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Evaluation of the acute and Subacute toxicity of aqueous leaves extracts of *Artemisia afra* on brain, heart and suprarenal gland in Swiss albino mice.

A thesis submitted to the Department of Anatomy, School of medicine, College of health science, Addis Ababa University, Addis Ababa, Ethiopia in partial fulfillment of the requirement for the Degree of Master of science (Msc) in anatomy.

BY: Ketema Mekonen

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Contents

List of Abbreviations

^{0}C: …….Degree Celsius

AAU: ……Addis Ababa University

ANOVA: ……..Analysis of variance

CAM: ……Complementary and alternative medicine

Cm: ……....Centimeter

EDM: ……Essential drugs and Medicinal policy

EPHI: ……Ethiopian Public Health Institute

Hrs: ……hours

LD_{50}: ……..Lethal doses of fifty percent

µm: …….Micrometer

mm^2 …….....Millimeter square

SE: ……..Standard Error

SPSS: ……..Statistical package for social sciences

Temp: ……...Temperature

TM: ……Traditional medicine

WHO: ……..World Health Organization

OECD: ……Organization for Economic Cooperation and Development

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Abstract

Having primary health care is a human right which is fulfilled by western country because of expansion of health infrastructure, and increased quantity and quality of health professionals. But many developing countries including Ethiopia are yet far from achieving this. In Ethiopia, the majority of population relay on traditional medicine as a source of health care. The most common sources of traditional medicine are plants. A.afra is one of these plants that are used to treat different aliments. The aim of the present study was to investigate the toxic effects of A.afra on brain, heart and suprarenal glands.

The study was conducted at Addis Ababa University, College of Health Science, School of Medicine, Department of Anatomy & Department of Physiology from Jan, 2014 to July, 2015. The plant was collected from Bale National park in Oromia Regional State. The plant was air dried and aqueous extract was prepared. In this research a total of 54 male and female mice of 8-12 weeks of age weighing 25-30g were used. The extract was given by oral rout in both acute and subacute study. The doses for acute toxicity study were 200mg/kg, 700mg/kg, 1200mg/kg, 2200mg/kg, 3200mg/kg, 4200mg/kg and 5000mg/kg of body weight, while for subacute toxcicy study a doses of 600mg/kg(low dose) and 1800mg/kg(high dose) of body weight were used.

LD_{50} was grounded to be greater or above 5000mg/kg which indicates that the plant is relatively safe. There were no observed signs of toxicity at the lower three doses, although mild toxicity sign was observed at the higher dose level in dose dependent manner. In the subacute study, two treatment groups 600mg/kg and 1800mg/kg and one control group containing both sexes were used. Weights of mice were measured weekly and individual mice were observed for possible toxicity sign. At the end of 28 days, the animals were scarified and organs were harvested and processed for microscopic examination. No toxicity signs were observed in all treatment groups. There were also no significant weight changes between the treated and control group. On microscopic examination of the brain, heart and suprarenal glands no sign of cellular injury was observed. From this study it can be concluded that A.afra is relatively safe in mice.

Key words: Traditional medicine, *A.afra*, Toxicity study, LD_{50}, Histopathology

1. Introduction

1.1. Traditional medicine

Human resource plays a major role in determining the economic development of every nation. Central to human resource development is improvement in the health status of the population that can be achieved via provision of medical care to those who need it (Dor and Van der Gaag, 1987). Furthermore, having primary health care service is a basic human right (WHO, 1978). As a result, providing medical care service has become the main agenda of all governments. The main challenge, however, is how to provide health care services to those who need it. For this challenge, expansion of health care facilities and increasing the quantity and quality of health personnel is obvious solution. While these had been enjoyed by the western long ago, it is yet far distant to a number of developing countries. As a result, traditional medicine played not only an alternative but also an immeasurable role in the health care delivery of developing countries.

Traditional medicine (TM) is an important and often underestimated part of health services. In some countries, traditional medicine or non-conventional medicine may be termed complementary medicine (CM). TM has a long history of use in health maintenance and in disease prevention and treatment, particularly for chronic disease. Traditional medicine is the sum total of the knowledge, skill, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness. The terms “complementary medicine” or “alternative medicine” refers to broad set of health care practices that are not part of that country’s own tradition or conventional medicine and are not fully integrated into the dominant health-care system (WHO, 2013).

Unlike the conventional medicine that mainly focuses only on disease causing pathogens, traditional medicine used holistic nature and culture-based approach. It includes diverse health practices, remedies, approaches, knowledge and beliefs incorporating plant, animal and mineral products, spiritual therapies and charms. Traditional health practitioners use a variety of approaches to diagnose, treat or prevent illness.

For many millions of people, herbal medicines, traditional treatments, and traditional practitioners are the main source of health care, and sometimes the only source of care especially in developing countries. In Africa up to 80% of the population uses TM to help meet their health care needs (WHO, 2002). Easy accessibility, efficacy on treatment and affordable cost in getting health services are main reasons in preferring traditional medicine to modern medication (Konno, 2004). Regardless of reasons for seeking out traditional & complementary medicine, interest has grown and will continue to grow around the world (WHO, 2013).

The Ethiopian condition is also the same to the rest of African countries, i.e. 80% of Ethiopian population is dependent on TM and traditional practitioner as a source of health care (WHO, 2013). Furthermore, medicinal plants are demanded in Ethiopia due to culturally linked traditions, the trust the communities have in the medicinal values of traditional medicine and relatively low cost in using them. The Ethiopian people's reliance on traditional medicine is also reflected by the fact that Ethiopian migrants in developed countries continue using them (Richard, 1997).

The health system of Ethiopia is primary health care based system which has different strategies to fulfill its goal of health for all. One of the strategies is incorporation of traditional medicine into the modern medicine. Furthermore, the health sector strategy of Ethiopia declares that incorporation of traditional medicine into the official health care system is advantageous for improving the health coverage of the country (MOH, 1995).

The practice of traditional medicine in Ethiopia consists of the use of herbs, bleeding, cauterization, steam bath, spiritual healing, holy water, bone setting and minor surgical procedures. Healers obtain their drugs mainly from natural substances and in descending order of frequency these constitute plants, animals and minerals. Leaves were the most commonly used and followed by roots to treat various health problems (Bayafers, 2000). Drugs are prepared in various dosage forms including liquids, ointments, powders and pills. Drugs are stored usually in containers such as bottles, papers, pieces of cloth, leaves and horns, and were kept anywhere at home. Herbal medicine is administered for patient by using different route. Oral application was the highest and most commonly used route of application followed by dermal (Gidey and

Samuel, 2011). Most knowledge on traditional medicinal plant is orally transmitted and few are available in written records.

In Ethiopia, medicinal plants have been used as traditional medicine to treat different human ailments by the local people from time immemorial. These medicinal plants are estimated to be over 700 species and most of them are confined to the southwestern region of the country (Abbink, 1995, Kebebew and Addis, 1996). One of the herbs used as treatment for various illnesses in different Ethiopian society is *Artemissia afra*. This research investigates if frequent use of *A. afra* has any histopathologic effect on brain, heart and suprarenal glands of mice.

Artemisia species

The genus Artemisia contains more than 400 species and most of its known species are found predominantly in Asia, Europe and North America (Mucciarelli and Maffei, 2002). The genus is widely used in many parts of the world either alone or in combination with other plants as herbal remedies for variety of human diseases. Artemisia species are most commonly used in traditional folk medicine, notably in treatment of malaria. The genus is known for the production of various types of seseqiterpenes, flavonoids and other bioactive metabolites including artemisinin which is the best known antimalarial compound from *A.anua* (Kelsey and Shafizadeh, 1979).

Artemisia species are one of the many traditional medicinal plants of Ethiopia for treatment of infectious and non infectious health problems. The most common species of genus Artemisia which are used as a traditional medicine in different Ethiopian community includes *A. absinthium, A. abyssinica, [A. annua] (exotic propagated through cultivation) and A.afra.*

Artemisia afra

Artemisia afra is a medium sized perennial herb plant, rarely exceeding 2 m high. The leaves are 6 cm long, gray-green, alternatively arranged and oval in shape. According to FAO report *A.afra* is a clump-forming perennial herb of the highland areas of Eastern and Southern Africa at an altitude ranging between 1500 and 3000m. It has been located in Ethiopia, Kenya, Zimbabwe, Tanzania, Zaire, Uganda, Zambia, Malawi, Angola and Republic South Africa (Maurice, 1993).

