide based gait that are observed in alcohol abusers are due to cerebellar atrophy (Shear et al., 1996).

Computed tomography (CT) has also shown that there is an enlargement of the basal cisterns, vermian and hemispheric atrophy (Victor et al., 1997). The most consistent investigation of alcohol related cerebellar change under this imaging technique was found to be dilatation of the fourth ventricle with an increase in both width and height (Vagas et al., 2003). Such dilatation of the fourth ventricle is suggested to be an early sign of chronic alcohol consumption with hemispheric atrophy (Shear et al., 1996).

1.4. PHARMACOLOGY OF KHAT

Khat (*Catha edulis* Forsk, Celastraceae family) is an evergreen tree, which grows at high altitudes extending from East to South Africa as well as Afghanistan, Yemen and Madagascar (Kennedy et al., 1983). It is known by a variety of names such as, “chat” in Ethiopia, “qat” in Yemen, “mirra” in Kenya and “khat” in English. Khat leaf contains three alkaloids namely, cathine, cathinone and norephedrine as well as sugar, tannins and vitamin C (Kalix, 1984). Cathinone and cathine are believed to be responsible for most of the pharmacological actions of khat. Cathinone, the active ingredient of khat, has similar structure and action with that of amphetamine. It is believed to contribute for the major pharmacological effects such as euphoria, alertness and anorexia. On the basis of such similar effects of cathinone and amphetamine, WHO in 1980 classified cathinone as a drug of abuse that can produce mild to moderate psychic dependence (Tariq et al., 2002).

1.4.1. PHARMACOKINETICS OF KHAT

Little was known about the pharmacokinetics of khat (WHO, 1980). However, recently since khat chewing becomes more common in western countries due to migration of people from

endemic khat chewing countries like Somalia, Ethiopia and access of air transport of khat, the pharmacokinetics of khat has obtained due attention (Rousseau et al., 1998). Its pharmacokinetics depends on the type of khat ingested. Khat with young leaves contain high concentration of cathinone that can be absorbed, distributed, biotransformed and excreted within a short time (Al-Motarreb et al., 2002).

1.4.1.1. ABSORPTION OF KHAT

Through chewing of khat leaves most of the alkaloids could be mixed with saliva and absorbed soon (Kalix, 1990). According to Stefan and colleagues (2003), khat has two phases of absorption but the rate of absorption might vary according to their potency. In the first phase of absorption, high concentration of alkaloids may be absorbed in the mucous membrane of oral cavity (Al-Motarreb et al., 2002). In the second stage, stomach and/or small intestine receive swallowed juice of khat (Kalix, 1984). Then, it gets rapidly absorbed from these sites into the blood stream (Kalix and Braenden, 1985).

1.4.1.2. DISTRIBUTION OF KHAT

Alkaloids together with other components of khat are distributed to different parts of the body through the blood stream. Among the alkaloids, cathinone has rapid and intense action because it is highly soluble in lipid and has access to pass the BBB to enter the brain (Zelger et al., 1980).

1.4.1.3. BIOTRANSFORMATION OF KHAT

The biotransformation of khat is variable because it has various components with different chemical properties (Brenneisen and Geisshusler, 1985). Cathinone, mainly present in young leaves, has short lifetime and it becomes transformed into norephedrine in the liver. As a result, the amount of norephedrine which is absorbed from khat is found to be high in the

plasma (Brenneisen et al., 1986). On the other hand, cathine cannot easily get biotransformed into other forms because it is an inactive alkaloid of khat (Kalix, 1984).

1.4.1.4. EXCRETION OF KHAT

According to Toennes and Kauert (2002), cathinone, which has shorter elimination half-life than cathine, can only be detected in blood about 10 hours after ingestion. It is eliminated almost exclusively in the form of norephedrine and only about 2% of it remains unchanged. The biotransformation of cathinone to norephedrine consequently increases the concentration of norephedrine to be excreted. Unlike cathinone, cathine has longer half- life and it is eliminated very slowly (Tariq et al., 2002).

1.4.2. PHARMACODYNAMICS OF KHAT

The pharmacodynamics of khat is mostly explained through the basic properties of its components. Cathinone is highly responsible for achieving sympathomimetic effects and CNS stimulation analogous similar to the effects of amphetamine (Schechter et al., 1984). These effects include elevated blood pressure, mydriasis, hyperthermia, anorexia, insomnia, alertness, elevated mood, psychosis, and talkativeness (Mekasha, 1984; Al- Mamary et al., 2002). In addition, other components of khat have their contribution for various clinical complications such as, constipations due to tannin (Halbach, 1972), impotence, respiratory problems (Kennedy et al., 1983).

1.4.2.1. EFFECT OF KHAT

The effects of khat chewing were reported in the literature as early as 1237 by the Arabian physician Naguib Ad din (Lebras and Fretillere, 1965), who proposed the use of khat for the treatment of depressive states. By the same year, other writers also reported that it was effective in blunting the sensation of hunger and fatigue (Krikorian, 1984; Lebras and Fretillere, 1965). At any rate, khat chewing has both pleasant and unpleasant effects, which can be explained by the pharmacodynamics of the alkaloids especially cathinone and cathine (Kalix and Braenden, 1985).

Consuming khat juice from young leaves stimulates brain and spinal cord (through synapses) resulting in the desirable effects (Kalix, 1984), which are perceived by addicted individuals. The pleasurable effects are relief from fatigue, euphoria, increased alertness and energy level, feelings of excitement, improved ability to communicate, enhanced imaginative ability and capacity to associate ideas and heightened self-confidence (Labras and Fretillere, 1965). In experimental animals on the other hand, ingestion of khat extract produces excitation and increased motor activity (WHO, 1980; Kalix and Braenden, 1985). It also increases metabolic rate and oxygen consumption, causes hyperthermia. These effects have been attributed to the cathinone, a sympathomimetic amine with properties similar to those of amphetamine (Kalix, 1992); although other less potent stimulant substances namely, norpseudoephedrine (cathine) and norephedrine are also present (Al- Motarreb et al., 2002). Cathinone, like amphetamines, exerts these sympathomimetic effects by penetrating intraneural sites (Kalix, 1992) and promoting the presynaptic release of neural dopamine (Kalix, 1990).

Chewing of khat can also causes unpleasant effects including serious impairment of sexual function, rapid heartbeat and rise in blood pressure through noradrenaline (norepinephrine)

release from peripheral neurons similar to amphetamine (Widler et al., 1994; Kalix, 1992).Undesirably, khat might also lead to malnutrition and increased susceptibility to infectious diseases such as tuberculosis because of its contribution to prolonged anorexia (Kalix, 1992). Further more, khat results in gastrointestinal tract (GIT) problems due to the high content of tannin in the leaves (Kalix, 1984; Kalix, 1992). The tannin causes constipation (Makonnen, 2000), periodontal disease, mucosal lesions, and a number of upper GIT disorders, such as esophageal carcinoma (Giannini et al., 1986; Halbach, 1972). In experimental animals, khat extract causes gastritis and duodentis (Kalix, 1992). Apart from these, chronic khat use causes neurodegenerative disease resulting in CNS problems (Carvalho, 2003). The symptoms for CNS problems are anorexia, insomnia (delayed bedtime), late wake up the next morning and low performance the next day, which might be due to the central and peripheral actions of cathinone and cathine in the khat leaves (Hassan et al., 2002).

The long-term effect of chewing khat causes cerebral hemorrhage as observed in laboratory animals (Halbach, 1972; Pantelis et al., 1989), which is suggested to be one of the causes for stroke (Couci et al., 1988). The consequence is quick degeneration of brain cells, which in turn leads to dizziness, loss of balance or coordination. In addition, magnetic resonance imaging (MRI) of khat-addicted individuals has shown the presence of a continuing diffuse, extensive abnormal signal in the white matter of both cerebral hemispheres with marked cortical atrophy (Morrish et al., 1999). This contributes for the development of psychiatric diseases and khat related psychoses (Alem and Shibre, 1997).

Over use of khat is normally recognized as a causative factor of khat related psychosis (Khattab Galal, 1995). According to Pantelis and collaborators (1989), in khat related psychosis, two types of reaction are commonly observed. First, there is a paranoid psychosis

with prominent delusions of persecution often associated with auditory hallucinations. This pattern most closely resembles the paranoid psychosis seen with amphetamines. Other first rank symptoms that are present in this subgroup of khat psychosis are found to be passivity while doing tasks (Alem and Shibre, 1997). The secondary psychiatric disorder related to khat chewing is typified by manic illness with grandiose (false impression) usually without hallucinations and with less common response of a depressive illness. However, such type of psychosis is a rarely occurring phenomenon (Halbach, 1972) and seems to occur only after consumption of exceptionally potent material (khat with high amount of cathinone and cathine) and /or in predisposed persons (Kalix, 1984)

Additional effect of khat on brain that is noticed through non-lethal dose administration of cathine is signs of excitation with loss of motor coordination (Halbach, 1972).

1.5. COMBINED EFFECT OF ETHANOL AND KHAT

The prevalence of concomitant use of khat chewing and drinking alcohol has sharply increased particularly in the young generation of Ethiopia (Mesfin et al., 1999). It appears that these individuals are attracted by the antagonistic effect of ethanol on the stimulant and insomnial effect of khat (Kennedy et al., 1983). However, drinking ethanol after khat chewing is found to exacerbate the risk factors of clinical complications of both ethanol and khat (Omolo and Dhadphale, 1987). The complications are fatal for those who are chronically addicted with khat and alcohol (Kassie et al., 2001). Though alternate use of khat and ethanol is serious and fatal (Pantelis et al., 1989), there is no research done on the combined effect of khat and ethanol.

Concomitant use of khat and ethanol may not only affect the health of individuals but also has serious socioeconomic consequences. The potential adverse socioeconomic problems that are observed in individuals who are chewing khat and drinking alcohol are diversion of income to purchase these drugs and neglecting their families' needs (Kenndey et al., 1983). Consequently, these individuals face family instability (Elm, 1983) and divorce (Baasher and Sadoun, 1983). Moreover, it appeared that they are encouraged to prostitution, absenteeism from work and criminal behavior (Elm, 1983; Ayana and Mekonen, 2004).

1.6. SIGNIFICANCE OF THE STUDY

According to the World Bank (2004) report, Ethiopia is an extremely poor country whose people live below poverty line and its citizens earn only 100 US dollar per annual per capita. The future progress and development of the country as well as growth of its economic standard to alleviate such economic problems relay on its youth. However, the current social situation seen in the country regarding the fast increase of chewing of khat coupled with drinking alcohol, will ultimately affect the health of the individuals involved in particular and the country's future in general. Hence, this issue requires attention and awareness by government, policy makers, and the society at large. The scientific investigation that could show the direct effect of these drugs on health condition of exposed experimental animals may provide evidence to create awareness and search solutions to alleviate the prevailing and the coming social problems that is caused by the drugs in the youth of this country.

Despite the seriousness of the problem, there is scarcity of biomedical research on the effect of khat and its combined effect when taken with alcohol and deserves investigation. Therefore, the present study is addressing this issue by looking at the effect of khat extract and ethanol on the histology of the cerebellar cortex of the rat.

2. OBJECTIVES OF THE STUDY

2.1. GENERAL OBJECTIVE

- To investigate the effect of ethanol, khat and combination of the two on cerebellar cortex

2.2. SPECIFIC OBJECTIVES

- To assess the body weight change of the rats after treatment
- To observe the brain and cerebellum weight changes at the end of administration
- To assess the microscopic changes of cerebellar cortex after ethanol and khat treatment
- To determine diameter, numerical density, total number and volume fraction of Purkinje neurons and volume of cerebellar cortex after treatment of ethanol, khat and combination of the two

3. MATERIALS AND METHODS

3.1. PLANT MATERIAL COLLECTION AND EXTRACTION

Khat leaves grown in Gelemso (Ethiopia) were purchased from a local market in Addis Ababa. Methods developed by Connor et al. (1999) and Makonnen (2000) 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 crushed 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 concentrated using a Rota-vapor under low pressure. The concentrated extractant was then poured on a petridish and subject 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 used. 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.

3.2. ANIMAL PREPARATION

Pregnant white Wistar rats were obtained from the Animal House of Pharmacology Department, Faculty of Medicine, Addis Ababa University and were housed in a standard plastic cage on straw bedding in a temperature controlled room (21 ± 1^0C) 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.

3.3. ANIMAL TREATMENT

Two groups of pups and a group of young rats were used in this work. The first group was composed of pups of PND 6, the second group was pups of PND 13 and the third group consisted of young rats of PND 30. These groups were further categorized randomly into control, ethanol treated, khat treated and combination of ethanol and khat treated groups; where each category contained (n=5 pups or young rats /group).

On each day of the experiment, all the three groups of 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 distilled water. Test substances as well as the vehicle were administered into the stomach via the mouth of the rats through a blunted feeding needle fitted to 2.0 ml and 5.0 ml syringe for pups and young rats, respectively every day for one month. The ethanol groups received 3ml/100 gm body weight of 20% ethanol and their control group received the same amount of vehicle (distilled water) according to their weight. The khat groups received 20 mg/100 gm of body weight khat suspension and their control received vehicle (2% Tween 80 in distilled water) corresponding to their body weight. The rats that would take combination of ethanol and khat received 20 mg/100 gm body weight of khat first and 20% of ethanol (3ml/100 gm of body weight) after an hour, and their control received only vehicle (2% Tween 80 in distilled water).

Unlike the other categories, the combined groups contained only the young rats, for the pups belonging to the first and the second groups died after treatment within two days interval, even at small doses.

3.4. ANIMAL DISSECTION

3.4.1. ANIMAL PERFUSION

The brain of rat has small size, on top of this, it is soft in the fresh state and liable for physical injury. To avoid such situation it was preferably fixed by perfusion before exposing the brain from the skull.

Prior to all steps of perfusion, the perfusate solution 4% formaldehyde (see appendix A.1.1) in 0.1M phosphate buffered saline (see appendix A.1.2.) at PH of 7.3 was prepared, (Miki et al., 2000). The animals were deeply anesthetized with diethyl ether. This was achieved by putting the animals in a tight dissector jar having cotton soaked with diethyl ether. The rats were then placed in supine position over an operating board and their limbs were stretched using pins. The thoracic cavity was opened by para-sagittal skin incision. The ribs were reflected and held laterally. An 18-gauge needle, which was attached to a clear plastic tube connected to the syringe with perfusate, was inserted into the left ventricle and tied with artery forceps (see fig. 1). To ease perfusion, the whole tube connections had been freed of air bubbles prior to connection. The right atrium was then opened with a pair of scissors to allow the blood and the fixative leave the body during perfusion. By applying the gravity method, the rat was perfused 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 (Zeman and Innes, 1963).

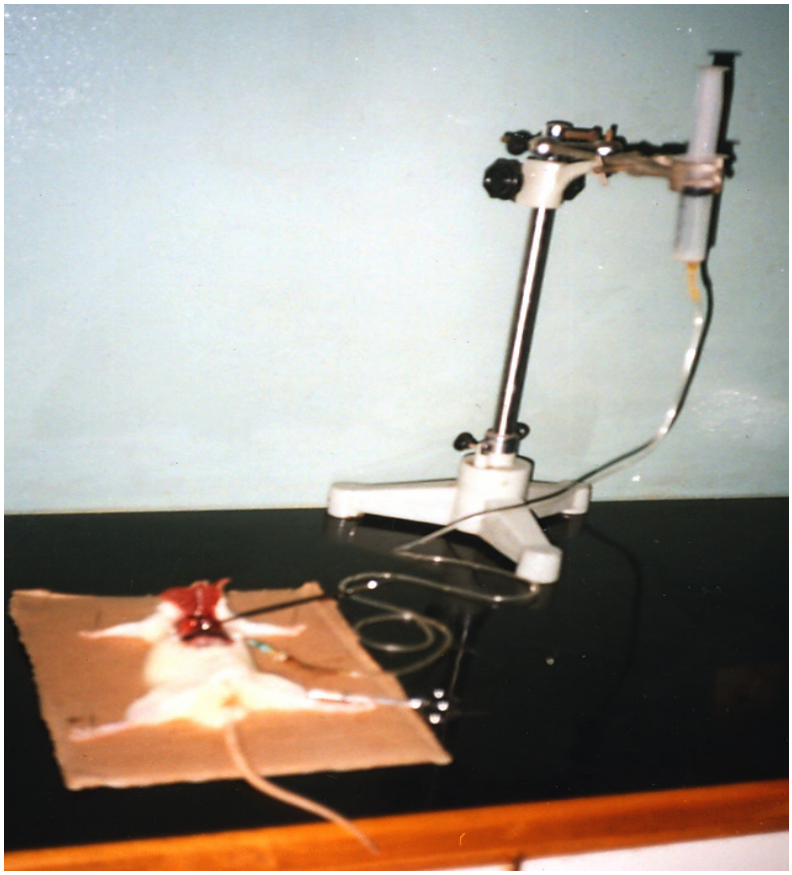


Figure 1: Brain perfusion by inserting needle, which was attached to a clear plastic tube connected to the syringe with perfusate, through the left ventricle

3.4.2. BRAIN DISSECTION AND FIXATION

After perfusion, the pins were detached from operating board and the rat was placed in a prone position. The skin located on the head region 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 sagitally until it reached to lambda by a small bone cutter. Continuously, the skull was cut sagitally from lambda to the foramen magnum. The dissected portion of the skull was reflected laterally to expose the brain as a whole. Finally, the brain was separated from spinal cord at the level of foramen magnum using a pair of scissors. Immediately thereafter, the entire brain was weighed (Adam Equipment electronic digital balance of 0.01 precision), immersed in fixative and kept in the refrigerator for 18 hours (Bancroft and Stevens, 1990).