In Ethiopia, *A. afra* commonly called "chuqne" and "chenabaria", and it is widely used in combination with other herbals as a remedy against headache, eye diseases, tinea capitis, haematuria and stabbing pain (Abebe and Ayehu, 1993). It is also used to treat infertility, febrile illness, common cold, devil and epilepsy (Desta, 1994). In many other parts of Africa, this plant is traditionally used for a wide variety of ailments including cough, cold, sore throat, influenza, asthma, indigestion, colic, constipation, gout and intestinal worms (van Wyk and Wink, 2004).

A. afra has recently attracted worldwide attention of researchers for its possible use in the treatment of chronic diseases like diabetes, cardiovascular diseases, cancer, and respiratory diseases. The possible uses in the treatment of these diseases are supported by different studies. Ethanol extract of *A. afra* has an arrest effect of cell cycle of cancer cell after 12 hr, 24 hr, and 48 hr exposure (Spies *et al*., 2013). Research done on diabetic rats showed that administration of aqueous extracts of *A. afra* decrease glucose level to near normal range. In the same research, *A. afra* also showed antioxidant activity on diabetic rats (Anthony and Taofik, 2013)**.** In another research, its aqueous extract had clearly demonstrated significant antioxidant activity (Taofik, 2012). It has also antihelmentic effect against parasitic gastrointestinal nematodes (Nthatisi *et al*., 2012). Studies using the aqueous extracts of *A.afra* have indicated that the plant has bronchodilator and anti-inflammatory activities (Harris, 2002), and preliminary tests done using the same type of extracts showed that *A.afra* may have narcotic analgesic and antihistaminic activity (Hutchings *et al*., 1996).

The mentioned pharmacological activities and any other pharmacological effects of *A.afra* must be linked to its chemical constituents. It has been shown that the plant contains volatile oil, terpenoids, coumarins, acetylenes, scopoletin, flavonoids (James, 2012). The volatile oil has also been shown to contain 1, 8-cineole, α-thujone, β-thujone, camphor and borneol as major constituents and to have definite anti-microbial and anti-oxidative properties. Thujon is well known to cause neurotoxicity with different neurological symptoms like dizziness, tremor, convulsion and hallucination (Olavi *et al*., 2013).

From the genus Artemisia, *A. absinthium* was known to contain high amount of thujon and historically this plant was used to produce a popular banned commercial drink called Absinthe during the 19th century. This drink was banned in different countries because of its association with a syndrome called *Absinthism* which had different neurological symptoms (Dirk, 2010). Since *A.afra* also contain thujon, excessive or prolonged consumption of this plant may cause neurotoxicity in human.

Whilst evidence-based studies indicating the efficacy of herbal drugs are still being unveiled, increasing evidence regarding side effects of herbal medicine has highlighted the demand for toxicological studies for herbal products (Vidushi, 2013) including *A.afra*. One research done in South Africa stated that acute oral administration of aqueous extract of *A.afra* to mice is non-toxic with LD_{50} of 8960mg/kg (James, 2012). In the same study, chronic administration of this extract orally in rat was relatively safe with minor intermittent diarrhea, salivation and partial hypo-activity.

Figure 1 Photographs of A.afra showing its leaves, as taken during the month June 2014

1.2. Anatomy of the mice nervous system

In both human and mice, the nervous system is organized into central and peripheral nervous system. The central nervous system consists of spinal cord and brain. The central nervous system is the main centers where correlation and integration of information occur. The central nervous system is composed of large numbers of excitable nerve cells and supporting cells called neuroglia. The peripheral nervous system includes cranial and spinal nerves with their associated ganglia. The cranial and spinal nerves are consists of bundles of nerve fibers or axons which conduct information to and from the central nervous system.

On gross examination, many striking differences are evident between the brains of an adult mouse and an adult human. The most obvious difference is the human brain is much greater in size, approximately about 1,400 g in adult (Ronald and Adel, 2005). While the mouse brain is quite small approximately weighing 0.4g (Piper and Suzanne, 2012). In both human and mice, the brain is subdivided into cerebrum, diencephalon, brain stem and cerebellum. The mouse cerebral cortex is lissencephalic which means it has no gyri and sulci, in contrast to the cerebral cortex of humans (Claudio *et al*., 2004). The human cerebral cortex is thrown into folds, or gyri, separated by fissures, or sulci which increase the surface area of the cortex. The human cerebral cortex can be organized by reference to surface topography (sulci and gyri), but the lissencephalic mouse brain does not enable comparable division.

Two groups of cells are found in the nervous tissue, neuron and neuroglia. The neurons are the functional and structural unit of nervous system. Neurons have cell bodies, one or more dendrites through which they receive input from other neurons and one axon that synapses on other neurons or non-neural tissues, such as the musculature. They have high metabolic rate, which makes them extremely vulnerable to certain global toxic insults that impair intracellular energy metabolism. Neuroglia assists neuron in performing their function. Six types of neuroglia are found, i.e. astrocyte, oligodendrocyte, microglia and ependymal cells in central nervous system and schewan cells and satellite cells in peripheral nervous system.

In general, based on their structure neurons are classified into:

- Unipolar or pseudounipolar neurons have a spherical cell body with single process that bifurcates.
- Bipolar neurons are spindle-shaped, with one process at each end of the cell.
- Multipolar neurons have one axon and many dendritic processes

Cell bodies often contain a highly developed rough ER organized into aggregates of parallel cisternae. In the cytoplasm between the cisternae are numerous polyribosomes, suggesting that these cells synthesize both structural proteins and proteins for transport and secretion. When appropriate stains are used, RER and free ribosomes appear under the light microscope as clumps of basophilic material called chromatophilic substance or Nissl bodies. The amount of chromatophilic substance varies according to the type and functional state of the neuron and is particularly abundant in large nerve cells such as motor neurons. The Golgi apparatus is located only in the cell body, but mitochondria can be found throughout the cell and are usually abundant in the axon terminals (Anthony, 2010).

Cerebrum

The telencephalon (cerebrum) originates as an outgrowth of the most rostral segments of the developing nervous system (Monuki *et al*., 2001, Redies and Puelles, 2001) and is the largest region to develop independently of the segmental template that patterns most of the central nervous system (CNS). The cortex does not deal directly with sensory afferents or motor neurons, instead receive pre-processed sensory information via thalamic relays, and controls behavior by modulating the activity of other CNS structures. Whereas the majority of the CNS is concerned with the efficient routing and processing of sensory and motor activity, the cortex is far more abstract. Its key function is to elaborate, integrate and analyze sensory information in the light of past experience, as well as to plan and oversee appropriate responses (Ferezou *et al*., 2007). In essence, the cerebral cortex is characterized by its handling of high-level information.

In both human and mice the cerebrum has two hemispheres which are separated by longitudinal fissure. Each cerebral hemisphere has an outer gray matter (cerebral cortex), subcortical white matter and deeply buried masses of gray matter called basal nuclei. The cerebral cortex contains cell bodies, dendrites and short unmylinated axons (Elaine *et al*., 2012).

MICROSCOPIC STRUCTURE OF CEREBRAL CORTEX

Microscopically, cerebral cortex can be divided into three regions based on phylogeny. The archicortex is the most primitive domain and consists of the olfactory bulbs, olfactory tracts, and olfactory cortex. The archicortex is quite large in proportion to the brain in rodents relative to humans. The paleocortex, the next oldest region, consists of the various derivatives of the limbic system: hippocampus, parahippocampus, and cingulate gyrus (in humans). Neocortex is the most evolutionarily recent form of cortical development, and it is functionally distinct from paleocortex and archicortex (Ronald and Adel, 2005). It is made by three types of cells and six distinct layers.

Cell Types

The mature mouse cortex consists of approximately 14 million neurons and 12 million glia cells (Herculano-Houzel *et al*., 2006). These direct counts contradict the historical dogma of glia greatly outnumbering neurons in other species including human. But they are much easier to reconcile with observations of single astrocytes that occupy cortical domains encircling dozens of neurons (Halassa *et al*., 2007). Three types of neuron i.e. Cajal-Retzius neuron, excitatory or principal neuron and inhibitory interneurons are found in the neocortex. The principal neuron sends axons and collaterals locally, across the cortex and to other parts of CNS. The inhibitory interneurons participate in local circuits, rarely projecting their axons for long distances.