3.5. SAMPLE SELECTION AND TISSUE PREPARATION

3.5.1. TISSUE SAMPLING

After fixation, the brain as a whole and cerebellum separately were weighed. Cerebellum was then cut according to stereological, multistage fractionator rules (Howard and Reed, 1998). 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 (Miki et al., 1999). 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 mm^2 (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 and sectioned with a thickness of 6 µm (Miki et al., 2000). About 400 sections were obtained from each block. For the purpose of stereological analysis every 20^{th} section of the tissues were collected in random fashion. The probability of any tissue to be selected is 1 /20(f1=20).

3.5.2. TISSUE PROCESSING

Tissue processing after fixation for routine histological section preparation encompasses four basic procedures, namely dehydration, clearing, impregnation and staining. 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-

60^0C, Dongnam petrochemical MFG. Co. Ltd, Korea). See appendix A.2. for detailed procedure.

As described in section 3.5.1, each tissue blocks were sectioned on Zeiss Microtome (Carl Zeiss Zunch AG, West Germany) and collected into egg albumin coated microscopic slide (see appendix A.3. for egg albumin preparation). Just after sectioning, the slides were put in a 60^0C oven for 8 hours (Bancroft and Stevens, 1990) to fix the tissue firmly on the slide. Subsequently, sections were deparraffinized and cleared with two changes of xylene, for 5 minutes each, and hydrated with decreasing alcohol concentrations (absolute alcohol, 90%, 70% and 50% of alcohol for 5 minutes each). The sectioned tissues were then stained with toluidine blue for 20 minutes (see appendix A.4. for toluidine blue preparation) and washed under running tap water to avoid over staining. Stained tissues were then dehydrated and cleared in a reverse direction to maintain the quality of stain. Finally, tissues were mounted in pertex (medite GmbH, Wollenweberstrasse12, D-31303 Burgdorf, Germany) and cover slipped. See appendix A.5. for detailed staining procedure.

3.6. MICROSCOPIC EXAMINATION

Slides were examined with a Zeiss binocular microscope (Carl Zeiss, Axiostar, Germany) fitted with x10 (to see change in folia and interfolia size) and x 40 (to examine neurons) 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). In addition, for stereological analysis randomly selected areas in the sections of cerebellum were photographed with x 2.5 (for estimation of volume) and x 25 (for estimation of numerical density and volume fraction).

3.7. STEREOLOGY

Stereology deals with a body of mathematical methods for the exploration of three-dimensional spaces when only two-dimensional sections through solid bodies or their projections are available (Mayhew and Gundersen, 1996). This mathematical method is reliable and unbiased to meet the goal of this investigation. It was done for the estimation of volume of cerebellar cortex, diameter of Purkinje neurons, numerical density of Purkinje neurons, total number of Purkinje neurons and volume fraction of these neurons. To estimate all those parameters, tissue samples were collected as described in section 3.5.1.

3.7.1. 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 (Pavlovic et al., 2003).

The mean diameter was calculated by using the formula,

$$D = \sqrt{a.b}$$ (Bedi and Warrant, 1988; Afework, 1988)

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

3.7.2. ESTIMATION OF VOLUME OF CEREBELLAR CORTEX

The most commonly used stereological method for estimating reference volume is the Cavalier method (Gundersen and Jensen, 1987). It was done by using exhaustive series of sectioned tissue in a fixed distance, T units. In this work, the volume of cerebellar cortex was

estimated by taking every 20th section of the tissue block sectioned at 6µm thickness. The sampling distance between every two successive sections was therefore 120µm. The serially sectioned representative sample of the tissues was photographed. A transparent calibrated point- counting grid (Grid P2) (from Agar Scientific Ltd.), was superimposed onto the picture (see appendix B.1.1.). The points overlie on the cerebellar cortex were counted. The area, which was represented by each point, was estimated by using point grid having known area associated with each point (a/p). Then, the volume of cerebellar cortex was estimated from the following formula (Howard et al., 1993).

$$\mathbf{V_{cc} = T.a/p.\ \sum P_i}$$

Where V_{cc}: Volume of cerebellar cortex

T: Fixed distance between parallel sections, in this case 120µm

a/p: Area associated with each point

P_i : Number of points landing within the cerebellar cortex transect on the i^{th} section

3.7.3. ESTIMATION OF NUMERICAL DENSITY OF PURKINJE NEURONS

The numerical density of neurons was estimated by the dissector method (Sterio, 1984). The dissector represents the ultimate minimalist approach to a three- dimensional probe. It consists of a pair of serial sections, a "reference" section and an adjacent section a "look-up" section, with known distance "h". According to the dissector method, neurons to be counted are those that appear in the reference section and not on the look-up section. This counting rule is unbiased estimator of numerical density (Mayhew and Gundersen, 1996). To apply this principle in the present study randomly selected area of cerebellar cortex from the reference section was photographed. A similar area of cerebellar cortex was also photographed from the look-up section. On the photographs the transparent point- counting grid (Grid F2 from Agar

Scientific Ltd., see appendix B.2.) was randomly thrown on the reference section and after that it was superimposed on the same area of look-up section. Then, neurons within the grid and those intercepted by the right vertical and top grid bars (acceptance line) were included in the count but those intercepted by the left vertical and bottom bar (forbidden line) were not counted. Counting was, therefore, made of the total number of Purkinje neurons appearing in the micrograph from the "reference" section, but not appearing in the corresponding micrographs of the "look-up" sections (Howard and Reed, 1998).

Then, the numerical density in any regions was calculated using the formula established by (Sterio, 1984; Mayhew and Gundersen, 1996).

$$N_A = \frac{1}{a/f.\ h} \cdot \frac{\sum Q^-}{\sum P}$$

Where, N_A: Numerical density of neurons

Q^- : Number of profiles seen on the reference section not on the look-up section

a/f: Area associated with each frame

h : Distance between sections

P: Number of frame associated points hitting the tissue

3.7.4. ESTIMATION OF TOTAL NUMBER OF PURKINJE NEURONS

Estimation of the total number of Purkinje neurons in cerebellum was calculated after estimation of the number of neurons per unit volume (numerical density) and volume of cerebellar cortex (Coggeshall and Lekan, 1996).

Thus, the total number of neurons was calculated by the following mathematical equation (Mayhew and Gundersen, 1996).

$N_n = N_A . V_{cc}$

Where N_n : Total number of neurons

N_A : Numerical density of Purkinje neurons

V_{cc} : Volume of cerebellar cortex

3.7.5. ESTIMATION OF VOLUME FRACTION OF PURKINJE NEURONS

The volume fraction of Purkinje neurons was estimated by throwing the grid (Grid P2 from Agar Scientific Ltd., see appendix B.1.2.) on the micrograph randomly and followed by counting points hitting the nerve cells separately and other parts of the tissue in general on the photograph. The volume fraction was then calculated using the following formula (Gundersen et al., 1987).

Vv (neuron, cerebellum) = ∑P(neuron) / ∑P (cerebellar cortex)

Where Vv: Volume fraction of Purkinje neurons

P (neuron) : Number of grid points falling within the image of Purkinje neurons

P (cerebellar cortex): Number of points falling within the image of cerebellar cortex.

3.8. STATISTICAL ANALYSIS

The data obtained from body weight, weight of brain and cerebellum; diameter of Purkinje, numerical density of Purkinje, volume of cerebellum and volume fraction of neurons of all groups were analyzed using version 10 statistical package for social sciences (SPSS). The significance difference among different categories of the same groups was tested by one way analysis of variance (ANOVA). All the data were presented as mean ± S.E.M. $P<0.01$ or $P< 0.05$ was considered statistically significant.

4. RESULTS

4.1. QUALITATIVE OBSERVATIONS ON RATS

At the end of each day of ethanol treatment, rats showed obvious signs of intoxications. Many of the intoxicated rats appeared to be asleep and depressed. All the rats, however, recovered from the symptoms of intoxication within a few hours after treatment. On the other hand, khat extract treated rats showed an increased motor activity and were restless. After a few hours of khat treatment, however, increased motor activity was followed by depression. Then, they returned to normal in around one and half hours. In the combined treated animals, khat extract enhanced motor activity but this was replaced by depression and the motor activity was also reduced as ethanol was administered after one hour of khat treatment.

At the end of the experiment (30 days), the animals were killed and all the parameters as described in the third section were studied, results obtained are summarized in table 1 and described in detail in subsequent paragraphs.

Table 1: Summery of results of the different parameters studied for each rat age groups and categories.

Parameters	**PND 6**				**PND 13**				**PND 30**			
	Control	ET	KT	CT	Control	ET	KT	CT	Control	ET	KT	CT
Body Weight												
Beginning	9.46	10.12	10.15	9.88	19.92	20.48	19.28	20.17	40.34	42.12	40.44	43.51
End	53.34	43.71	41.74	died	78.33	62.76	57.12	died	142.4	115.8	103.61	114.46
Brain wt.	2.10	1.55	1.70	died	2.19	1.85	1.97	died	2.42	1. 91	2.06	1.94
Cerebellum wt.	0.27	0.15	0.17	died	0.31	0.26	0.27	died	0.34	0.26	0.28	0.27
Mean diameter of Purkinje	16.96	12.10	15.02	died	17.03	16.22	16.48	died	17.47	16.28	16.87	16.63
Volume of cerebellum	1.0792	0.5573	0.968	died	1.2373	1.1387	1.1819	died	1.3604	1.2377	1.3323	1.2733
Numerical density	225000	255000	234000	died	219000	225000	222000	died	199500	210000	201000	207000
Total number of Purkinje	237900	147300	223400	died	262000	246200	254200	died	274300	262200	268800	265100
Volume fraction	0.0124	0.0140	0.0131	died	0.0088	0.0093	0.0090	died	0.0077	0.0084	0.0079	0.0081

4.2. BODY WEIGHT MEASUREMENT

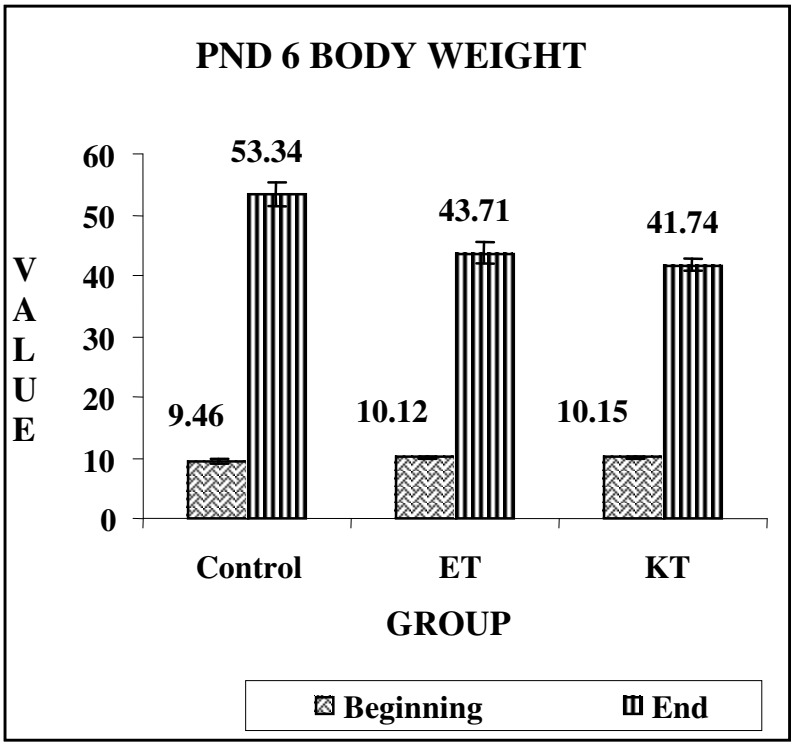
The body weight of the animals of PND 6, PND 13 and PND 30 was recorded just at the beginning of treatment and at the end of the experiment at which the animals were sacrificed for histological examination as indicated in Bar graph (Graph 1a-c). At the beginning of the treatment there was no statistically significant difference in the body weight among different categories within each age group. At the end of the experiment, the body weight of the rats of all groups was increased. However, the increment was different for each categories of the same age group.

The body weight increment for PND 6 rats 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 of PND 6 was increased 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 ($P>0.05$).

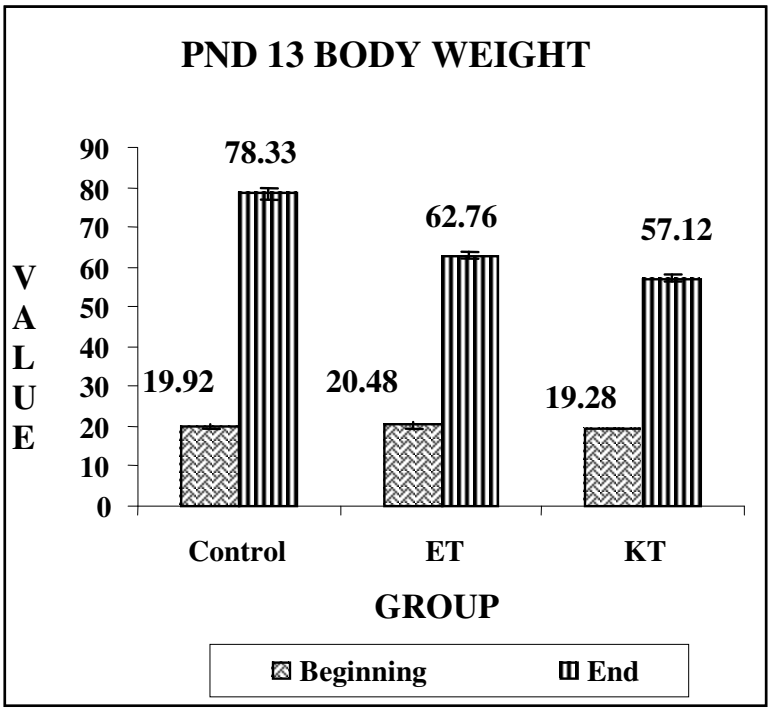
At the end of the treatment the body weight of the PND 13 groups was increased by 74.57%, 67.37% and 66.25% for the control, ET and KT rats, respectively. The result indicated that the body weight of the control was increased by 19.88% and 27.08 % as it was compared with the ET and KT rats, respectively. There was a statistically significant difference in the weight of all the groups ($P<0.01$). No statistically significant difference ($P>0.05$) was observed between the experimental groups though the weight of KT group was less than ET by 8.99%.

At the end of the experiment, the body weight of the PND 30 rats increased by 71.67%, 63.63%, 60.97% and 61.99% for the control, ET, KT and CT categories, respectively. As was observed from the result, the body weight of the control increased by 18.67%, 27.24% and 19.62% when compared with the age match ET, KT and CT, respectively. It was statistically significant at the level of $P<0.01$. Among the treated groups the average body weight of KT (103.61± 0.79) treated rats were less than those treated with ET (115.8±0.81) and CT (114.46± 0.15) categories. However, the mean body weight difference among the treated groups was not statistically significant ($P>0.05$).

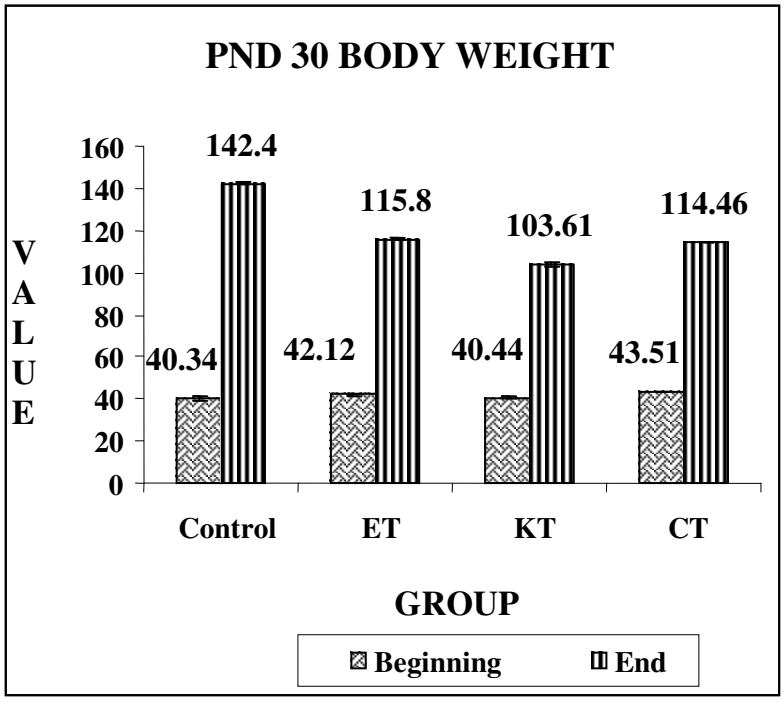
Graph 1a-c: Average body weight (g) of rats treated with ethanol, khat and combination of ethanol and khat and their age matched controls at the beginning of the experiment and after 30 days of treatment for PND 6, PND 13 and PND 30.



Graph 1a



Graph 1b



Graph 1c

4.3. BRAIN WEIGHTS

For the PND 6 rats, the average brain weight of the control was 2.1 gm., where as that of ET and KT were 1.55 gm and 1.7 gm, respectively (see table 2). This depicted that the brain weight of the control was significantly greater by 26.19% and 19.05% as compared with the ET and KT ($P<0.01$). 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, part of brain, of the ET (0.15 ± 0.01) was less than the control (0.27 ± 0.01) and KT (0.17± 0.01) animals. It was significantly decreased by 44.44% and 11.76% to their age matched control and KT groups, respectively ($P<0.01$). Moreover, the cerebellar weight of KT rats decreased by 37.04% from that of control. This was statistically significant ($P<0.01$).

The brain weights of rats of PND 13 were 2.19± 0.01 for the control, had 1.85 ± 0.02 for ET and 1.97± 0.01 for KT rats, (see table 2). These were analyzed by one-way ANOVA and ethanol and khat treatments significantly decrease brain weight by 15.53% and 10.04%, respectively ($P<0.01$) as compared with the weight of their corresponding controls. Furthermore, the weight of cerebellum, part of brain, of the control (0.31± 0.01) was greater than those of the ET (0.26± 0.01) and KT (0.27± 0.01) by 16.13% and 12.9%, respectively and this was statistically significant ($P<0.01$). In addition, the cerebellar weight was greater in KT than in ET groups though it was not statistically significant ($P>0.05$).

In PND 30 groups, the brain weight of the control was greater than those of the ET, KT and CT rats by 21.07%, 14.88% and 19.83%, respectively. The result was statistically significant ($P<0.01$). Among the experimental groups, the brain weight of KT was greater than ET and CT by 7.28% and 5.83% respectively. However, the result was significantly different only for ET rather than from the CT groups. Besides, the cerebellar weight , part of brain, of the

control was increased by 23.53%, 17.65% and 20.59% as compared with those of ET, KT and CT, respectively and the difference was statistically significant at the level of $P<0.01$. Among the experimental groups of animals, the cerebellar weight was greater in KT than ET and CT groups by 7.14% and 3.57%, respectively. It was greater in CT groups by 3.7% from the ET. However, the difference in the cerebellar weight among the different experimental animals was not statistically significant ($P>0.05$).

Table 2: Mean $\pm$ S.E.M. the whole brain and its part cerebellar weights (g) of rats treated with ethanol, khat and combination of khat and ethanol and their age matched controls after 30 days of treatment for PND 6, PND 13 and PND 30.

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
PND 13		
Control	2.19 ± 0.01	0.31 ± 0.01
ET	1.85 ± 0.02	0.26 ± 0.01
KT	1.97 ± 0.01	0.27 ± 0.01
PND 30		
Control	2.42 ± 0.02	0.34 ± 0.01
ET	1.91 ± 0.01	0.26 ± 0.01
KT	2.06 ± 0.01	0.28 ± 0.01
CT	1.94 ± 0.01	0.27 ± 0.01

4.4. MICROSCOPIC OBSERVATION OF CEREBELLAR CORTEX

Cerebellum was fixed and processed for light microscopic examination. Sections from the cerebellum of different group of animals were stained with toluidine blue. This helped to differentiate the white matter from grey matter. It clearly revealed the folia and interfolia of cerebellum. It also showed the three layers of cerebellar cortex. Furthermore, it assisted to identify the neurons, neuroglial cells and blood vessels.

In the molecular layer nerve fibers, a few dispersed neurons and lightly stained neuroglial cells were distinctly observed. 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, nucleus which is surrounded by Nissle substance was clearly observed. In most cases nucleus was located centrally. The Purkinje neurons contained one or two nucleoli where as in other layers mostly neurons contain 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 groups as studied by light microscope were as described below.

Post natal day 6 Rats

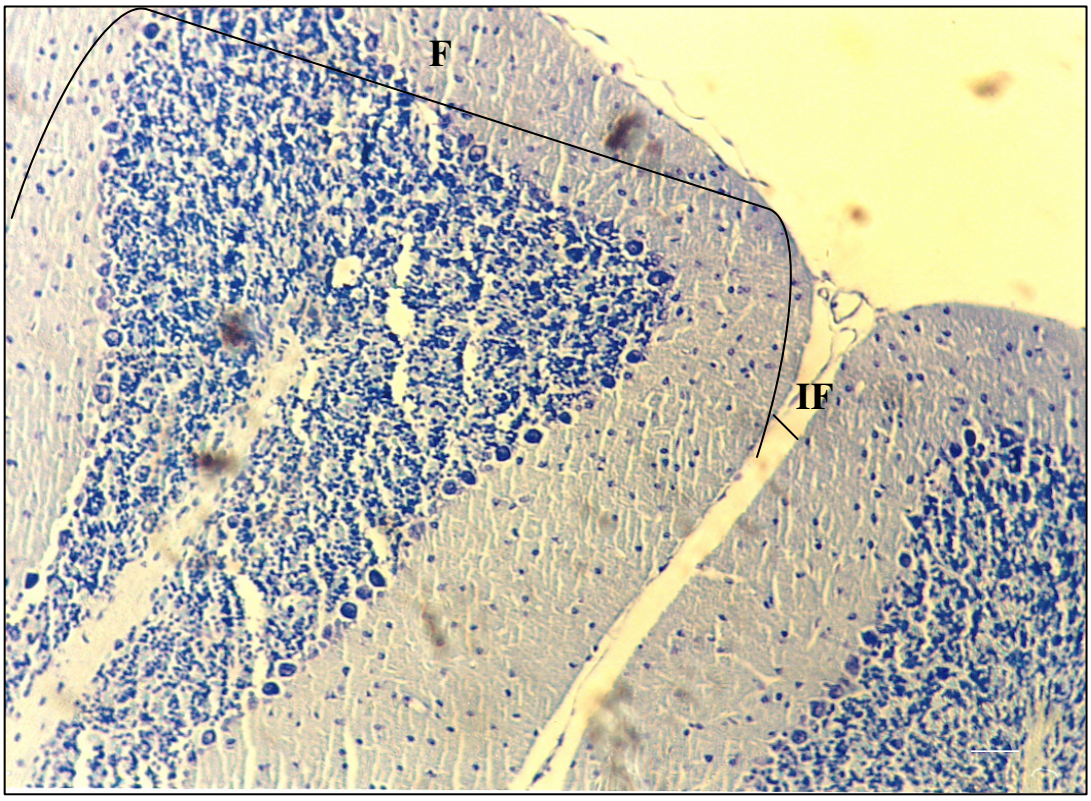
At the beginning of the experiment the PND 6 age groups comprised four categories, however, the combined (khat and ethanol) treated groups of rats died after two days of treatment similar to those of PND 13. Therefore, further investigations on the combination of khat and ethanol treated category in these two age groups were not carried out.

In the three categories of PND 6, Purkinje neurons with nucleus were seen clearly. In the control, ET and KT rats the nucleus in most case was located centrally (fig. 2a, 2b and 2c). On

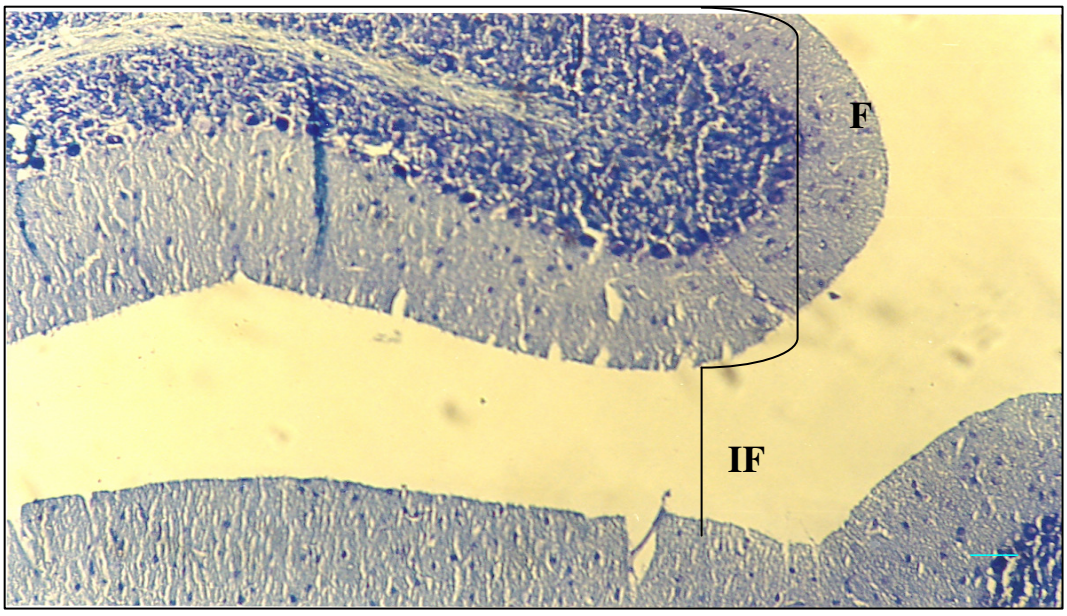
the other hand, the nucleus was also found eccentrically in the ET groups and a few neurons lack nucleolus in their nucleus (fig. 2b). The cytoplasm of the neurons was surrounded by Nissl substance and was dissoluted in the ET. Similarly, in some Purkinje neurons of KT animals the Nissle substance was dissoluted and some of the nuclei were pushed peripherally (fig. 2c). The dendrites of Purkinje neurons were also evident and projected to the molecular layer. In the molecular layer, dispersed neurons and neuroglial cells were observed. In the granular layer, different sized granule neurons were seen. Moreover, unlike the control and KT rats, the folia in the ET ones were smaller and interfolia were wider (fig. 6). Blood vessels were also observed in all categories.

PND 13 and 30 Rats

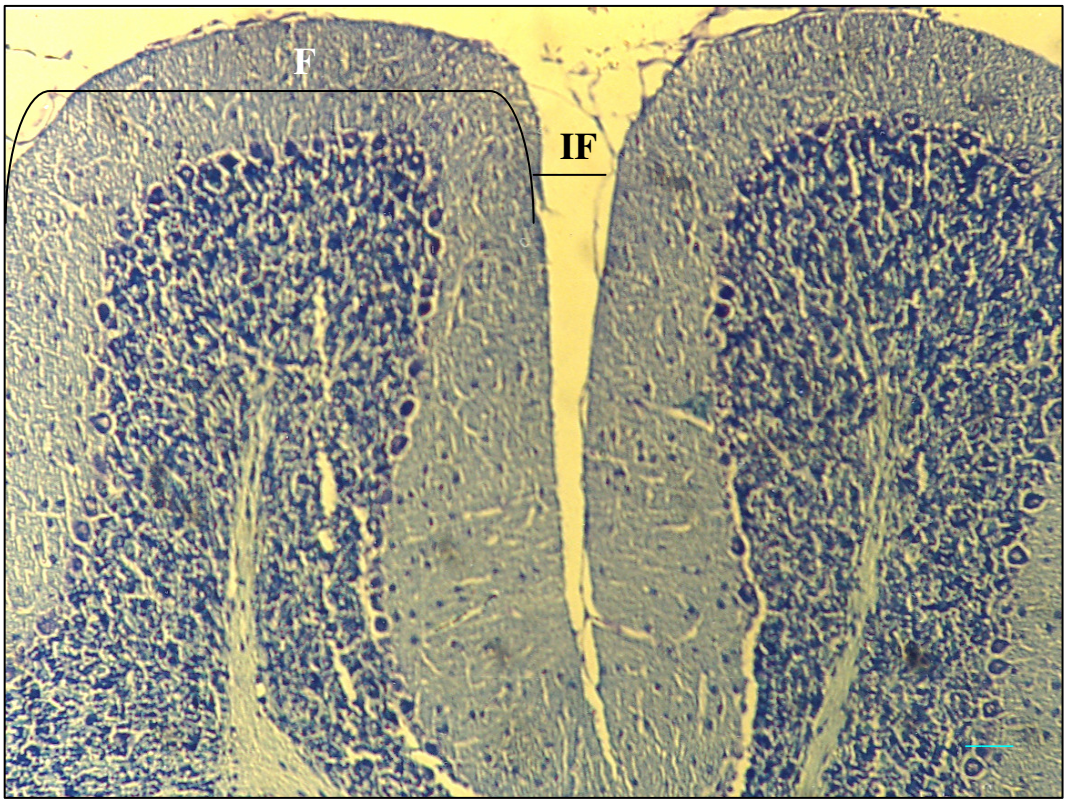
In both PND 13 and PND 30 age groups, there was no detectable morphological difference among the different categories as well as between the two age groups of rats (fig. 3a-c and 4a-d). The Purkinje neurons possessed centrally located nucleus. Densely stained Nissle substance was surround the cytoplasm of these neurons. Furthermore, the dendrites of Purkinje neurons were projected to the molecular layer. In the molecular layer unlike the granular layer, neurons were dispersed. In this layer, neurons with clear nuclei and nucleoli were clearly seen. In addition to neurons, glial cells were also observed. The granule neurons had variable size and were deeply stained. In some granule neurons, nucleus and nucleoli were clearly observed. Blood vessels were also evident. In these groups, the size difference of the folia and interfolia among the different categories was not detected.