Cajal-Retzius (C-R) neurons are restricted to layer I and their axons descend toward the boundary with layer II and make extensive tangential ramifications with numerous ascending processes (DeFelipe and Jones, 1988). These neurons are principally important in structuring the developing cortex by secreting reelin (Nishikawa *et al*., 2002). Around 97% die in later development (Chowdhury *et al*., 2010). Their adult distribution is irregular, tending to concentrate in the primary motor and sensory regions (Bielle *et al*., 2005).

Principal neurons constitute 62 to 85% of all neocortical neurons, and are abundant throughout all regions and layers except layer I (DeFelipe and Farin˜as, 1992). In addition to their local connectivity, principal neurons are the dominant projection cells of the cortex. They send axons down through inferior layers to the deep cortical white matter, which continue to other parts of the cortex or the CNS. Principal neurons include pyramidal cell, star pyramid and the

polymorphic cells of layer VI and small spiny stellate cells of layer IV (DeFelipe and Farin˜as, 1992).

Pyramid cells are usually identified by a pyramidal or ovoid soma in layers II/III to upper VI, as well as a prominent ascending apical dendrite and several basal dendrites. They typically have an axon arising from the base of the soma or a basal dendrite, descending through and extending out of the cortex (Spruston, 2008). The apical dendrite often reach layer I in human but in mouse cortex frequently terminate in layer II/III or layer IV for the deepest pyramidal neurons (Ledergerber and Larkum, 2010). The axons of pyramidal cells are principally descending and many travel through white matter to targets in other cortical or subcortical regions (Feldman, 1984).

Spiny stellate cells resemble pyramidal cells, although they lose their apical dendrites as they mature (Nieuwenhuys, 1994). Spiny stellate cell distribution is limited to layer 4 of primary sensory regions (visual, auditory and somatosensory), and some associated secondary regions. Their nuclei pack together to form a "granular layer" seen in Nissl stains (White, 1989). Spiny stellate dendritic fields vary, some stratifying tangentially, and others ascending or descending radially for one layer (Guellmar *et al*., 2009). Their axons nearly always emerge from the descending side of the soma, but often recurve and ascend with arc-like collaterals, first noticed by Ramo´nyCajal (Staiger *et al*., 2004).

Interneurons are distributed across all layers of the mouse neocortex, giving rise to axon terminals that densely innervate both principal neurons and interneurons (Solberg *et al*., 1988). The axons of interneurons are far more convoluted and torturous than the relatively straight branches of pyramidal axons, making specific targeted contacts rather than the spine mediated "plug in" connections typical of excitatory communication (Huang *et al*., 2007).

Basket cells are the most common interneuron (Fishell, 2007). They are usually large-bodied cells, although "nest" and "small" subtypes have been identified (Wang *et al*., 2002). They are found in layers II/III to VI. Basket cells have four to ten sparsely spinous dendrites that spread radially and tangentially for millimeters, extending through all cortical layers in some cases (Jones and Hendry, 1984).

Chandelier cells were the only major short-axon type neuron not observed by Ramo´n yCajal (DeFelipe and Jones, 1988). They are rare but widespread in the rodent cortex, usually being found in layer II/III (Woodruff *et al*., 2009, Zhu *et al*., 2004). Their cell bodies are generally small and round, and their dendritic arbours vary considerably between multipolar and bitufted morphology. Other interneurons include Martinotti cells and neurogliaform cells (DeFelipe and Jones, 1988).

Layers of Neocortex

The mouse neocortex is bordered by the medial pallium (archicortex) on the medial side and the lateral pallium (allocortex) laterally. It has a surface area of ~120 to 130 mm^2 in area and a volume of 112 mm^3 (Gaglani *et al*., 2009). The neocortex consists of glia and radially extending neurons in tangential strata, defined by their cellular composition and characteristic connectivity.

The division of the neocortex into layers has been the outcome of extensive cytoarchitectonic and myeloarchitectonic studies. Although several such studies are available, the most widely used are the cytoarchitectonic classification of Brodmann and the myeloarchitectonic classification of the Vogts. According to these two classifications, the mammalian neocortex is divided into six layers. In mice, layers I, II/III, IV, V, and VI are named by homology with other mammals (DeFelipe and Jones, 1988). In mice a thin additional layer is found deep to layer VI and has nomenclature VII (Reep, 2000). These seven cortical layers sit on top of a dense plexus of axons and glia referred to as white matter which is subdivided into deep cortical white matter and the internal and external capsules converging on the cerebral peduncles near the midbrain.

Layer I (External Plexiform, Molecular or Superficial layer)

Layer I is a thin plexus on the outermost rim of the cortex which lies directly below the pia mater and is almost devoid of neurons (Peters and Jones, 1984). It consists largely of axons running across the cortical surface, sparsely distributed inhibitory and Cajal-Retzius neurons, glia, and the apical dendrites of pyramidal neurons whose somata lie in deeper layers (Chowdhury *et al*., 2010). Layer I is principally a meeting ground for modulatory influences on the neurons of deeper layers (Shlosberg *et al*., 2003).

Layer II/III (Supragranular Pyramidal layer)

Layer II/III has a varied population of neurons predominantly small pyramidal cells and is chiefly involved with local and corticocortical connectivity. Compared to primate layers II and III, the mouse cortex has thinner and less differentiated supragranular layers (Hutsler *et al*., 2005). Accordingly, layer II/III is treated as a single layer in the mouse cortex (Bureau *et al*., 2006). Layer II/III pyramidal cells project to adjacent and nearby parts of the cortex, as well as across the corpus callosum (Fame *et al*., 2011). In the motor cortex, they also drive the sub-cortical projection neurons of layer V (Weiler *et al*., 2008). Among the interneurons of layer II/III are the chandelier cells, which regulate pyramidal axon firing (Woodruff *et al*., 2009) and basket cells which modulate the excitability of pyramidal cells via synapses on the soma (Czeiger and White, 1997). Also numerous in supragranular layers are neurogliaform, bipolar, and bitufted interneurons (Miyoshi *et al*., 2010).

Layer IV (Granular layer)

In sensory regions, layer IV is prominent and is the principal destination for thalamocortical fibers (Frost and Caviness, 1980). In these regions, spiny stellate cells crowd into layer IV (Lefort *et al*., 2009), forming a tightly packed band of nuclei for which granular cortex or konio cortex is named. Ramo ´n y Cajal at one stage called this the “layer of the medium pyramids” and stated that mice lack a granular layer but later described granule layers in various rodent sensory regions (DeFelipe and Jones, 1988).

Layer V (Deep Pyramidal layer)

Layer V contains the largest pyramidal neurons of the cortex, which project their axons to a variety of cortical and sub-cortical targets (Larsen *et al*., 2007). A smaller population of corticocortical callosal projection neurons is distributed across the entire layer (Ramos *et al*., 2008). Axons from layer V target various subcortical structures, including the striatum, midbrain and pontine nuclei, the brainstem and the spinal cord. On the basis of these projections, the layer is often subdivided into layers VA and VB (Anderson *et al*., 2010). In motor and frontal regions, layer VB can be further divided into superficial and deep populations (Caviness, 1975).

Layer VI (Polymorphic or Multiform layer)

Layer VI is also primarily an output layer, where the bulk of corticothalamic fibers originates, and receives a strong thalamic projection in return (Thomson, 2010). By influencing the thalamic drive to other regions, these projections may be a major corticocortical communication pathway in rodents (Lam and Sherman, 2010). Layer VI has also been found to project to adjacent and distant ipsilateral cortical regions, as well as to local cells in other layers (Thomson, 2010) which may modulate incoming thalamic activity (Lee and Sherman, 2009). Interneurons, principally basket cells, form an inhibitory network for layer VI principal neurons (Zhang and Descheˆnes, 1997). Layer VI is typically thick and often occupies a large fraction of the cortical depth in mice. Ramo ´n y Cajal named it the "layer of the polymorphic cells" (DeFelipe and Jones, 1988) for its wide range of neuron morphologies, which can be inverted, horizontal and fusiform.

Layer VII (Subgriseal layer)

This layer is found below layer VI and separated by a cell-poor stratum. This layer is less well-known because it is named inconsistently in the literature as: upper subplate neurons, border neurons, the deep cortical band, deep/lower layer 6, layer 6b and subgriseal neurons (Chen *et al*., 2009, Clancy and Cauller, 1999). In mice, the rostral lateral edge of layer VII merges with the claustrum, although in other species these structures are distinct (Reep, 2000).

1.3. Anatomy of heart in mice and human

The cardiovascular systems in humans and mice, with the exception of cardiac size, heart rate, and small variations in the anatomy, are very similar. In both species, this system is consists of heart, artery, vein and capillary. The heart is fundamentally a hollow muscular organ that is primarily responsible for pumping blood with sufficient pressure and volume to perfuse the body's tissues.