2a: PND 6 - Control group



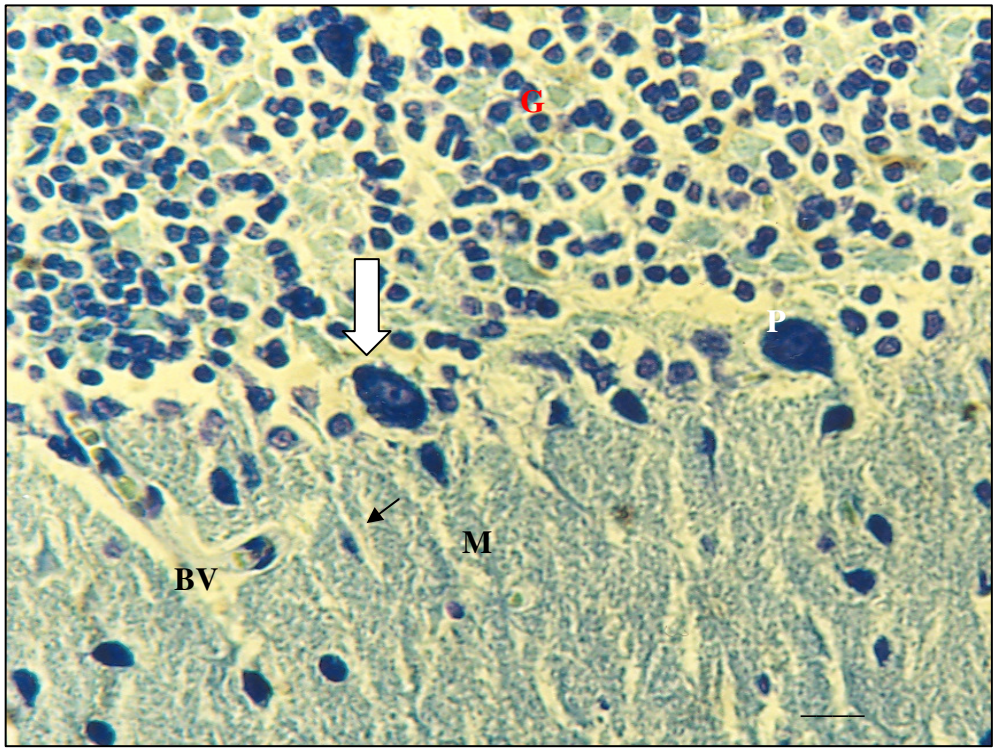
2b: PND 6 - Ethanol Group



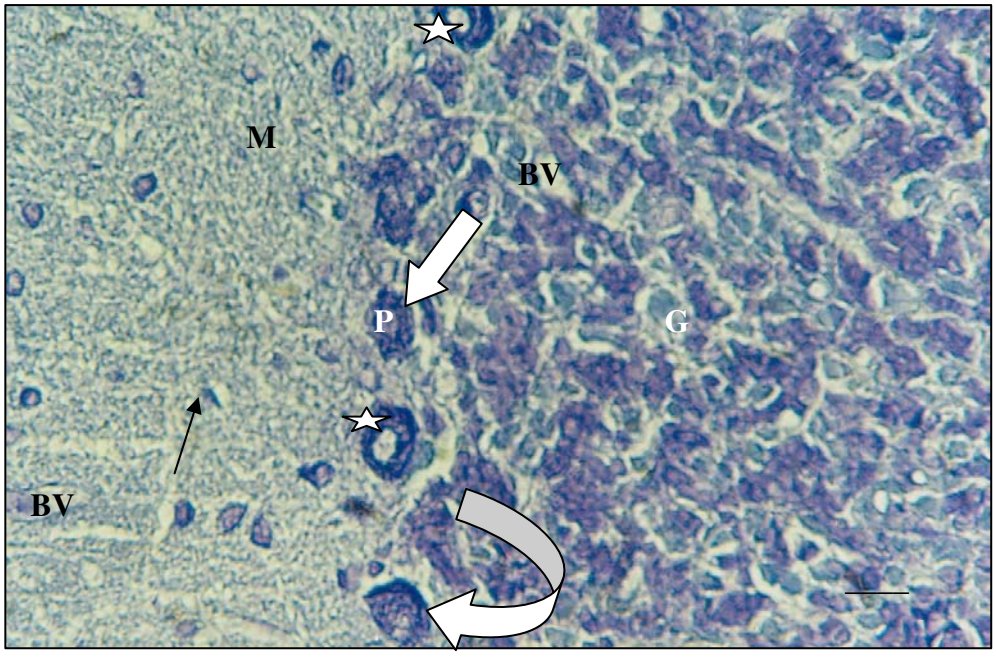
2c : PND 6 - Khat Group

Figure 2a-c: Photomicrographs of toluidine blue-stained paraffin sections of cerebellar cortex for PND 6 group illustrating the width of folia (F) and interfolia (IF). In the control (2a) and khat treated (2c) rats the respective width of folia and interfolia are almost equal. In the ethanol treated (2b) rats the interfolia (IF) is wider and thickness of folia (F) is smaller.

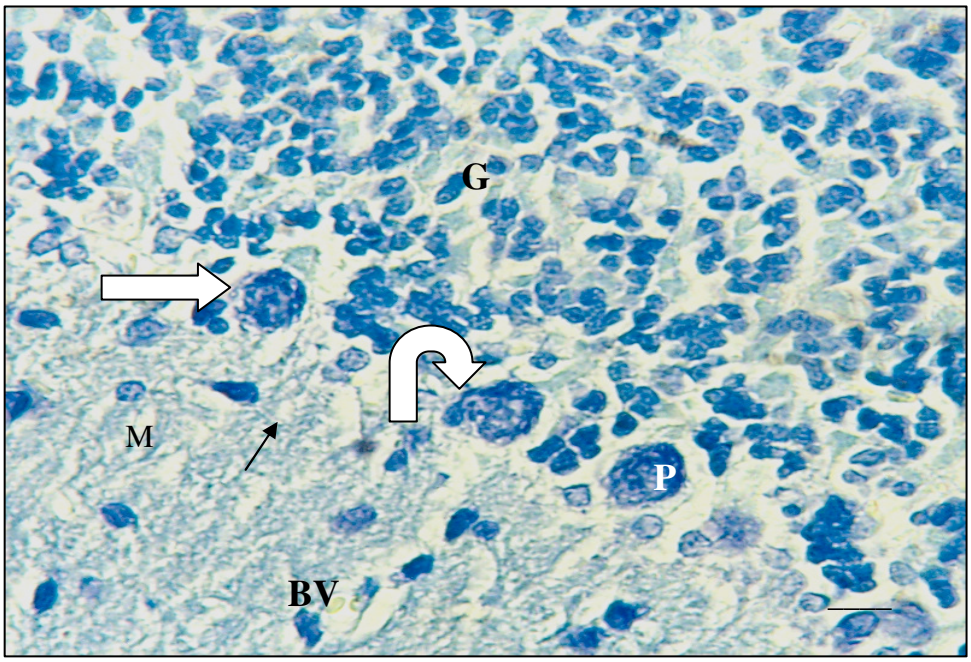
Bar = 21µm: x 140



3a : PND 6 - Control Group



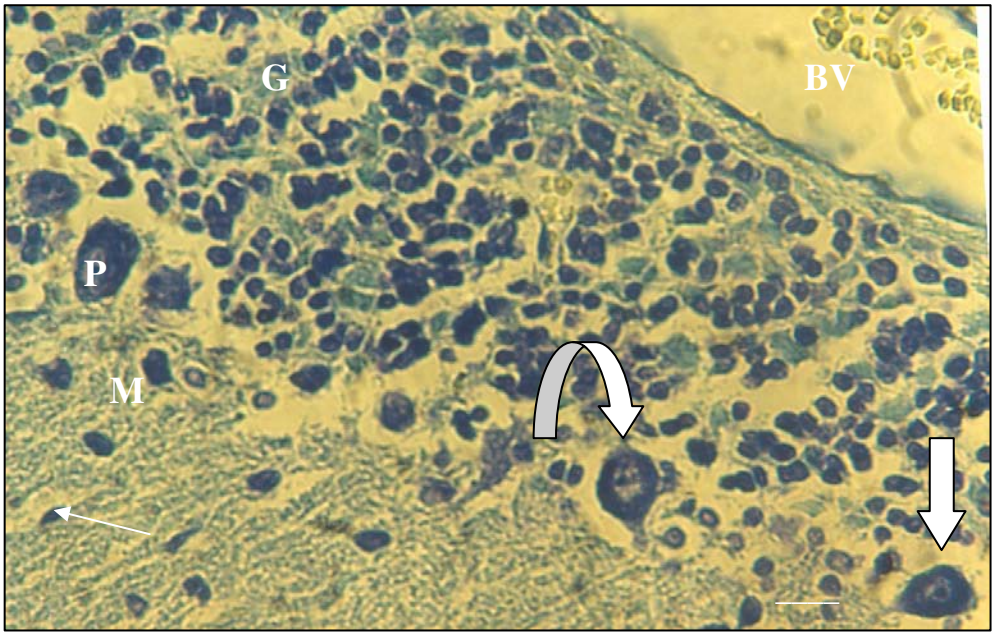
3b: PND 6 - Ethanol Group



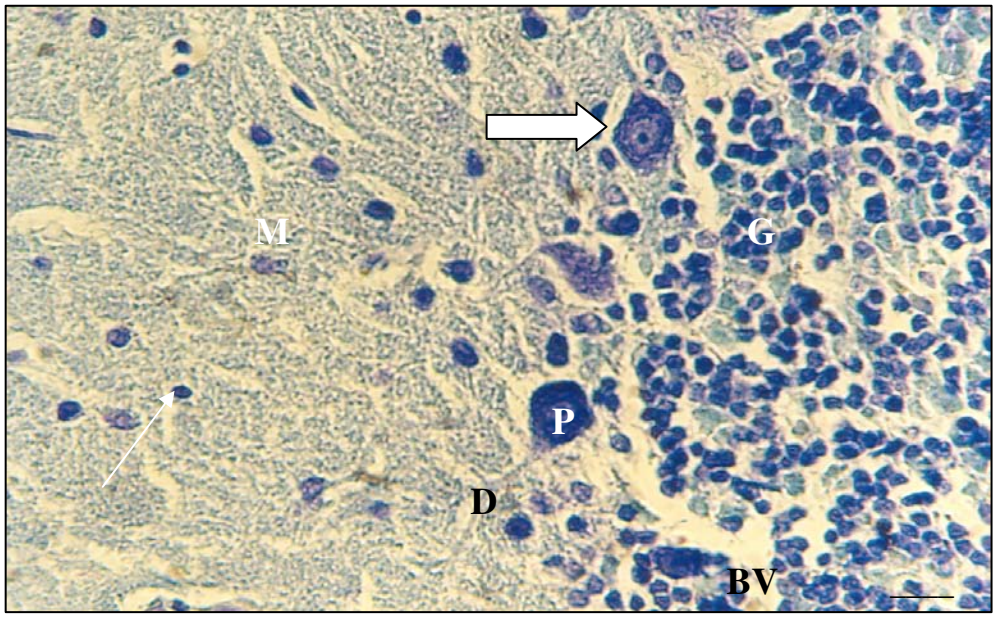
3c: PND 6- Khat group

Figure 3a-c: Photomicrographs of toluidine blue- stained paraffin sections of cerebellar cortex for PND 6 groups illustrating molecular layer (M), Purkinje cell layer (P) and granular layer (G). Neurons with clear profile and nucleoli, typical of those used for size determination in all categories are marked by thick white 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 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 is pushed peripherally (white curved arrow).

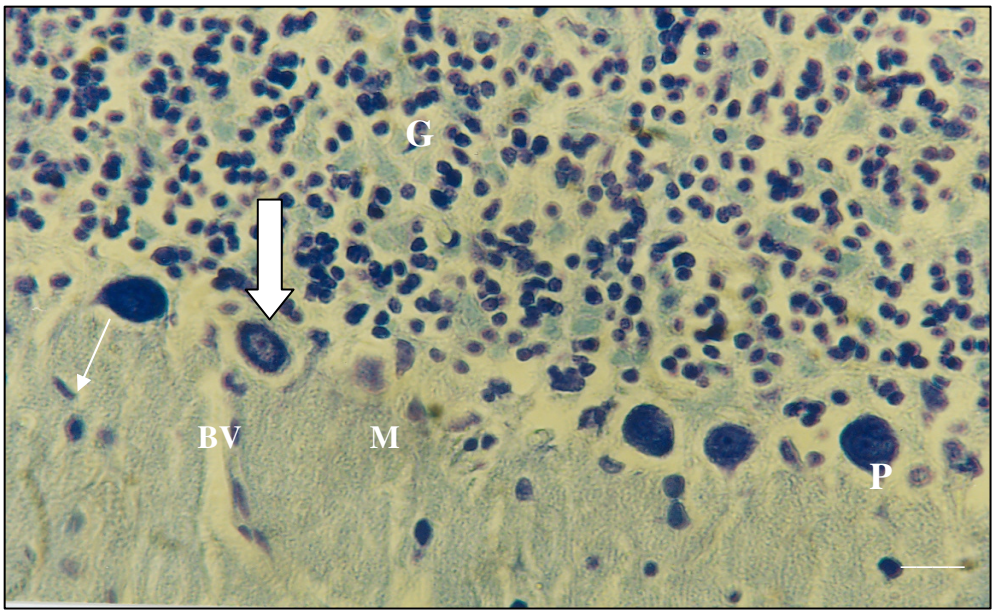
Bar = 17µm: x 590



4a: PND 13- Control Group



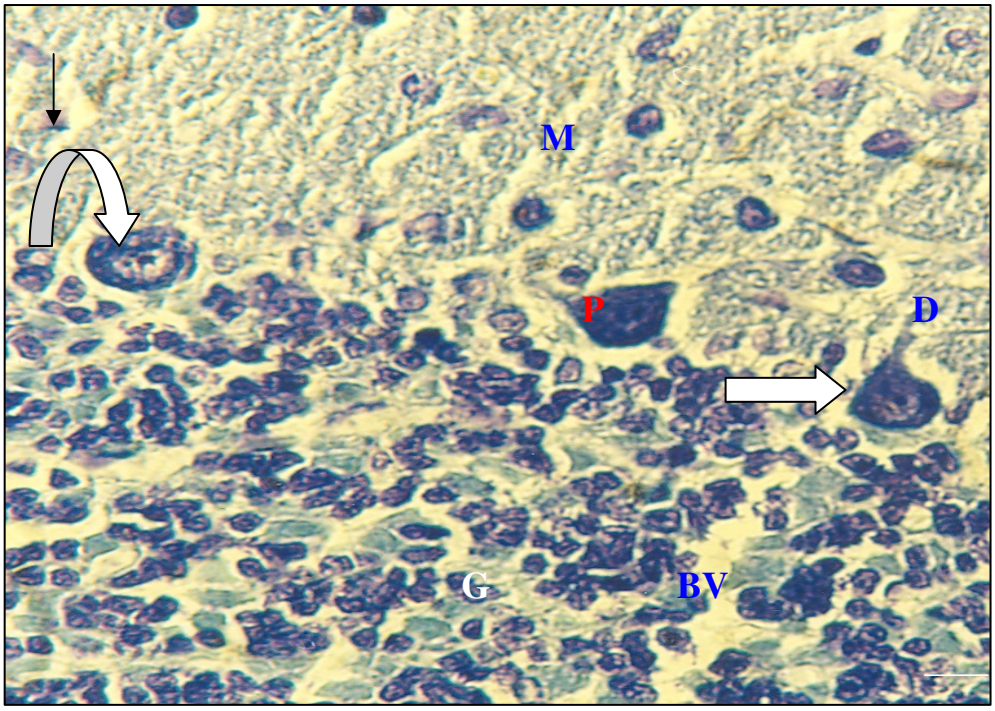
4b: PND 13 - Ethanol Group



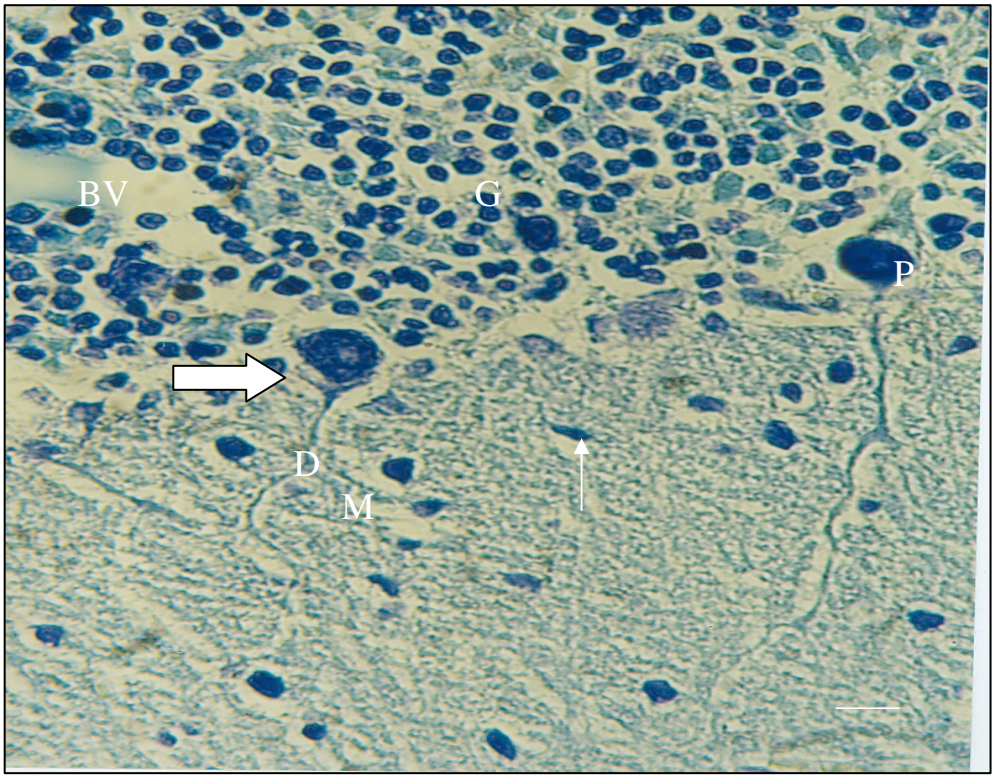
4c: PND 13 - Khat Group

Figure 4a-c: Photomicrographs of toluidine blue- stained paraffin sections of cerebellar cortex for PND 13 group illustrating molecular layer (M), Purkinje cell layer (P) and granular layer (G). Neurons with clear profile and nucleoli typical of those used for size determination in all categories are marked by thick white arrow. Neurons with two nucleoli can be seen (white curved arrow) and their dendrite is also seen (D). Neuroglial cells can be seen in the molecular layer (thin arrow). In addition, blood vessels (BV) were evident. In the control (4a), ethanol treated (4b) and khat treated (4c) rats, normal histological structure was observed.

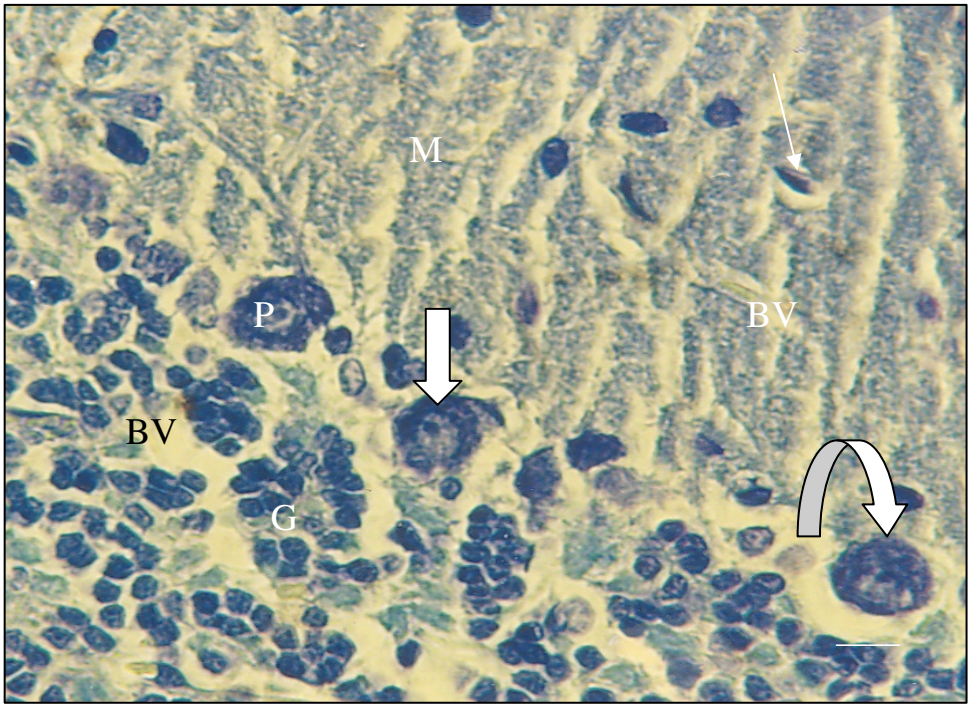
Bar = 17µm : x 590



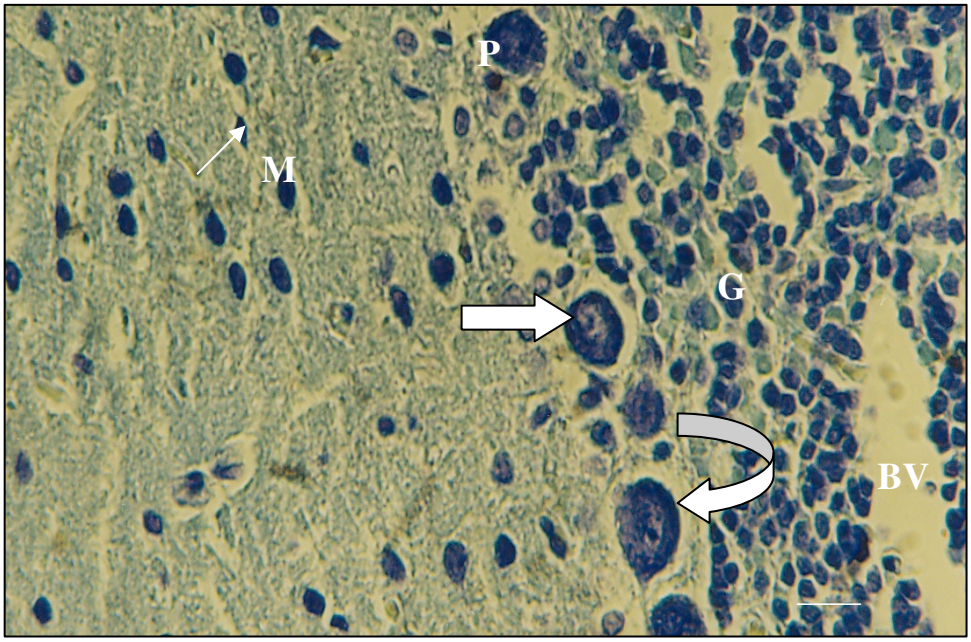
5a: PND 30 - Control Group



5b: PND 30 - Ethanol Group



5c: PND 30 -Khat Group



5d: PND 30 - Combination of Ethanol and Khat Group

Figure 5a-d: Photomicrographs of toluidine blue- stained paraffin sections of cerebellar cortex for PND 30 group illustrating molecular layer (M), Purkinje cell layer (P) and granular layer (G). Neurons with clear profile and nucleoli typical of those used for size determination in all categories are marked by thick white arrow. Neurons with two nucleoli can be seen (white curved arrow) and their dendrite is also seen (D). Neuroglial cells can be seen in the molecular layer (thin arrow). In addition, blood vessels (BV) were evident. In the control (5a), ethanol treated (5b), khat treated (5c) and combination of ethanol and khat treated (5d) rats, normal histological structure was observed. Bar =17µm : x 590

4.5. ESTIMATION OF MEAN DIAMETER OF PURKINJE NEURONS

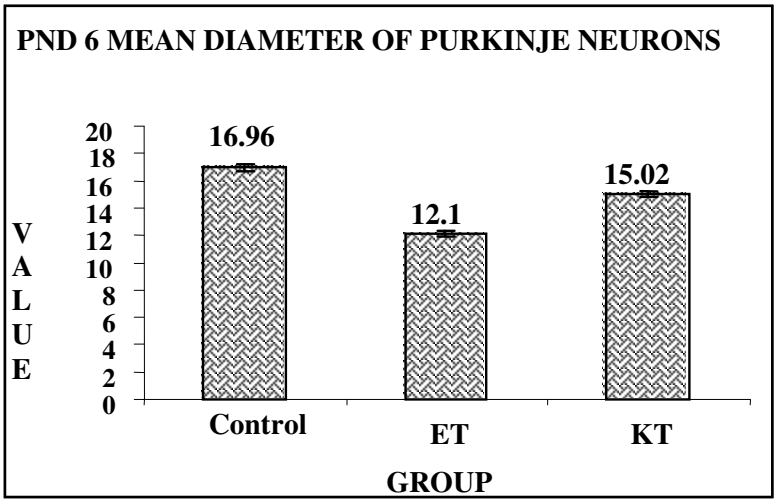
The size of Purkinje neurons was estimated from their mean diameter (D). The diameter of cell bodies of Purkinje neurons having clear nuclear profile was measured and expressed as mean $\pm$ S.E.M. The estimates of these neurons for PND 6, 13 and 30 rats after 30 days are as presented with Bar graph (graph 2a - c).

For PND 6 rats, the average diameter of Purkinje neurons of control groups was 16.96 micrometer (µm) where as it was 12.10 µm and 15.02µm for ET and KT categories. The mean diameter of the control was greater by 28.66% and 11.44% from those of the ET and KT animals, respectively and the difference was statistically significant at the level of $P< 0.01$. Furthermore, the size of Purkinje neurons was significantly greater in the KT than ET groups by 19.44%. Thus, the mean size of Purkinje neurons of animals exposed to ethanol was smaller than those animals exposed to khat extract and from the control groups. These differences were further reflected by *Post-hoc* analysis using *Scheffe* test.

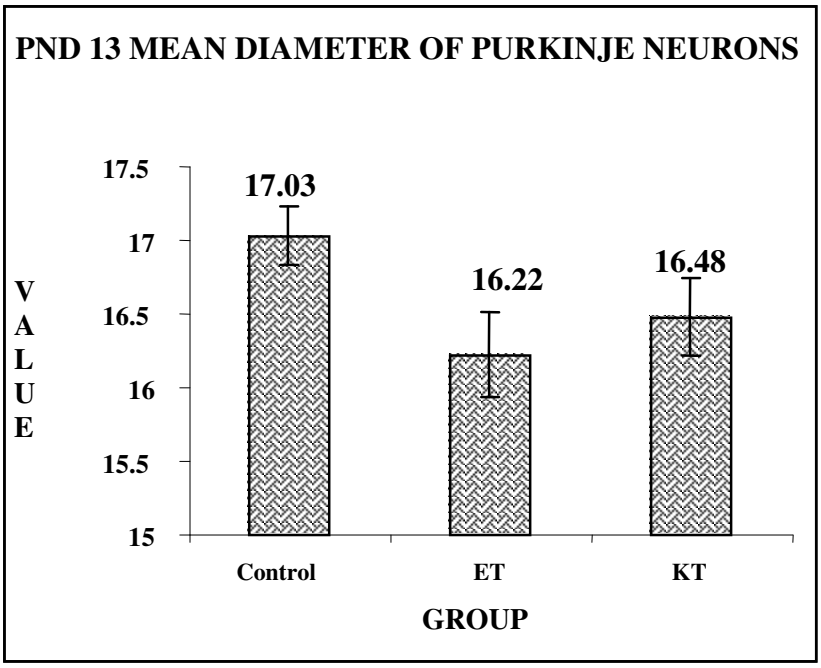
After 30 days of khat and ethanol treatment, the diameter of Purkinje neurons of 13 PND groups of animals was measured and analyzed by one-way ANOVA. The result revealed that the mean diameter of Purkinje neurons of the control groups had 17.03 ± 0.02, the ET had 16.22 ± 0.29 and KT had 16.48 ± 0.26. The result depicted that the mean diameter of Purkinje neurons of the control was greater by 4.76% and 3.23% as compared to ET and KT groups, respectively. These were, however, not statistically significant (P>0.05). Similarly, it was smaller in the ET than the KT animals by 1.58% although this was also statistically non-significant.

The mean diameter of the control, ET, KT and CT in the PND 30 groups were 17.47± 0.22, 16.28± 0.27, 16.87± 0.26 and 16.63± 0.13, respectively. This shows that the mean diameter of Purkinje neurons of the control was greater by 6.81%, 3.43% and 4.81% from ET, KT and CT, respectively. However, the differences in diameter were not statistically significant (P>0.05). Among the experimental groups of animals, the mean diameter of Purkinje neurons was greater in KT rats as compared with those of ET and CT categories by 3.5% and 1.42%, respectively. It was also greater in CT categories than ET by 2.10%. However, the mean diameter differences among the four categories of 30 PND rats were not statistically significant (P>0.05).

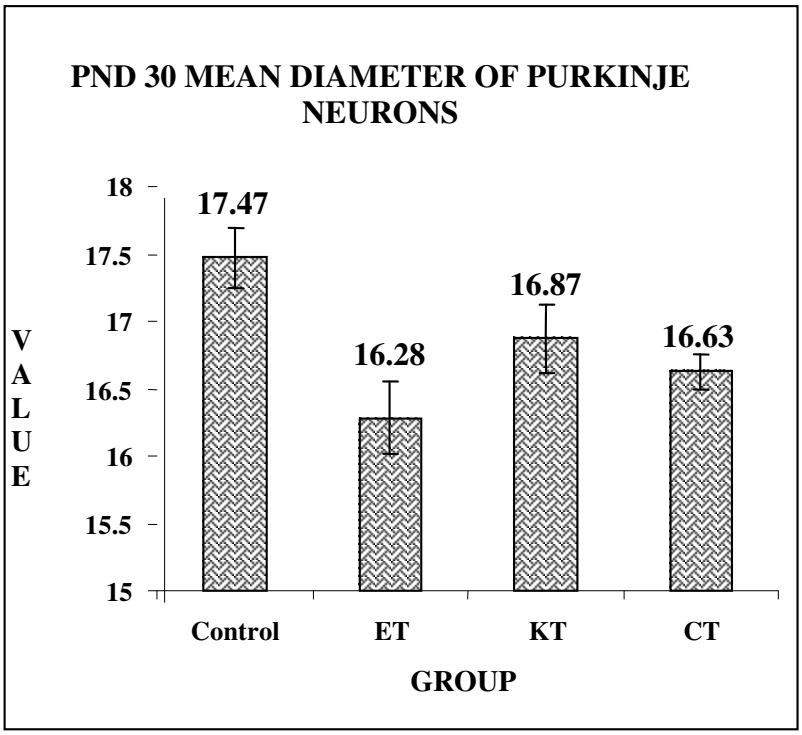
Graph 2a- c: Mean ± S.E.M. of Purkinje neurons diameter (µm) of rats treated with ethanol, Khat and combination of khat and ethanol and their age matched controls after 30 days of treatment for PND 6, PND 13 and PND 30.



Graph 2a



Graph 2b



Graph 2C

4.6. ESTIMATION OF VOLUME OF CEREBELLAR CORTEX

The volume of cerebellar cortex (mm^3) of the control, ET, KT and CT groups for each of PND 6, PND 13 and PND 30 rats as determined by the Cavalieri principle using micrographs as shown in figure 6 for PND 6 and the same type micrograph was used for the rest of the groups.

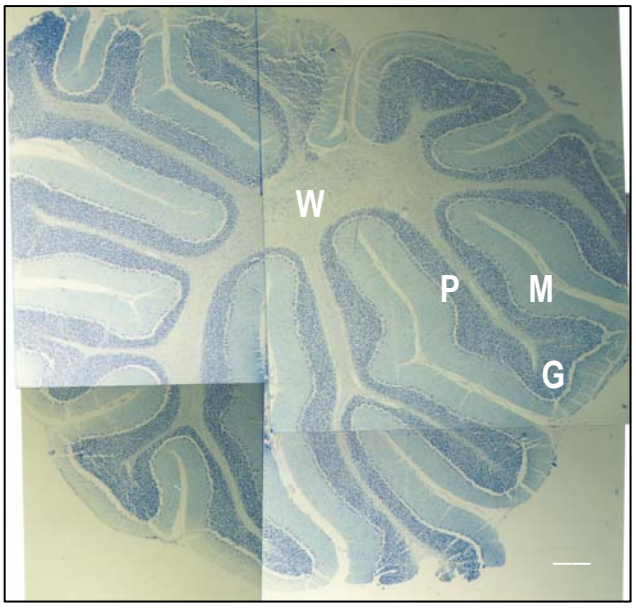


Figure 6: Photomicrograph of toluidine blue-stained paraffin sections of cerebellum of PND 6 used for determination of volume of cerebellar cortex. It was prepared using four photographs of the same tissue. The Purkinje layer (P) is arranged in a single row at the junction of molecular (M) and granular (G) layers. Adjacent granular layers are separated by white matter (W). Bar=28μm : x 36

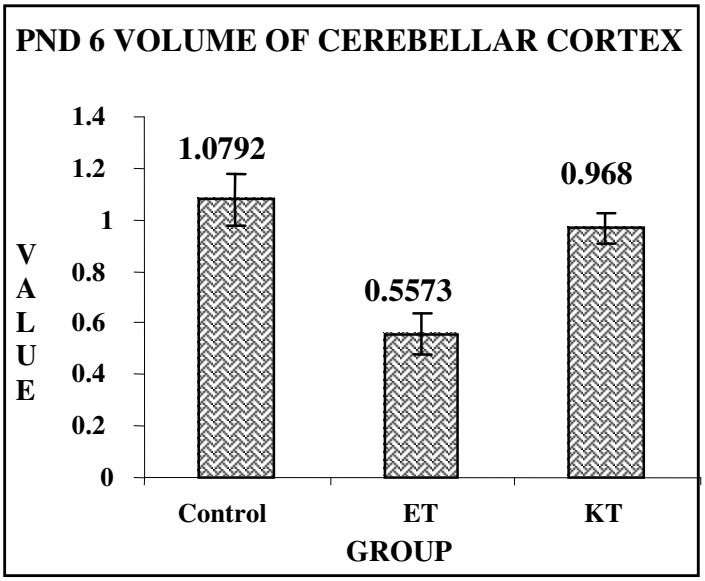
The volume of cerebellar cortex of PND 6 was estimated to be 1.0793 mm^3 in the control, 0.5573 mm^3 in the ET and 0.9680 mm^3 in KT groups. Hence, the volume of the cortex was smaller in ET than the control by 48.36%. This was statistically significant at $P<0.01$. The volume of cerebellar cortex of KT was also less by 10.30% as compared with the control; however, this was not statistically significant at $P> 0.05$. In addition, the volume of the cortex of ET was significantly reduced by 42.43% from those of KT group.