The mouse heart sits in a very thin pericardial sac, typically only a few cell layers thick. Distinct layers are difficult to distinguish. The pericardial sac is continuous with the mediastinum and attaches to the heart at the base; the inner layer is continuous with epicardium of the heart. Unlike humans, mice have little or no epicardial fat (a small amount is present at the base of the heart), and there is no distinct interventricular groove in mice (Cheryl, 2014).

As is true in all mammals, both species have four chambered hearts: left and right atria, a dominant left ventricle, and a thinner-walled right ventricle. The two chambers on the right side of the heart (right atrium and ventricle) are responsible for receiving deoxygenated blood from the body and distributing it via the pulmonary trunk to the lungs for gas exchange to occur. The two chambers on the left side of the heart (left atrium and ventricle) are responsible for receiving oxygen rich blood from the lungs and pumping it out to the body through the aorta. Each atrium serves primarily as a reservoir for blood with only a small amount of pumping action, which assists with ventricular filling. The right and left ventricles are the major pumping chambers for providing blood to the pulmonary and systemic circulations, respectively

Although the mouse heart is obviously far smaller than the human heart, the ratio of heart to body weight is similar in both species, as is the relative thicknesses of the right and left ventricular walls. The left ventricular free wall is approximately 1500–1800 μm thick, with the right ventricular free wall typically approximately one-third as thick (Claudio *et al*., 2004).

The mouse heart is about the same size as a pencil eraser, weighs ~100–200 mg and beats 500–800times/min. The human heart weighs about 250–300 g and beats an average of 60–70times/min (Wessel and Sedmera, 2003). Because the mice body is parallel to the ground, the mouse heart does not rest on the diaphragm like the human heart and, therefore, has more room to move around within the pericardial cavity. As such, the mice heart has more of an ellipsoidal, "rugby ball" shape (Wessel and Sedmera, 2003).

A prominent anatomic variation of the heart between humans and mice is found in the venous supply component of the atria. In the human heart the left atrium receives oxygenated blood from four pulmonary veins. In the mouse, however, the pulmonary veins join in a pulmonary confluence behind the left atrium, which in turn empties via a single foramen into the dorsal wall of the left atrium. Another anatomical difference in the atrial anatomy between the two species relates to the venous drainage into the right atrium.

The mouse has both a left and right anterior vena cava; humans have only the latter. During the embryo stage both species have a left and a right anterior vena cava. Postnatally, the left anterior vena cava regresses and becomes nonfunctional in the human. In the mouse, however, the left anterior vena cava remains functional throughout the animal's life. Together with the right anterior vena cava, the left anterior vena cava delivers blood from the head back to the heart.

They both join together with the posterior vena cava to bring the blood into the right atrium (Wessel and Sedmera, 2003).

The heart is composed of three layers: (1) inner endocardium, (2) middle myocardium, and (3) outer epicardium or visceral pericardium. The outer surface of the heart is covered by a delicate, thin mesothelium. Immediately below this layer lies the epicardium, a thin layer composed of fibrous connective tissue and small blood and lymphatic vessels (Elwell and Mahler, 1999). The mice myocardium is composed of cardiac muscle cells with centrally located nuclei. It has a rich vascular supply composed of thin-walled arterioles, venules, and a well-organized capillary network (Michael *et al*., 2004). The endocardium is lined with endothelium and is continuous with the intima of the blood vessels entering and leaving the heart (Elwell and Mahler, 1999). In mice, the subendocardial connective tissue is scant or absent but it is found in human heart (Piper and Suzanne, 2012).

1.4. Anatomy of suprarenal glands in mice and human

These glands are located above the antero-rostral poles of the kidneys, the right suprarenal glands being closer to the kidney than the left (Frith, 1996) and the venous drainage differs between the two sides. The overall weight and appearance of the gland differs between the male and the female, with the left gland typically weighing more than the right gland (Beamer *et al*., 1983). The weight in the female is about 25% greater than in the male, while the gland is said to be more opaque in the female. This is due to the presence of an increased amount of lipid in it (Hummel *et al*., 1975). In the mouse, the adrenal cortex comprises about 80% of the total volume of the gland, while the medulla occupies about 20% of the gland (Matthew *et al*., 2010).

In humans and most mammals three zones can be distinguished in the cortex, but the cortex of the mouse suprarenal glands have only two zones: zona glomerulosa and zona fasciculata. There is no discernible zona reticularis in the mouse adrenal (Nyska and Maronpot, 1999). The cells in zona golmerulosa are small, and this region of the cortex is relatively narrow and located just beneath the capsule of the gland. The cytoplasm of these cells is basophilic. Their mitochondria tend to be round or ovoid in shape, and possess tubular cristae. They also possess round perinuclear lipid droplets of uniform size (Frith, 1983).

The zona fasciculata is wide and contains cells that are arranged in columns and their cytoplasm is eosinophilic. These cells also possess large number of lipid droplets within their cytoplasm and round mitochondria. It has also been noted that considerable numbers of cells that store vitamin A are scattered throughout this zone. In a study done by Hirosawa, It is noted that vitamin A was stored within lipid droplets in the cytoplasm of fibroblast-like cells that were mostly distributed in the region between the capsule and the medulla. The highest incidence of these cells, however, was located in the adrenal cortex, principally in the zona fasciculata, whereas only relatively low levels were seen in the zona glomerulosa (Hirosawa and Yamada, 1978).

In young mice, the cortex exhibits a unique zone, the X-zone, which is formed by basophilic cells that surround the medulla. The X-zone appears at approximately 10 days of age and disappears at sexual maturity in male mice or first pregnancy in female mice (Howard-Miller, 1927).

The adrenal medulla is located in the most central part of the gland, and is completely surrounded, except at its hilar region, by adrenal cortical tissue. It tends to be reddish in color, and is largely composed of chromaffin cells. Chromaffin cells are involved in the storage and secretion of adrenaline and noradrenaline. The adrenal medulla also contains sympathetic ganglion cells, venules, and capillaries. The cytoplasm of the medullary cells is granular and more basophilic than that of the cortical cells, and contains large numbers of chromaffin-positive secretory granules. Two types of chromaffin cells have been recognized in the adrenal medulla, largely based on their staining characteristics, and are said to be related to the two catecholamines secreted by this region of the gland (Eränkö, 1951).

In the adult mouse, a high proportion of these cells appears to be adrenaline secreting, while only about one third to one quarter are noradrenaline secreting (Tischler and Sheldon, 1996). The latter are principally located in the region of the medulla closest to the adrenal cortex. The density of the two types of granules within the chromaffin cells differs, so that those that are involved in the storage of adrenaline are moderately dense, while those that store noradrenaline are extremely dense. Sympathetic ganglionic cells are distributed throughout the medulla.

2. Statement of the problem

Practices of traditional medicine vary greatly from country to country, and from region to region, as they are influenced by factors such as culture, history, personal attitudes and philosophy. In many cases, their theory and application are quite different from those of conventional medicine. Long historical use of many practices of traditional medicine, including experience passed on from generation to generation, has demonstrated the safety and efficacy of traditional medicine. However, scientific research is needed to provide additional evidence of its safety and efficacy.

The major challenges of any pharmaceutical scientist are serious problems with the overall quality, safety and efficacy of herbal products. Preservation and dosage measurement are serious problems in developing countries. The label claim and other information provided for the use of any herbal preparation may be far from what is in the `bottle'. Different orthodox medicines may be added to a herbal preparation with the hope that one of the added drugs may cure the user's ailment. Just because an herb is natural does not mean that it is safe, and claims of remarkable healing powers are often not supported by reliable evidence. Unfortunately, most countries do not have regulatory policies that can effectively protect their citizens from the identified problems.

Despite its existence and continued use over many centuries, and its popularity and extensive use during the last decade, traditional medicine has not been officially recognized in most countries. Consequently, education, training and research in this area have not been accorded due attention and support. The quantity and quality of the safety and efficacy data on traditional medicine are far from sufficient to meet the criteria needed to support its use worldwide. The reasons for the lack of research data are due not only to health care policies, but also to a lack of adequate or accepted research methodology for evaluating traditional medicine. It should also be noted that there are published and unpublished data on research in traditional medicine in various countries, but further research in safety and efficacy should be promoted, and the quality of the research should be improved. *A. afra* is also one herbal medicine without adequate information and research on its safety and efficacy. So in this study, toxicity of *A.afra* on three organs (brain, heart & suprarenal glands) was assessed. Brain and heart were selected because of their high metabolic need which makes them prone to any noxious agent. Suprarenal glands were selected as a representative organ for endocrine glands and because of its high blood supply.

3. Significance of the study

Many plants are highly toxic. Herbal medicine probably presents a greater risk of adverse effects and interactions than any other complementary therapy. Serious adverse events after administration of herbal products have been reported, and in most cases, the herbs involved were self-prescribed and bought over the counter or were obtained from a source other than a registered practitioner (Andrew *et al*., 2000). In Ethiopia, most traditional healers are not registered and this makes the problem of developing toxic effect of herbal medicine worse. Furthermore, in Ethiopia most traditional medicine practioners are not trained and mostly traditional medicine practice are passed orally from generation to generation without any record which cause lack of complete information on side effect of herbal medicine. Therefore traditional medicine practioners prescribe herbs without considering its side effects and the patient condition. *A.afra* is one of the most commonly used herbal medicines in Africa including Ethiopia. This herb is commonly administered nasally, orally and topically. Several studies on *A. afra* have been done especially its importance in treatment of helmentic infection, control of diabetes, anticancer and antioxidant effects. But research related to toxic effects of *A.afra* in different organ is insufficient and incomplete including brain, heart and suprarenal glands.

Neurotoxicity is the term applied to a toxic effect on any aspect of the central or peripheral nervous system. Effects may be functional (behavioral or neurological abnormalities), biochemical, physiological, or morphological. It has been suggested that over one-third of chemicals may be neurotoxic (John and Howard, 2006)**.** High metabolic rate of the brain which leads to disproportionate shares of the total cardiac output and blood-borne oxygen supply(approximately 15% and 20%, respectively) even though the brain mass represents only about 2% of the total body mass (Heiss, 1981). This tremendous metabolic rate makes the brain as a whole especially vulnerable to neurotoxicants that disrupt intracellular energy production (Nicklas, 1992). A high rate of oxidative metabolism rate is a property also shared by the heart. Toxicants that injure the brain often injure the heart, and vice versa. Suprarenal glands are reported to be the most common endocrine organ associated with chemically induced lesions (Ribelin, 1984).Furthermore, the histopathologic effect of *A. afra* on the brain, heart and suprarenal glands are not completely understood. Generally, this research will fill the gap in knowledge on histopathologic effects of *A. afra* on the brain (cerebral cortex), heart and suprarenal glands by then it can be used as a baseline for further toxicity study in higher animals.

4. Objectives

4.1. General objectives

- To investigate toxic effects of aqueous leaves extract of *A.afra* on different organs of mice after acute and subacute administration.

4.2. Specific objectives

- To determine the LD_{50} of aqueous leaves extracts of *A.afra.*
- To assess the effects of aqueous leaves extracts of *A. afra* on the body weight.
- To evaluate the histopathological effect of aqueous leaves extracts of *A.afra* on cerebral cortex of mice after subacute administration.
- To evaluate the histopathological effect of aqueous leaves extracts of *A.afra* on heart of mice after subacute administration.
- To evaluate the histopathological effect of aqueous leaves extracts of *A.afra* on suprarenal glands of mice after subacute administration.

5. Material and method

5.1. Study design:-laboratory –based experiment

5.2. Study area

The study was conducted at Addis Ababa University, College of Health Science, School of Medicine, Department of Anatomy (*Histology Laboratory*) and Department of Physiology.

5.3. Study period

The study was conducted from Jan. 2014-July, 2015.

5.4. Collection and extraction of plant materials

A.afra was collected from Bale National Park, 400km southeast of Addis Ababa and 150 km east of Shashemene in the Oromia regional state during the month of September, 2014. The plant was identified and identification number was given by Ethiopian National Herbarium (392/NKI/PHARM). The plant leaves were dried in shade so as to bring down the initial large moisture content to enable its prolonged storage life. The powdered dried leaves 400 g of *A.afra* were macerated with water for 2hrs with intermittent agitation by orbital shaker. Then, the supernatant part of agitated materials was decanted and filtered with 0.1 mm^2 mesh gauze from the un-dissolved portion of plant. The filtrate of plant was freeze-dried to form crude extract. The yield of the extract was 43 g (10.75%). The extract was kept in a desecrator at room temperature until used for the experiment.

5.5. Selection of animal species

The animals used in this study were bred and reared at the animal house of the EPHI and transported to Tikur Anbessa, Collage of health Science, Laboratories of physiology. Experiments were conducted on 54 healthy adult male and female mice aged 8-12 weeks weighing 25-30g. Grouping of mice were done randomly. The animals were kept in separate aluminum cages and provided with bedding of clean paddy husk. All animals had a free access to standard pellet diets and tap water. The mice were acclimatized to laboratory conditions for one week prior to the experimental protocol to minimize any nonspecific stress (OECD, 2001).

5.6. Method of extract administration

The test substance was administered in a single and repeated dose by gavage for acute and subacute studies respectively. For the acute toxicity study, prior to dosing food but not water was withheld for 3hr to increase absorption of the extract. Following the period of fasting, the animals were weighed and the dose was calculated according to the body weight for each animal. The test substance was then administered. After the substance has been administered, food was withheld for 2 hours (OECD, 2001).

For chronic study, each animal was weighed in each group and their total weight was calculated. Then, for each group dose was calculated according to the group weight. For each mouse within the group, the drug was given according to their weight after preparing stock solution.

5.7. Acute toxicity Study and LD_{50} Determination

Acute toxicity test was started with low initial dose of 200mg/kg. This dose was selected in reference to previous efficacy study of *A.afra* which showed significant antidiabetic activity at 200 mg/kg in diabetic rat (Taofik and Anthony, 2013). Additional six higher doses of 700mg/kg, 1200mg/kg, 2200mg/kg, 3200mg/kg, 4200mg/kg and 5000mg/kg were used. A total of eight group of mice were used (seven treated and one control) each consisting of 3 female adult albino Swiss mice. All groups of mice fasted 3hr prior to administration. At the end of the fasting period, the body weight of each mouse was recorded before dosing and the total body weight was calculated for each group. For each group, dose and stock solution was also calculated and prepared. Then the extract was administered for each mice in treated groups (G I to G XII) according to body weight. The control group (Group VIII) received water (the vehicle). The administration procedure was done by gavages using a ball-tipped stainless steel feeding needle to all treated and control animals.

The treated and control groups were observed continuously for 3 h and then every 24 hours for the next 14 days; and any signs of toxicity and mortality were recorded. The presence or absence of toxic signs like increased motor activity, tremors, ptosis, lacrimation, exophthalmos, piloerection, salivation and depression were observed during the study. The body weight of each mouse was recorded at the 7^{th} day and 14^{th} day. The differences in the body weight were also recorded.

Lethal doses for fifty percent of the mice (LD_{50}) for aqueous leaf extracts of *A.afra* were determined using Protocol for LD_{50} determination (OECD, 2001). On the 14th day of treatment, all mice were sacrificed with anesthetic diethyl ether. Comprehensive gross pathological observations were carried out on the brain, heart and suprarenal glands to check for any signs of abnormality and presence of lesions.

5.8. Subacute toxicity study

The study was carried out using 15 female and 15 male mice which were grouped into six groups. The 15 female mice were grouped into three groups with five mice in each group. Similarly, the 15 male mice were grouped into three groups with five mice in each group. For both sex, two groups were given the extract of *A.afra* for 28 days. One group was given vehicle i.e. water. The doses for experimental groups were 600mg/kg (low dose) and 1800mg/kg (high dose). The low dose was selected in reference to efficacy study of *A.afra* in treatment of malaria which shows 400 mg/kg as effective dose (Moges *et al*., 1998). But this does was modified to 600 mg/kg based on clinical observation of acute toxicity studies (OECD, 2008).

Individual weights of mice were taken shortly before the test substance is administered and weekly thereafter using digital electronic balance. Weight changes were calculated and recorded. Mice were observed individually one time during the first 30 minutes after dosing, four times with one hour interval for the first 4 hours and daily thereafter for 28 days. All observations are recorded with individual records being maintained for each animal (OECD, 2008).