The volume of cerebellar cortex of the control in PND 13 groups of rats was greater by 7.97% and 4.48% than those of ET and KT animals, respectively. This was not statistically significant ($P>0.05$). In addition, it was greater in the KT than ET by 3.66%, although, it was not statistically significant at the level of $P> 0.05$.

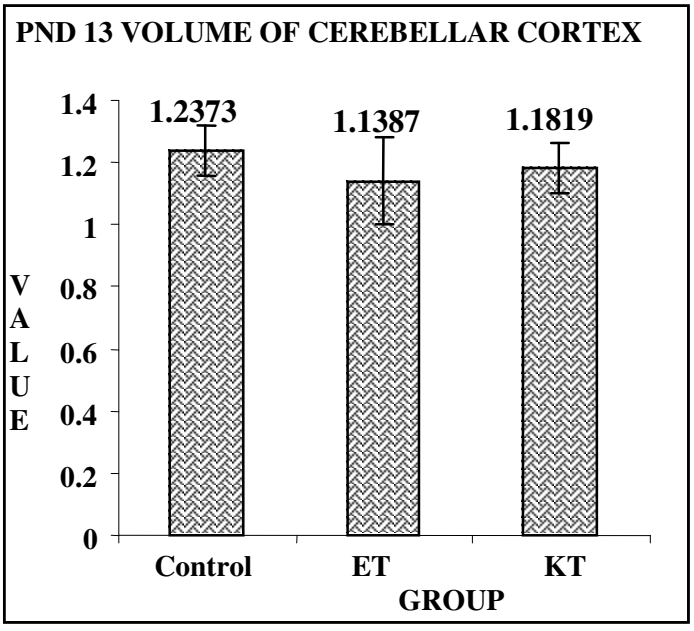
The volume of the cerebellar cortex of PND 30 was found to be 1.3604 mm^3 in the control groups, 1.2377 mm^3 in ET, 1.3323 mm^3 in KT and 1.2733 mm^3 in CT. This indicated that the volume of the cortex of the control was greater by 9.02%, 2.07% and 6.40% than ET, KT and CT groups, respectively. However, this was not statistically significant ($P>0.05$). In addition, it was greater in the KT than ET and CT by 7.10% and 4.43%, respectively. It was also less in ET than CT by 2.8%. One-way ANOVA of this age groups depicted that there was no statistically significant difference among these different experimental animals. In addition, for multiple comparisons the data were tested by *Post hoc* analysis using *Scheffe* test and non-

significant deficit was observed in volume of the cortex of the three experimental groups as compared with their age matched control and among the experimental groups themselves.

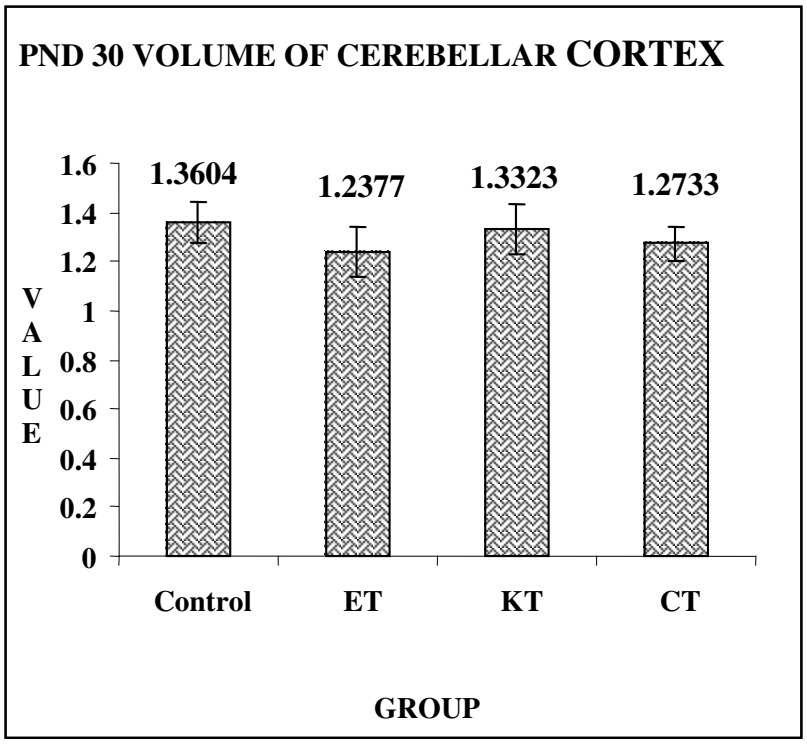
Graph 3a-c: Volume of cerebellar cortex (mm^3) of rats treated with ethanol, khat and combination of ethanol and khat and their age matched controls after 30 days of treatment for PND 6, PND 13 and PND 30.



Graph 3a



Graph 3b



Graph 3c

4.7. ESTIMATION OF NUMERICAL DENSITY OF PURKINJE NEURONS

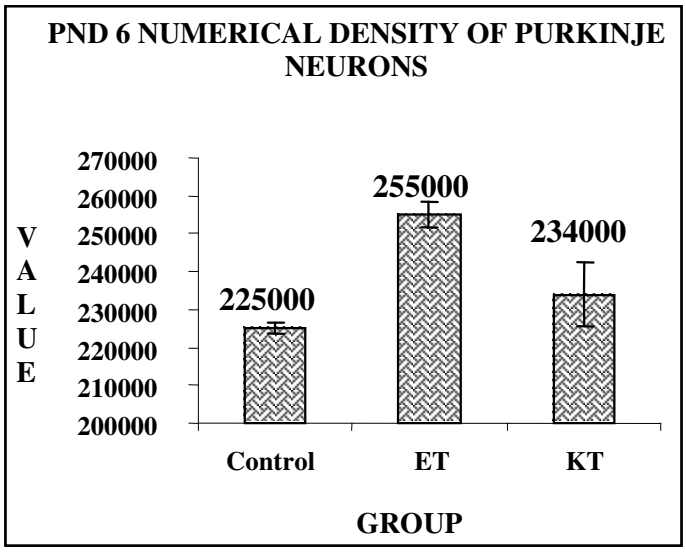
The numerical density of Purkinje neurons as determined by the physical dissector method for different categories of PND 6, PND 13 and PND 30 groups of the rats is shown with Bar graph (graph 4a-c).

The numerical density of Purkinje neurons at PND 6 was 225000/ mm^3 in the control, 255000/ mm^3 in the ET and 234000/ mm^3 in KT groups. This indicated that the numerical density of Purkinje neurons was greater in the ET than those of control and KT by 11.76% and 8.24%, respectively. It was statistically significant at $P< 0.05$. Furthermore, the numerical density of these neurons was less in the control than KT by 3.85%, although it was not statistically significant ($P>0.05$).

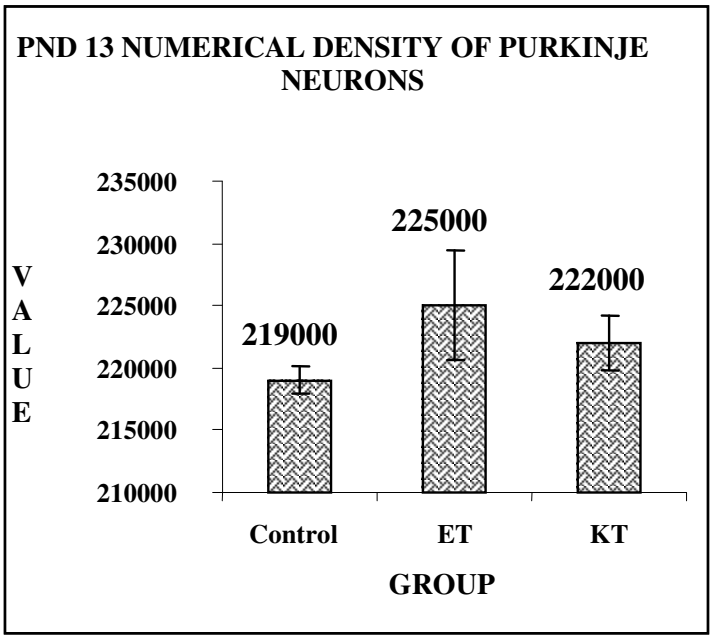
The numerical density of Purkinje neurons of the ET at PND 13 was greater by 2.67% and 1.33% than the age matched control and KT, respectively. It was smaller in the control than KT by 1.35%. However, one way ANOVA depicted no statistical significant difference at P>0.05 was observed not only among the control and different experimental groups but also between the experimental groups of animals. Such non-significant difference in numerical density was also reflected by *Post hoc* analysis using *Scheffe* test.

The numerical density of Purkinje neurons at PND 30 was greater in ET than those of control, KT and CT rats by 5%, 4.29% and 1.43%, respectively. This was not statistically significant at P>0.05. In addition, the numerical density of Purkinje neurons was smaller in the control than KT and CT by 0.75% and 3.62%, respectively. It was also smaller in the KT than CT by 2.9%. However, one-way ANOVA has shown non- significant difference in numerical density of Purkinje neurons among these different categories. The non- significant diffence in numerical density among the four categories was also reflected by *Post hoc* analysis using *Scheffe* test

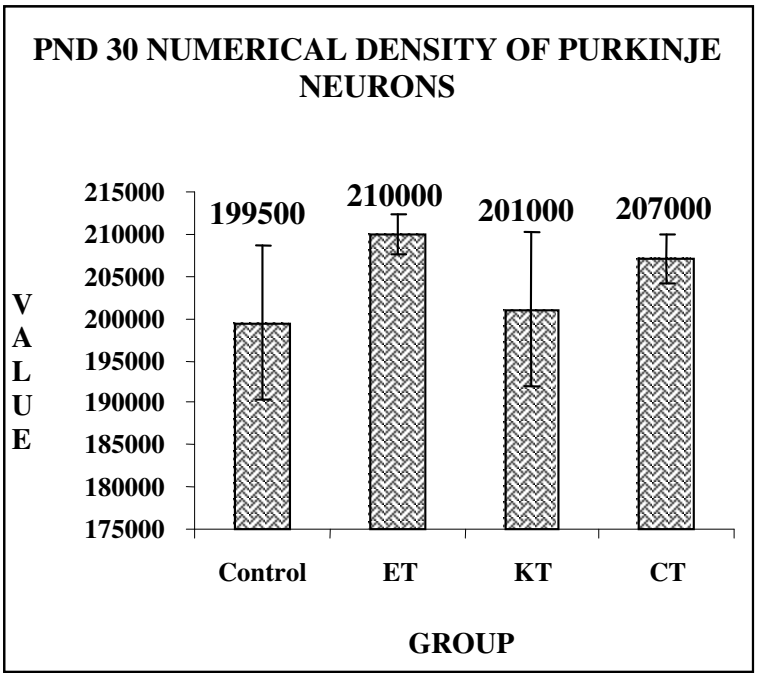
Graph 4a-c: Numerical Density of Purkinje neurons (per mm^3) of rats treated with ethanol, khat and /or combination of ethanol and khat and their age matched controls after 30 days of treatment for PND 6, PND 13 and PND 30.



Graph 4a



Graph 4b



Graph 4c

4.8. ESTIMATION OF TOTAL NUMBER OF PURKINJE NEURONS

The total number of Purkinje neurons of cerebellum as determined by multiplying the volume of cerebellar cortex with the numerical density of Purkinje neurons in the different categories of the same age groups is indicated with Bar graph (graph 5a-c).

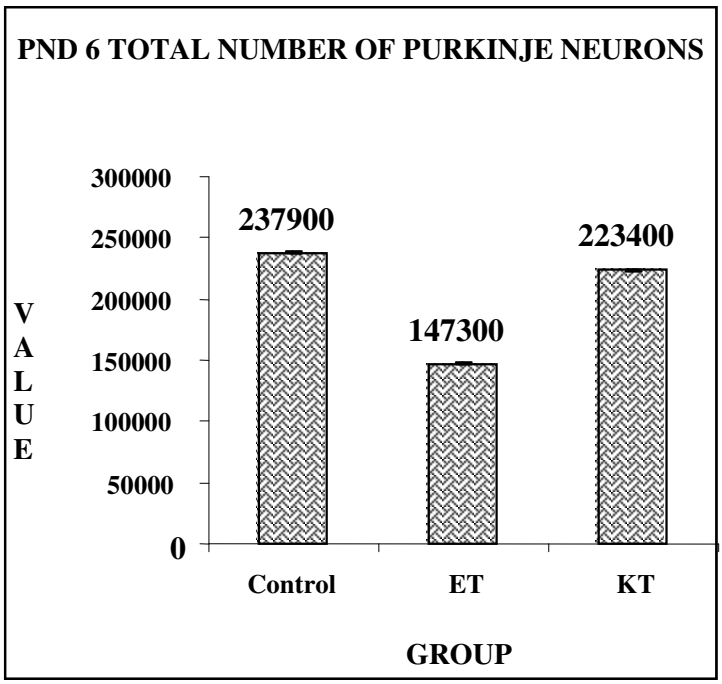
In the PND 6 group, the total number of Purkinje neurons was estimated to be 237859.2 in the control, 147279.6 in the ET and 223430.4 in the KT. This showed that the total number of Purkinje neurons was greater in the control by 38.08% and 6.07% as compared with ET and KT, respectively. The difference was statistically significant between the control and ET, but not between control and KT. In addition, the total number of Purkinje neurons was greater in the KT than ET by 34.08% and it was statistically significant ($P<0.05$). For multiple comparisons the data were analyzed by the *Post hoc* analysis using *Scheffe* test. This indicated

significant difference on the total number of Purkinje neurons of ET from those of the age matched control and KT groups.

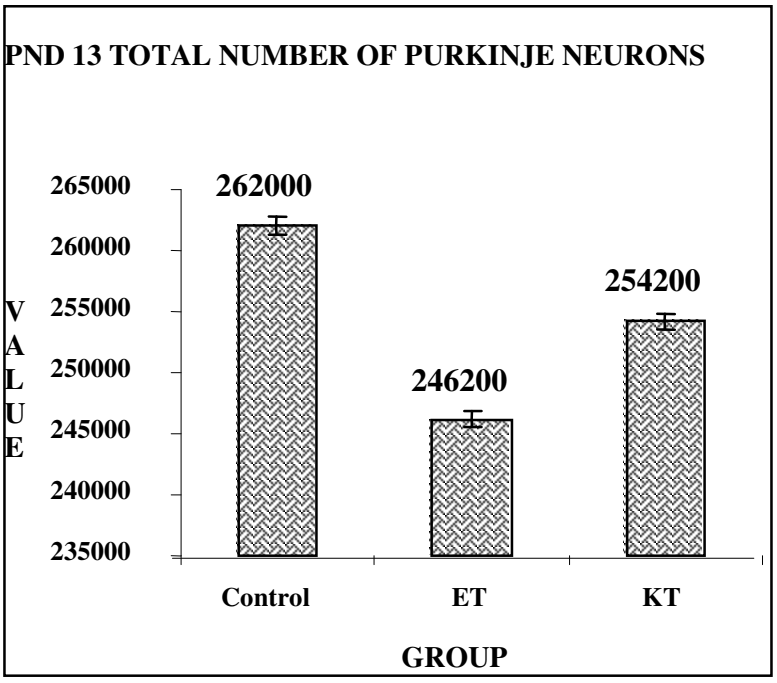
The total number of Purkinje neurons at PND 13 was greater in the control than ET and KT by 6.03% and 3%. However, this difference was not statistically significant at P>0.05. In the experimental groups, the total number of neurons was less in the ET than KT by 3.15%, although it was not statistically significant (P>0.05). The *Post hoc* analysis with *Scheffe* test for multiple comparisons also depicted non- significant difference in all categories of PND 13 groups of animals.