For screening possible neurobehavioral toxicity of *A.afra* in cage and open field observation was done daily. Behavior is the adaptive response of an organism to internal or external stimuli and, as such, represents the integrated end product of multiple neuronal subsystems. Thus, evaluation of behavior can serve as an indicator of the status of the functional components of the nervous system. In addition, since behavioral testing is noninvasive, it can provide a longitudinal assessment of the neurotoxic effects of an agent, including persistence, delayed onset, or recovery. Typically, a functional observational battery (FOB) is employed to assess a wide range of neurobiological functions, including sensory, motor, and autonomic components. In the present study FOB comprising a series of assessments designed to measure motor, sensory and autonomic function was used (Michael and Carol, 2014). To avoid bias observation and scaling was done by colleagues (See Annex IV).

At the end of the test, animals were weighed and then humanely killed using diethyl ether and cervical dislocation (OECD, 2008). Organs of interest namely the brain, heart and suprarenal glands were carefully dissected out and weighed in grams. The relative organ weight of heart and brain was calculated by using the animal weight as a denominator. But for suprarenal glands the brain was used as a denominator.

$$\textbf{Relative organ weight} = \frac{\textbf{absolute organweight(g)}}{\textbf{body weight of mice on sacrifice}} \times \textbf{100}$$

Gross pathological examinations were done for toxicological lesion on. For the brain, frontal lobes of cerebrum were taken by trimming (coronal section) the brain at the level of Bregma. Both the right and left suprarenal glands were taken as a sample. The heart was sectioned longitudinally through ventricle and atria from the base to the apex and the left atria and ventricle were taken for histopathological investigation. Sample tissues were taken and placed in a labeled test tube containing 10% buffered formalin.

The tissue samples of the various groups of mice in 10% neutral buffered formalin were withdrawn after 24 hrs of fixation and then thoroughly rinsed over several changes of tap water. They were then dehydrated with increased concentration of ethanol (70% and 90% and absolute alcohol). After dehydration the tissues were cleared by xylen. Next, the tissues were impregnated in paraffin wax. Embedding the tissue samples into tissue blocks were done by putting tissue samples in squares of metal plates with proper orientation of the specimens and carefully pouring molten paraffin over them followed by waiting to harden at room temperature (See Appendix II).

Tissue blocks were sectioned with a thickness of 5µm using Leica rotary microtome (LEICA RM 2125 RT, Germany). Ribbons of the tissue sections were gently collected using a piece of brush and laid onto the surface of a water bath heated at 40^0C. After the sections were appropriately spread on the water bath, they were placed over tissue slides. The slides were arranged in slide racks and were placed in an oven with a temperature of 60^0C for 10-15 minutes to facilitate the adhesion of the specimens onto the glass slides. The specimens were allowed to cool and stained using Hematoxylin and Eosin staining method as shown in the Appendix III.

Stained tissue sections of brain, heart and suprarenal glands were carefully examined under binocular compound light microscope (LEICA DM 750, Germany). Tissue sections from the treated groups were examined for any evidence of histopathological changes with respect to those of the controls. After examination, photomicrographs of selected samples of brain, heart and suprarenal glands sections from both the treated and control mice were taken under a magnification of x20 objective by using (EVOS XL, USA) automated built-in digital photo camera.

5.9. Data processing and analysis

All data were organized and analyzed by SPSS version 21 statistical software. The values of body and organ weight including relative organ weights were analyzed and the results were expressed as M ±SEM (standard error of mean). Difference between the treated and control group were compared using one-way ANOVA followed by Dunnett's T test to assess level of significance. P values <0.05 were considered statistically significant.

6. Results

6.1. Acute toxicity study

The crude extract of *A.afra* was administered orally to mice in the treatment groups I, II, III, IV, V, VI, and VII with doses of 200mg/kg, 700mg/kg, 2200mg/kg, 3200mg/kg, 4200mg/kg and 5000mg/kg, respectively; while the control group (Group IX) received the vehicle (distilled water). The acute toxicity study of aqueous extract of *A.afra* in mice did not show any mortality even with the highest dose which was 5000mg/kg. No signs of toxicity were observed on the lower four doses i.e. 200mg/kg, 700mg/kg, 1200mg/kg, and 2200mg/kg. However sign of mild toxicity like anxiety and piloerection were seen at the doses of 3200, 4200mg/kg and 5000mg/kg. These symptoms disappeared after some washing periods in the two weeks observation. Moreover, there was a gradual increase in body weight of treated mice of groups I to VII and control mice as shown in Table 1. The body weight changes in both the treated and control group were insignificant.

Table 1 Body weight changes of male mice treated with a single doses of crude extracts of *A.afra* in doses of 200mg/kg, 700mg/kg, 1200mg/kg, 2200mg/kg, 3200mg/kg,4200mg/kg and 5000mg/kg as compared to control.

Group	Dose (mg/kg)	Mean initial body weight(g)	Mean body weight at the end of week 1 (g)	Mean body weight on day 14 (g)
I	**200**	27.5±1.4(0.68)	31.7±1.7(0.19)	34.2±1.4(0.78)
II	**700**	27.9±1.2(0.24)	31.2±1.5(0.23)	34.2±1.1(0.05)
III	**1200**	29±0.8(0.58)	31.7±0.5(0.52)	33.9±1.5(0.33)
IV	**2200**	27.4±0.8(0.27)	29.4±1.2(0.92)	31.7±1.4(0.51)
V	**3200**	29.1±0.6(0.57)	31.6±1.2(0.69)	33.6±1.3(0.17)
VI	**4200**	28.5±0.9(0.77)	29±0.3(1)	29.1±1.1(0.05)
VII	**5000**	29.1±0.3(0.82)	29.4±0.9(0.57)	30.4±1.3(0.5)
Control	**Vehicle**	28.4±0.7	29.2±0.6	30.5±0.9

6.2. Subacute toxicity study

6.2.1. Effects of A.afra aqueous extract on behavior and body weight of mice

Even though this research was concerned to investigate morphological change in cerebral cortex after administration of aqueous extract of *A.afra*, the presence or absence of behavioral change was checked subjectively using modified observational testing Batteries (Brad and Mark T, 2011, Michael and Carol, 2014). During the period of 28 days of subacute toxicity evaluation, mice that were treated orally with the repeated doses of the formulation of 600mg/kg and 1800mg/kg body weight showed no noticeable changes in their general behavior as compared to the control group (See table 2). For detailed evaluation and scoring of individual mice on some selected sign see Annex V. Moreover, there was no toxicity related death throughout the period of study.

Table 2 Neurobehavioral neurotoxicity evaluation of Male and Female mice treated with 600mg/kg and 1800mg/kg doses as compared with those of control group in consecutive four weeks observation.

Neurologic symptom & sign	Male			Female		
	600mg/kg	1800mg/kg	Control	600mg/kg	1800mg/kg	Control
Ataxia/abnormal gait	-	-	-	-	-	-
Hunched posture	-	-	-	-	-	-
Pain response (tail pinch)	+	+	+	+	+	+
Convulsions	-	-	-	-	-	-
Depression of open field activity	-	-	-	-	-	-
Anorexia	-	-	-	-	-	-
Irritability/aggressiveness	+	+	+	-	-	-

Present (+) and absent (-)

There were gradual weight gain in both control and treated groups. There was no significant difference in terms of weight gain between treated and control groups. There was also no significant difference between male and female groups (See Table 3& 4).

Table 3 Mean body weights of Male and Female mice treated with 600mg/kg and 1800mg/kg doses as compared with those of the control group in consecutive four weeks measurement.

Week	Male			Female		
	Control	600mg/kg	1800mg/kg	Control	600mg/kg	1800mg/kg
1	27.7±0.8	27.1±0.9 (0.644)	29.0±0.5 (0.538)	28.5±0.9	27.7±0.97 (0.428)	28.5±0.6 (0.692)
2	30.2±1.3	30.6±0.8 (0.732)	32.0±0.9 (0.717)	33.1±0.97	29.8±0.9 (0.962)	31.8±0.7 (0.187)
3	34.9±1.5	34.7±0.6 (0.921)	34±0.8 (0.787)	36.9±0.98	31.3±1.1 (0.846)	36±0.6 (0.828)
4	36.6±1.4	35.9±1.8 (0.284)	37±0.6 (0.507)	37.6±1.3	34.1±1.3 (0.339)	38.9±0.5 (0.275)

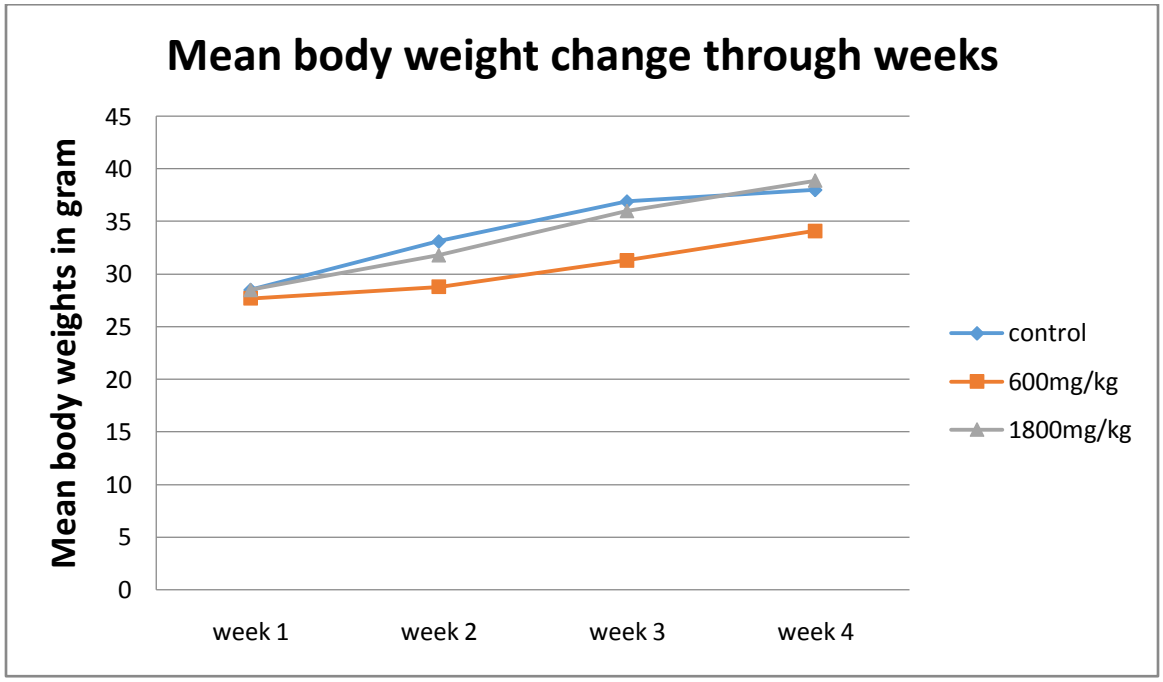

Figure 2 Comparison of body weight change between female mice treated with 600mg/kg and 1800mg/kg as compared to control

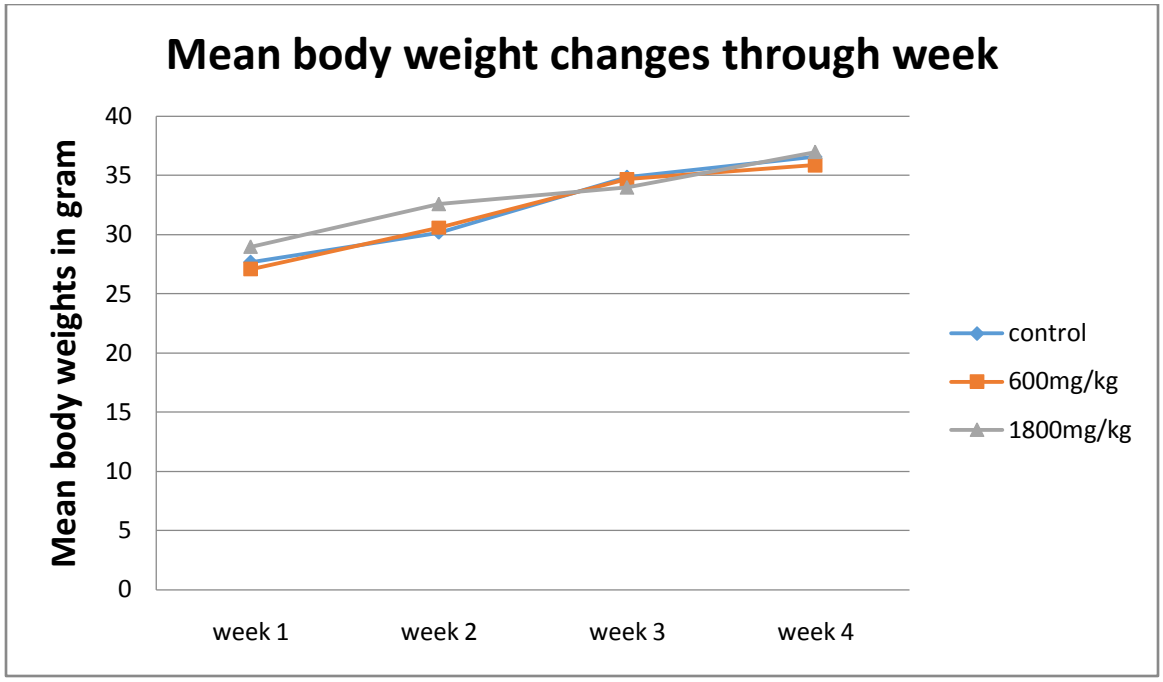

Figure 3 Comparison of body weight changes between male mice treated with 600mg/kg and 1800mg/kg as compared to control

6.2.2. Effects of A.afra aqueous extract on gross pathology and relative organ weight of mice brain, heart and suprarenal glands

On gross examination of brain, heart and suprarenal glands, no abnormal gross pathological findings like dark & white spot and necrosis were observed in any of the treatment and control groups. There were no significant organ weight and relative organ weight change between treated and control groups in both sexes (See Table 5&6).

Table 4 Absolute and relative organ weights of female mice orally treated with different dose of aqueous extract of *A.afra* for 4 weeks as compared to control

Group	Brain		Heart		Suprarenal glands	
	Absolute weight(g)	Brain /lean weight (%)	Absolute weight(g)	Heart /lean weight (%)	Absolute weight(g)	Adrenal/brain weight (%)
600mg/kg	0.43±0.02 (0.93)	1.26±0.02	0.15±0.007 (0.97)	0.44±0.01	0.01±0.001 (0.95)	2.32±0.001
1800mg/kg	0.45±0.003 (0.325)	1.16±0.02	0.16±0.02 (0.78)	0.41±0.008	0.01±0.002 (0.33)	2.22±0.001
Control	0.45±0.002	1.2±0.04	0.15±0.003	0.41±0.004	0.01±0.004	2.22±0.001

Table 5 absolute and relative organ weights of male mice orally treated with different doses of aqueous extract of *A.afra* for 4 weeks as compared to control

Group	Brain		Heart		Suprarenal glands	
	Absolute weight(g)	Brain /lean weight (%)	Absolute weight(g)	Heart /lean weight (%)	Absolute weight(g)	Adrenal/ brain weight (%)
600 mg/kg	0.43±0.02 (0.89)	1.21±0.02	0.15±0.01 (0.75)	0.42±0.02	0.01±0.002 (0.97)	2.32±0.003
1800mg/kg	0.44±0.01 (0.56)	1.19±0.03	0.17±0.01 (1.23)	0.46±0.01	0.01±0.004 (0.57)	2.27±0.004
Control	0.44±0.2	1.2±0.02	0.15±0.01	0.41±0.01	0.01±0.003	2.27±0.004

6.2.3. Effects of aqueous extract on histology of cerebral cortex, heart and suprarenal glands

Microscopic examination of cerebral cortex of mice treated with 600mg/kg and 1800mg/kg of aqueous extracts of *A.afra* indicated no structural disturbance as compared to control. No sign of individual neuron death and focal lesion i.e. pyknosis, karyorrhexis and karyolysis and eosinophilic cytoplasm were observed in male and female mice. No sign of inflammation like lymphocytic infiltration were observed in male and female mice (See Fig 4). The architecture of cerebral cortex was identical between control and treated mice.

On microscopic examination of the heart of treated and control female and male mice, no architectural difference was observed. In both mice treated with 600mg/kg and 1800mg/kg of extract no sign of myocardial cell injury i.e. pyknosis, karyorrhexis, karyolysis, vacuolation, focal necrosis and fibrosis was observed. No sign of inflammation (leukocytic infiltration) were observed in female and male treated mice groups (See Fig 5).

On examination of suprarenal glands section, no sign of toxicity were observed on both the cortical and medullary region. The architecture of the cortex and medulla of mice treated with 600mg/kg and 1800mg/kg of extract is identical with control. In both treated and control mice, two cortical zones are observed with some mice showing additional zone(X-zone). But no

cortical lesion like degeneration (vacuolar or granular), necrosis or hemorrhage was observed (see Fig 6).