The mean total number of Purkinje neurons at PND 30 groups was estimated to be 274305 in the control, 262185 in the ET, 268800 in the KT and 265055 in the CT. The result indicated that the total number of Purkinje neurons was greater in the control than the ET, KT and CT by 4.41%, 2.01% and 3.35%, respectively. However, this was not statistically significant at P>0.05. In addition, among the experimental groups the total number of Purkinje was smaller in ET category than the KT and CT categories by 2.46% and 1.09%, respectively. It was also greater in the KT than CT by 1.39%. However, one-way ANOVA revealed that there was no statistically significant difference among these treated animals. Such non- significant difference among the four categories of PND 30 was also reflected by the *Post hoc* analysis using *Scheffe* test.

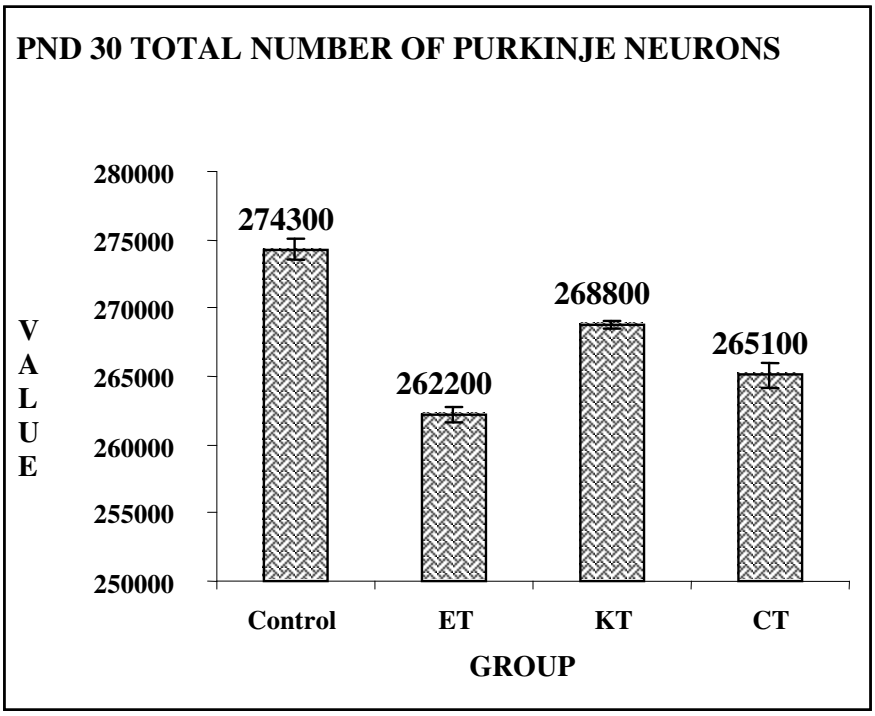
Graph 5a- c: Total number of Purkinje neurons of rats treated with ethanol, khat and combination of ethanol and khat and their age matched controls after 30 days of treatment for PND 6, PND 13 or PND 30.



Graph 5a



Graph 5b



Graph 5c

4.9. ESTIMATION OF VOLUME FRACTION OF PURKINJE NEURONS

The volume fraction of Purkinje neurons which was estimated by dividing points be positioned on the Purkinje neurons by points overlie on the whole cerebellar cortex is shown in Bar graph (Graph 6a-c). This was done to assess the volume fraction difference among different categories of the same age groups.

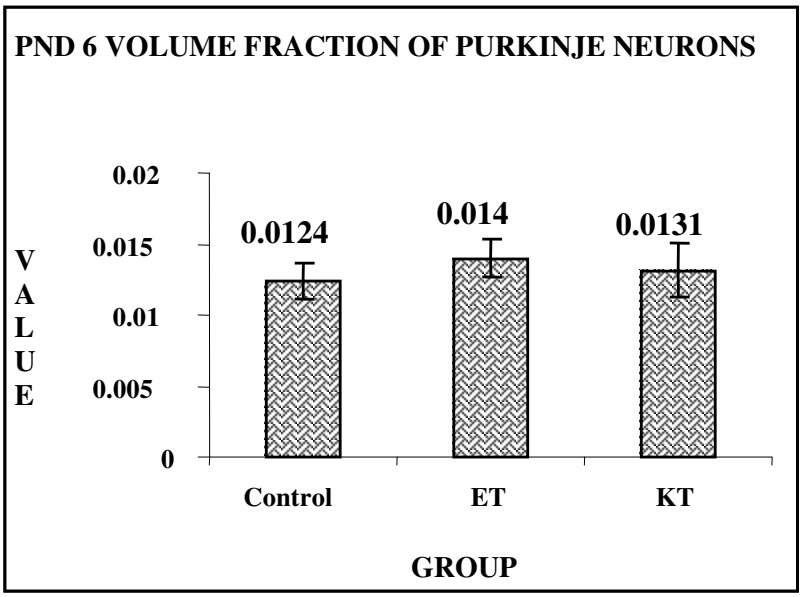
The volume fraction of control group of PND 6 was smaller than the ET and KT by 11.43% and 5.34%, respectively. It was statistically significant only between the control and ET at $P<0.05$, however, no statistically significant different was observed between the control and KT ($P>0.05$). In addition, the volume fraction of ET was greater than KT by 6.43%, although it was not statistically significant ($P>0.05$).

The mean volume fraction at PND 13 groups of animals was estimated to be 8.8×10^{-3} in the control, 9.3×10^{-3} in the ET and 9.0×10^{-3} in the KT. This indicated that the volume fraction

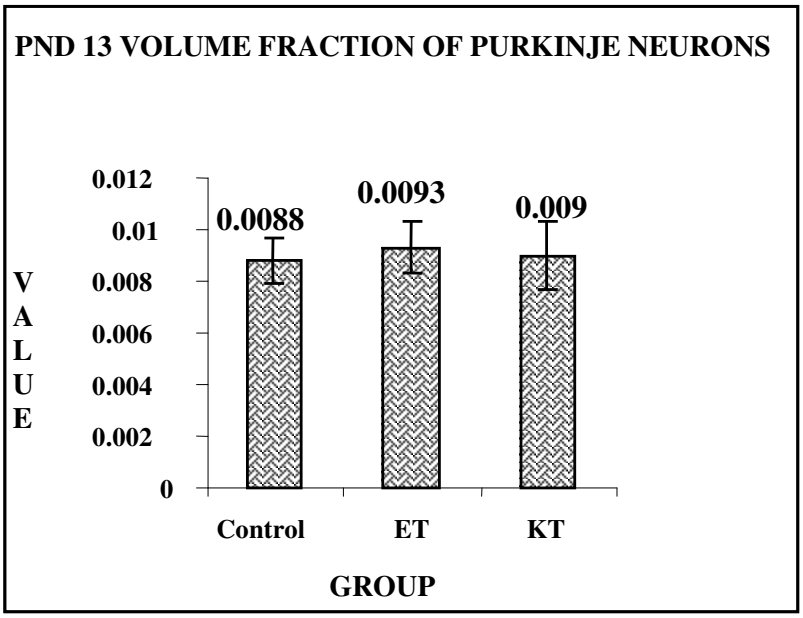
of Purkinje neurons is smaller in the control than ET and KT by 5.38% and 2.22%, respectively, though it was not statistically significant (P>0.05). It was greater in the ET than KT by 3.23% but was not statistically significant at P>0.05. The non- significant difference among different categories of PND 13 was also reflected by *Post hoc* analysis using *Scheffe* test.

The average volume fraction of Purkinje neurons at PND 30 was estimated to be 7.7×10^{-3} in the control, 8.40×10^{-3} in the ET, 7.9×10^{-3} in the KT and 8.1×10^{-3} in the CT. This revealed that the volume fraction of the control was less than those of the ET KT and CT by 8.33%, 2.53% and 4.94%, respectively. However, the difference was not statistically significant at P>0.05. In addition, among the experimental groups the volume fraction of Purkinje neurons was greater in the ET than KT and CT by 5.95% and 3.57%, respectively, although there was not statistically significant difference. It was also greater in the CT than KT by 2.47% but was not statistically significant. In addition to one- way ANOVA, for multiple comparisons the data were analyzed by *Post hoc* analysis using *Scheffe* test and non- significant difference among the four categories of the PND 30 groups were observed.

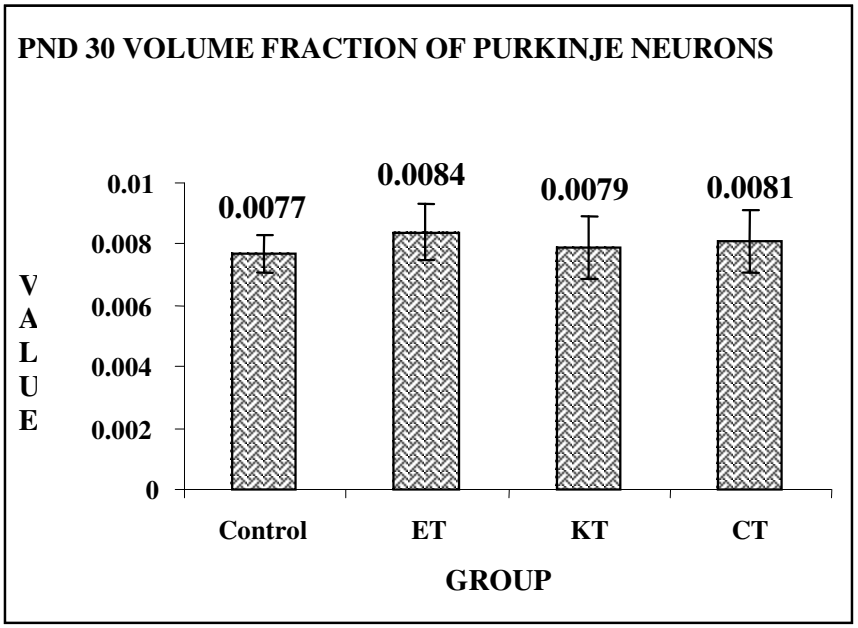
Graph 6a-c: Volume fraction of Purkinje neurons rats treated with ethanol, khat and combination of ethanol and khat and their age matched controls after 30 days of treatment for PND 6, PND 13 or PND 30



Graph 6a



Graph 6b


PND 30 VOLUME FRACTION OF PURKINJE NEURONS
VALUE
0.01
0.008
0.006
0.004
0.002
0
0.0077
0.0084
0.0079
0.0081
Control
ET
KT
CT
GROUP

Graph 6c

5. DISCUSSION

The use of drugs other than from therapeutics is motivated by many factors including peer pressure, self-medication for psychological problems such as anxiety and depression, and dependence (APA, 1994). Therefore, the strong association between alcohol consumption and khat chewing are likely to be attributable to multiple factors, including their pharmacological actions (Mesifin et al., 1999). The use of alcohol and khat has chronic health complications and also affects family relation and economic condition of an individual (Ayana and Mekonnen, 2004). Among these drugs, effect of ethanol was studied by several investigators (Miki et al., 1999; Miki et al., 2000; Olney et al., 2002). According to their findings, ethanol consumption has marked effect on different organs of the body especially on central nervous system (CNS). On the other hand, there is lack of specific and comprehensive information on the histological change of different systems particularly CNS with khat. However, epidemiological and clinical studies related to psychosis (Alem and Shibre, 1997), blood pressure (Widler et al., 1994), sexual functions (Mwenda et al., 2003) and gastric disorders (Giannini et al., 1986) are available. Therefore, the present study tried to provide histological evidence on the effect of ethanol and khat on a part of CNS, cerebellar cortex. The basic parameters used to indicate the effect of these drugs are microscopic observations, body weight, brain and cerebellar weights and estimation of diameter of Purkinje neurons. All these were supported by unbiased stereological analysis including, numerical density of Purkinje neurons per unit volume, total number of Purkinje neurons of cerebellum and volume fraction of Purkinje neurons and volume of cerebellar cortex.

Administration of combination of khat and ethanol resulted in death of rats of PND 6 and PND 13 age groups within 2 days of treatment. As found from their autopsy, there was sever and extensive hemorrhage in the posterior region of the brain. It is thought that this may be the

main cause of death as there were no other observable pathological changes in other tissues. This is interesting as PND 6 and PND 13 are comparable with human fetus of second and third trimester, respectively (Miki et al, 2000; Hernandez-Gonzalez and Juarez, 2000). It is not known whether a similar effect is manifested if a pregnant woman takes ethanol and khat simultaneously at higher doses and adversely affects the pregnancy. Therefore, it is a matter that deserves attention and future investigations.

Cerebellar cortex has three distinct layers, namely, molecular layer, Purkinje layer and granular layer. The molecular layer consists of neurons, glial cells, blood vessels and nerve fibers projected from Purkinje neurons. In this study it was found that, the Purkinje layer is composed of flask shaped Purkinje cells with their dendrite. A few of these neurons are binucleoulated and most of them are uninucleolated. The inner granular layer is composed of compacted granule cells and blood vessels. Eventhough the aforementioned morphological features are generally comparable in all age groups of rats, there are also differences which were observed microscopically. In the ethanol treated animals for Post natal day (PND) 6 groups, the nucleus were found pushed peripherally and their Nissle substance was dissolute. On top of these, there were neurons without nucleolus in their nucleus. In rats which received khat starting from their early life (PND 6), the nucleus of some Purkinje neurons was also pushed peripherally and their Nissle substance was also dissolute. It is known that, displacement of nucleus to the periphery, absence of nucleolus in the nucleus and dissolution of Nissle substance are signs of neuron degeneration (Carpenter and Sutin, 1983; Tavares et al., 1987; Lynch, 1996). The above-mentioned features of the nucleus and Nissle substance observed in this study may also indicate the degeneration of neurons that are caused by the ethanol and khat.

In the present study of ethanol treated (ET) rats the folia was narrow and the interfolia was wider as compared with the age matched control and khat treated (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 (Victor and Laurens, 1978; Torvik and Torp, 1986). It is found that, neuropathologically, atrophic changes in cerebellum as a result of alcohol abuse are localized particularly in the cerebellar vermis (Pentney and Dlugos, 2000; Torvik and Torp, 1986). The lesion is characterized by a decrease in the volume of molecular and granular layers, gliosis (damage of neurons accompanied by hypertrophy and hyperplasia of astroctyes) of the granule cells and a decrease in the number of Purkinje cells (Victor et al., 1997; Crews, 1999). This leads to shrinkage of the anterior part of the vermis and reduction of the volume of cerebellar cortex consequently results in narrowing of folia and widening of interfolia. It is frequently seen both in chronic alcoholic individuals and in the fetal alcohol syndrome (Jones and Jones, 1980). The mechanism of these neuronal lesions and cerebellar atrophy, however, remains uncertain (Cavanagh et al., 1997). It is suggested that, it may possibly involves hypoxia due to spasm of cerebral blood vessels (Altura et al., 1983), fluidization of membranes (Sun and Sun, 1985), and focal accumulation of toxic aldehyde due to intraneural ethanol metabolism (Kerr et al., 1989). In addition, acetaldehyde is a toxic substance, which can penetrate the blood brain barrier (BBB) and affect different parts of the brain and neurons (Brien and Loomis, 1983). The accumulation of acetaldehyde increased in the following order: brain hemispheres, striatum, brain stem, hypothalamus and cerebellum (Zimatkin et al., 1998). 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 dependent producing drugs (Kril and Harper, 1989), but the mechanism is uncertain.

Unlike the effect of ethanol and khat during early life (PND 6), no morphological change in the cerebellar cortex was detected in all categories of animals of PND 13 and PND 30 and no study was available. Thus, it needs further study.

In addition to the histological observations of cerebellar cortex, the effect of khat, ethanol and combination of the two was also assessed by measuring the body weight, weight of the brain as a whole and cerebellar separately and neuron diameter as well as by estimating volume of cerebellar cortex, numerical density, volume fraction and total number of Purkinje neurons using unbiased stereology.

The relation between alcohol consumption and total body weight remains a puzzle for nutritionists (Westerterp- Plantenga and Verwegen, 1999). However, in the present study, ingestion of high amount of alcohol for 30 days caused total body weight loss in all age groups of rats ($P<0.01$) and it is in agreement with Miki et al (1999). This is because ethanol intake to the level of intoxication suppresses appetite unlike the moderate intake (Poppit et al., 1996). Similarly, intake of khat extract significantly ($P<0.01$) reduced the body weight of rats belonging to all age groups. This observation is in agreement with other study on the effect of khat (Hassan et al., 2002). This seems to be due to the amphetamine like effect of cathinone, one of its active components, through stimulation of the release of norepinephrine in CNS, which acts centrally to activate the satiety centers (center for a condition of being fully satisfied) (Patel, 2000). It consequently, delays gastric emptiness and suppress appetite (Heymann et al., 1995; Halbach, 1972). The combination of khat and ethanol intake simultaneously for 30 days also significantly decreased the body weight of rats at $P<0.01$. However, the body weight of the rats taking combination of the two (114.46 ± 0.15) was greater than those taking khat alone (103.61 ± 0.79). Hence, body weight loss due to khat

chewing might be reduced by ethanol consumption after chewing. It is likely that, ethanol counteract the action of active component of khat. Therefore, it enhances gastric emptying and promotes food consumption.

In the present study, both ethanol and khat treatment causes a significant reduction in weight of the whole brain and its part , cerebellum as compared to their age match control during early life (PND 6), respectively by 26.19% and 44.44%; 19.05% and 37.04%. However, there was no direct relationship with body weight, because the body weight of KT rats was smaller than the body weight of ET rats unlike to the brain and cerebellar weight, part of brain. This finding was in agreement with that of Miki et al (2000). It is thought that, the brain tissues during PND 6 and second trimester of human fetus are vulnerable to dependent inducing drugs such as ethanol and khat consumption (Amha, 1983; Carvalho, 2003). Furthermore, consumption of ethanol and khat alone as well as their combination also significantly reduced total brain weight at $P<0.01$ even in the advanced stage (PND 13 and 30). The functional implications of this reduction in the brain weight especially in the cerebellar weight are uncertain (Miki et al., 1999). However, Thomas and coworkers (1998) found that rats exposed to high amount of ethanol on PND 4 and 5 or 8 and 9 had a deficit in motor coordination as tested on a parallel bar apparatus (an apparatus used to diagnose the motor coordination). In addition, it was found to be correlated with the extent of the loss of cerebellar Purkinje neurons (Nairn et al., 1989; Mayhew, 1991).

For quantitative explanation, this study has gone through estimation of Purkinje neurons diameter and unbiased stereological analysis such as, estimation of volume of cerebellar cortex, numerical density of Purkinje neurons per unit volume, volume fraction and total number of Purkinje neurons in all the three age groups. These parameters were practically

applicable to observe the impact of ethanol and khat and their combination on Purkinje neurons in particular and cerebellar cortex in general. They were also used by several researchers in similar works related to ethanol intake (Bedi and Warren, 1988; Miki et al., 1999; Miki et al., 2000). In order to implement this unbiased stereology a random sampling method was used.

The mean diameter of Purkinje neurons in all categories of the three age groups (PND 6, 13 and 30) was assessed to examine the effect of ethanol and khat ingestion on these particular neurons. Their mean diameter among the controls showed progressive increment with age (16.96 ± 0.25 for PND 6, 17.03 ± 0.2 for PND 13 and 17.47 ± 0.22 for PND 30). It was greater than their age matched treated rats, which was 12.10 ± 0.21 (ET) and 15.02 ± 0.20 (KT) of PND 6; 16.22 ± 0.29 (ET) and 16.48 ± 0.26 (KT) of PND 13; 16.28 ± 0.27 (ET), 16.87 ± 0.26 (KT) and 16.63 ± 0.13 (CT) of PND 30. This might be accounted by the increase in functional activity of the components of the cytoplasm. Despite the fact that the mean size of Purkinje neurons of the treated groups is smaller than their control, it was significantly reduced only in the ethanol and khat treated rats during early life (PND 6) at $P<0.01$. This is attributed to their vulnerability towards the metabolite of alcohol (acetaldehyde). It is also likely that, they are susceptible towards cathinone and cathine of khat as it has been observed in the previous studies (Brien and Loomis, 1983; Amha, 1983; Fadda and Rossetti, 1998; Tariq et al., 2002). The exposure towards these dependence-inducing drugs after PND 6 can also affect these neurons but it is largely dependent on the duration and amount of ethanol and khat being administered (Holford, 1987; Carvalho, 2003). In general, in advanced age (PND 13 and 30) the mean size of Purkinje neurons of ethanol treated animals was smaller than khat and combination of ethanol and khat treated rats but it was not significant ($P> 0.05$). This might indicate the severity of ethanol on neurons.

It was also noted in this study that the volume of cerebellar cortex of the control groups was increased successively with age (1.0792 mm^3, 1.2373 mm^3 and 1.3604 mm^3 respectively at PND 6, 13 and 30). It is suggested that, there is an increment of the amount of neuroglial cells with increasing age (Jensen, 1980). Furthermore, in all categories of the same age groups, there was difference in the volume of cerebellar cortex, i.e. the volume in the ET and KT rats was less than that in the control of similar age. However, the volume difference between ET rats and control of PND 6 was statistically significant at $P< 0.01$. PND 6 in rats or second trimester in human fetus is the crucial period of brain development. Thus, it appears that exposure of animals or pregnant women to high amount of ethanol for sometime at this period might severely affects the brain development and causes shrinkage of cerebellum (Rosenbloom et al., 1995). The severity of cerebellar shrinkage is largely dependent on exposure time and age of individuals (Victor et al., 1997). Hence, the effect of ethanol on cerebellar cortex in the present study was non- significant as the age advanced at $P>0.05$. This might be explained as the cerebellum matures it becomes relatively resistant to at least some of the adverse effects of ethanol. As to the effect of khat and combination of khat and ethanol on cerebellar cortex, this study depicted for the first time that there was no significant effect on volume of the cortex ($P>0.05$). It is thought that khat might not affect other neurons of cerebellar cortex and as a result the cortex is not shrunk. In addition, with increasing age, neurons and other non- neuronal cells might be structurally resistant towards dependence provoking drugs like combined use of khat and ethanol and hence did not reduce the volume extensively.

The numerical density of Purkinje neurons per unit volume of cerebellar cortex was estimated using physical dissector method. It progressively decreased with age because the size of

Purkinje neurons and volume of cerebellar cortex was increased and hence the neurons became dispersed. In addition, the neural package density in the ethanol administered groups was greater than the rest of the experimental groups as well as their age matched controls and was significant only for PND 6 rats ($P<0.05$). The numerical density difference among various groups in the present study and other related studies is difficult to interpret because the density can be influenced both by a change in number and size of neurons and/or by a change in the volume of cerebellar cortex (Bedi, 1987; Kril and Harper, 1989). The explanation usually offered for the marked increment of numerical density in the ET rats is that ethanol exposure during early life or during a period of synaptogenesis (a period at which development takes place) delays the maturation of the dendritic arborization (branching) of neurons and cause a deficit in the cortical volume (Pentney and Dlugos, 2000; Michaelis, 1990). This results in the formation of package of neurons in a smaller volume of tissue thus giving an increased numerical density of Purkinje neurons of ET and it is in agreement with the findings of Mitra and Mukherjee (2001). In addition, the numerical density can be explained in accordance with number and size of neurons. Ethanol and khat consumption during early life causes significant decrement of Purkinje neurons size and this might contribute for significant increment of neural package density. This means that, as the size of neurons gets smaller, they became crowded in a smaller volume of the cortex; consequently, the number of neurons included in the counting frame is increased. However, this was not observed in KT rats, despite khat decreased neuron size. It is most likely that, khat might not delay dendritic arborization and/ or did not significantly decrease the volume of cerebellar cortex or due to some other factors. In PND 13 and 30, the size of neurons had direct relation with numerical density, in a way that in all categories of the two age groups ethanol, khat and combination of ethanol and khat did not significantly change the size and number of neurons and hence no significant change in numerical density. It is likely that, as age of the rats progressively increased neurons might be

protected from the adverse effect of ethanol, khat and combination of the two.

The results of the numerical density in all age groups were supported by the estimation of volume fraction of Purkinje neurons in the cerebellar cortex that indicated the relative proportion of neurons to that of other non- Purkinje neurons as well as non- neuronal cells in the cortex. The volume fraction progressively decreased with age and it was found to be 0.0124, 0.0088 and 0.0077, respectively at PND 6, 13 and 30. It is suggested that, the small sized neurons during early age (PND6) are found more closely grouped together in a smaller volume of cerebellar cortex and increase volume fraction of Purkinje neurons. On the other hand, with increasing age, the size of neurons increased and larger Purkinje neurons became more spread out and separated by a greater amount of neuroglial cells in the cortex. Consequently, neurons became dispersed throughout the cortex and decrease volume fraction of Purkinje neurons. In the three age groups, the relative proportion of Purkinje neurons of cerebellum was greater in the ethanol administered rats than the other age matched categories of rats. There was, however, significant difference only in PND 6 groups ($P<0.05$). This may be attributed to shrinkage and volume reduction by ethanol.

Eventhough neuronal loss as a result of ethanol exposure largely depends on the duration of exposure and level of blood alcohol concentration, BAC (Bonthieus and West, 1990). In this study the BAC level was not measured but the time of exposure was determined and was the same in all animals. And, as ethanol was administered to all age groups of ET rats, the animals were intoxicated for a few hours after treatment. This indicated the presence of high BAC. The administration of 3 ml/100 gm body weight of 20% ethanol induces marked loss of Purkinje neurons in PND 6 groups, while no significant loss of Purkinje neurons of cerebellum was observed in PND 13 and PND 30 rats ($P>0.05$), which is similar to KT of the three age groups

and CT of young animals (PND 30). This suggests that Purkinje neurons are particularly vulnerable to ethanol at the early life (PND 6). In the current study, ethanol exposure during PND 13 and 30 did not cause significant loss of Purkinje neurons of cerebellum and it is in line with Miki et al., (1999). It is suggested that Purkinje neurons of rat cerebellum are somewhat protected against ethanol intake and its metabolites, like acetaldehyde, at least in terms of their capability to resist this effect (West at al., 1986). In addition, Purkinje neurons are also protected against khat; however, as it is taken for a long time it might affect them. This is because khat contains a mutagenic and carcinogenic component that includes polyphenol such as tannins (Bichel and Batch, 1968), which could damage Purkinje neurons. This might be exacerbated by concomitant use of khat and ethanol. Hence, it is likely that use of ethanol after khat chewing for a longer time might severely affects neurons.

In the present work, ethanol administered rats at PND 6 and PND 13 had about 147300 and 246200 of Purkinje neurons, respectively and it was in agreement with Miki et al., (1999) where ethanol treated rats of PND 4-9 and PND 10-15 had a total of 128,000 and 254,000 Purkinje neurons, respectively. This signifies that ethanol treatment during early life kills and decreases the total number of Purkinje neurons. However, Pentney and Dlugos (2000) stated that alcohol does not kill and reduce the total number of Purkinje cells of cerebellum rather decreases the total number of synapses on dendritic arbors of these neurons, and hence impairs the brain by damaging the ability of neurons to communicate with others.

In any way, the neural death can be induced by excess activity of certain neurotransmitters, including glutamate (Kroemer et al., 1997). This phenomenon, which is called excitotoxicity, may contribute to alcohol related damage to the developing brain (Michaelis, 1990). Under certain conditions, when glutamate interacts with the N- methyl- D- aspartate (NMDA)

receptor, it causes calcium to flow into the signal receiving neurons (Pang and Geddes, 1997). This calcium influx is a powerful regulator of the activity and function of a neuron. In the fetus, the calcium influx generated at the NMDA receptor is an important signal in neuron development and synapse formation (Michaelis, 1990). Excessive activation of NMDA glutamate receptor, however, can lead to dangerously high calcium accumulation inside the neuron (Choi, 1995) and consequently leads to cell death (Pang and Geddes, 1997).

6. CONCLUSIONS

From this study the following conclusions can be drawn:

- Administration of combination of ethanol and khat killed all the rats of PND 6 and 13.
- Ethanol and khat consumption resulted in morphological change in the cortex of the younger rats (PND 6) than the older ones (PND 13 and 30).
- Ethanol, khat and combination of the two reduced the total body weight as well as brain and cerebellar weight in all age groups of rats.
- Ethanol and khat reduced the diameter of Purkinje neurons of cerebellar cortex of PND 6 but not in the advanced age (PND 13 and 30); in addition combination of ethanol and khat didn't reduce the neurons diameter in the young rats (PND 30).
- Ethanol reduced the volume of cerebellar cortex, total number of Purkinje neurons and increased numerical density as well as volume fraction of Purkinje neurons of PND 6 rats.
- Exposure of ethanol in advanced age (PND 13 and 30), khat in all age and combination of ethanol and khat in young (PND 30) neither reduced the volume of cerebellar cortex and total number of Purkinje neurons nor increased numerical density and volume fraction of Purkinje neurons.

7. RECOMMENDATIONS

From the present investigation, the following recommendations can be given.

- ☞ The mechanisms of action of khat and ethanol for the observed effect should be studied.
- ☞ The cause of death of the combined treatment of khat and ethanol should be investigated.
- ☞ Epidemiological studies are required to examine whether concomitant use of khat and ethanol has similar significant effect in pregnant women.
- ☞ The effect of ethanol and khat in neuronal and non- neuronal cells of other regions of CNS and other neurons of cerebellum needs further study.
- ☞ Whether the present observation could be extrapolated in human beings should be investigated.

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9. APPENDICES

Appendix A: Tissue Preparation

A.1 Solutions for Tissue Processing

A.1.1. 4 % Formaldehyde in 0.1M Phosphate Buffer

The 4 % formaldehyde is prepared by adding 50 ml of 40 % formalin to 450 ml of 0.1M phosphate buffer solution.

A.1.2. 0.1M Phosphate Buffer at PH 7.3

Solution A:

Potassium dihydrogen orthophosphate……7.8 gm

Distilled water……..250 ml

Solution B:

Disodium hydrogen orthophosphate.....7.07 gm

Distilled water……..250 ml

Add solution A to solution B until a P^H of 7.3 is reached.

A.2. Tissue Processing

- 70 % Alcohol…….....2 hrs
- 90 % Alcohol……..2 hrs
- Absolute Alcohol……....1 ½ hrs
- Absolute Alcohol 2…… …...1 ½ hrs
- Absolute Alcohol 3…….1 ½ hrs
- Absolute Alcohol 4…….. …..Over night
- Xylene 1…….....1 ½ hrs
- Xylene 2……... ….2 ½ hrs
- Wax 1……..1 ½ hrs
- Wax 2…… ….2 ½ hrs
- Wax 3…… ….Overnight
- Embedding

A.3. Preparation of Albumin Coated Glass Slides

- Dissolve 50 ml of egg albumin in 50 ml of glycerin in 1:1 ratio.
- Dip slides, arranged in a clean slide rack, into the solution for 2 minutes.
- Leave the slides to air dry for 10 minutes.

A.4. Toluidine blue stain preparation

Staining solutions:

Toluidine blue stain…….1 gm

Distilled water ……...100 cm^3

Acetic acid…….0.25 cm^3

A.5. Toluidine blue Staining Procedure

- Rinse sections in water.
- Stain sections for 20-30 minutes in 0.1 % toluidine blue made in distilled water.
- Rinse in distilled water.
- Dehydrate in successive concentrations of 50%, 70%, 90% and two 100% alcohol.
- Clean in two changes of xylene, mount with a mountant and coverslip.

Appendix B: Setting of stereological grids

The following sets of grids were used in the stereological study of this investigation and all of them were obtained as a set of photocopiable masters from Agar Scientific Ltd.

B.1 Point Grids

In each case the grids (**P2**) are quadratic and a multiple of the fundamental inter-point spacing of the grid, $\Delta\mathbf{x}$, is shown. On the grid, after measuring the distance between a known number of grid spacing, the calculation of the area per point **(a/p)** of the grid, corrected for magnification (M) will be obtained from $\mathbf{a/p = \Delta x^2/M^2}$.

B.1.1. Point-counting Grid P2 (for estimation of volume of the cerebellar cortex).

B.1.2 Point-counting Grid P2 (for estimation volume fraction of Purkinje neurons within the cerebellar cortex).

B.2 Sampling Frames

In the case of sampling frames **F2**, the frames are square and a multiple of the frame width, Δ**x**, is shown. The calculation of the area per frame-associated point **(a/f)**, corrected for magnification will be obtained from $\mathbf{a/f = \Delta x \,.\, \Delta y \,/\, M^2}$. The sampling frame, **F2** was used for estimation the numerical density of Purkinje neurons per unit volume of cerebellar cortex.