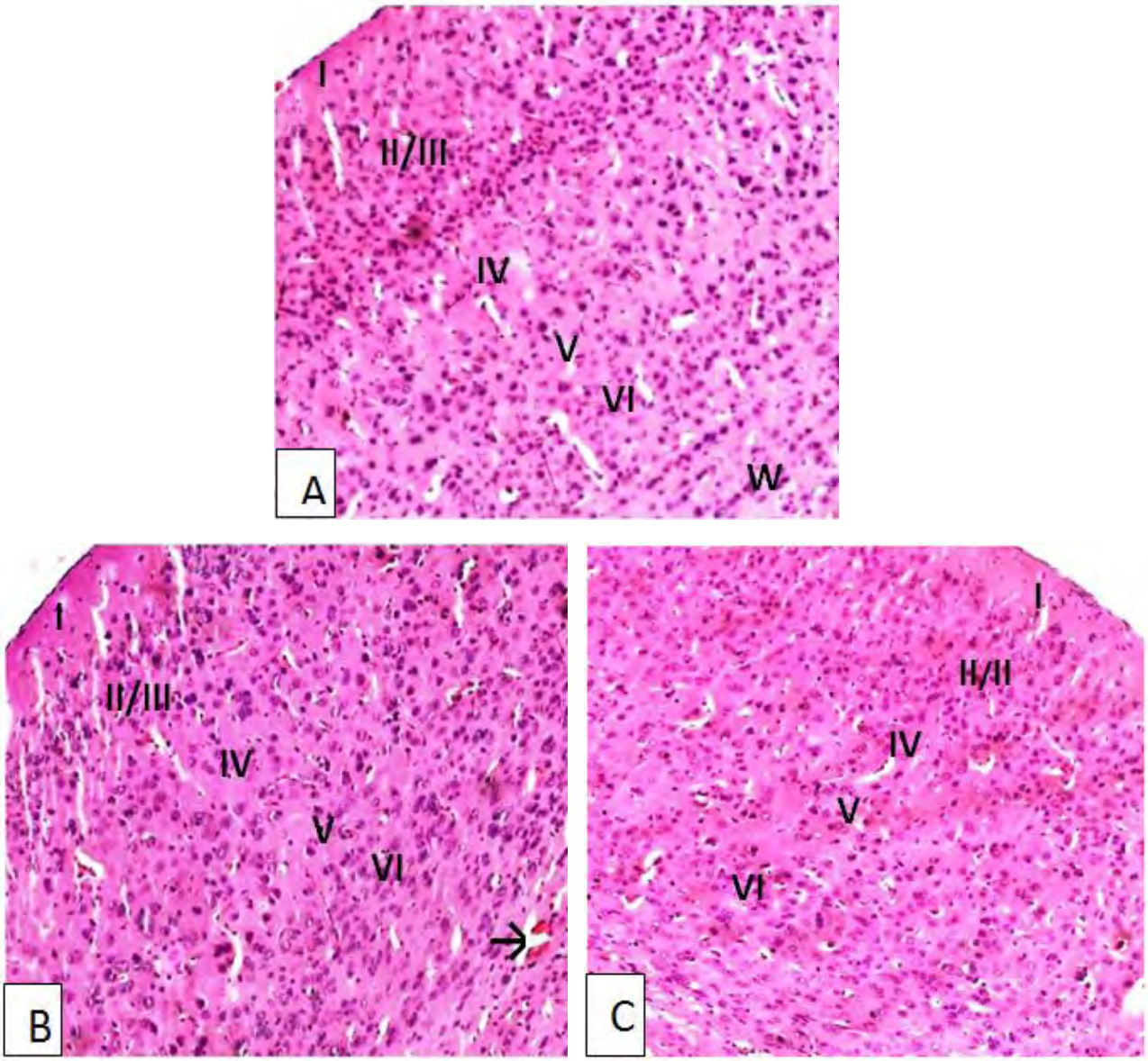

Figure 4 Photomicrographs of sections of cerebral cortex of female mice control (A) as compared to mice treated with 600mg/kg body weight/day (B) and 1800mg/kg of body weight/day (C) of aqueous extract of *A.afra* (A). I= Superficial (molecular) layer, II/III= Supragranular Pyramidal layer, IV= Granular layer, V= Deep Pyramidal layer and VI = Polymorphic or Multiform layer (Stained with H&E, X200).

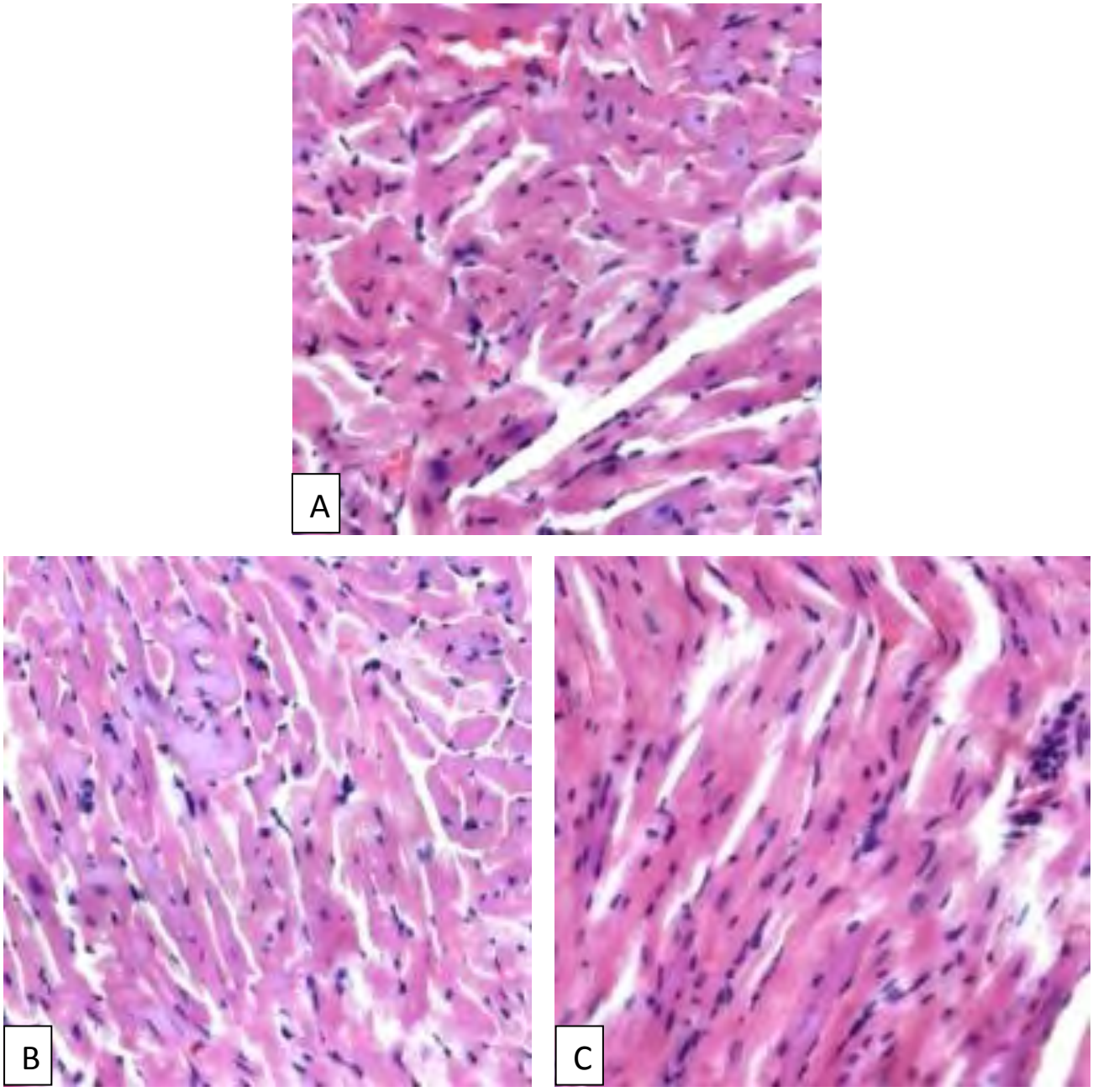

Figure 5 Photomicrographs of sections of heart of female mice control (A) as compared to mice treated with 600mg/kg body weight/day (B) and 1800mg/kg of body weight/day (C) of aqueous extract of *A.afra* (A). (Stained with H&E, X300)

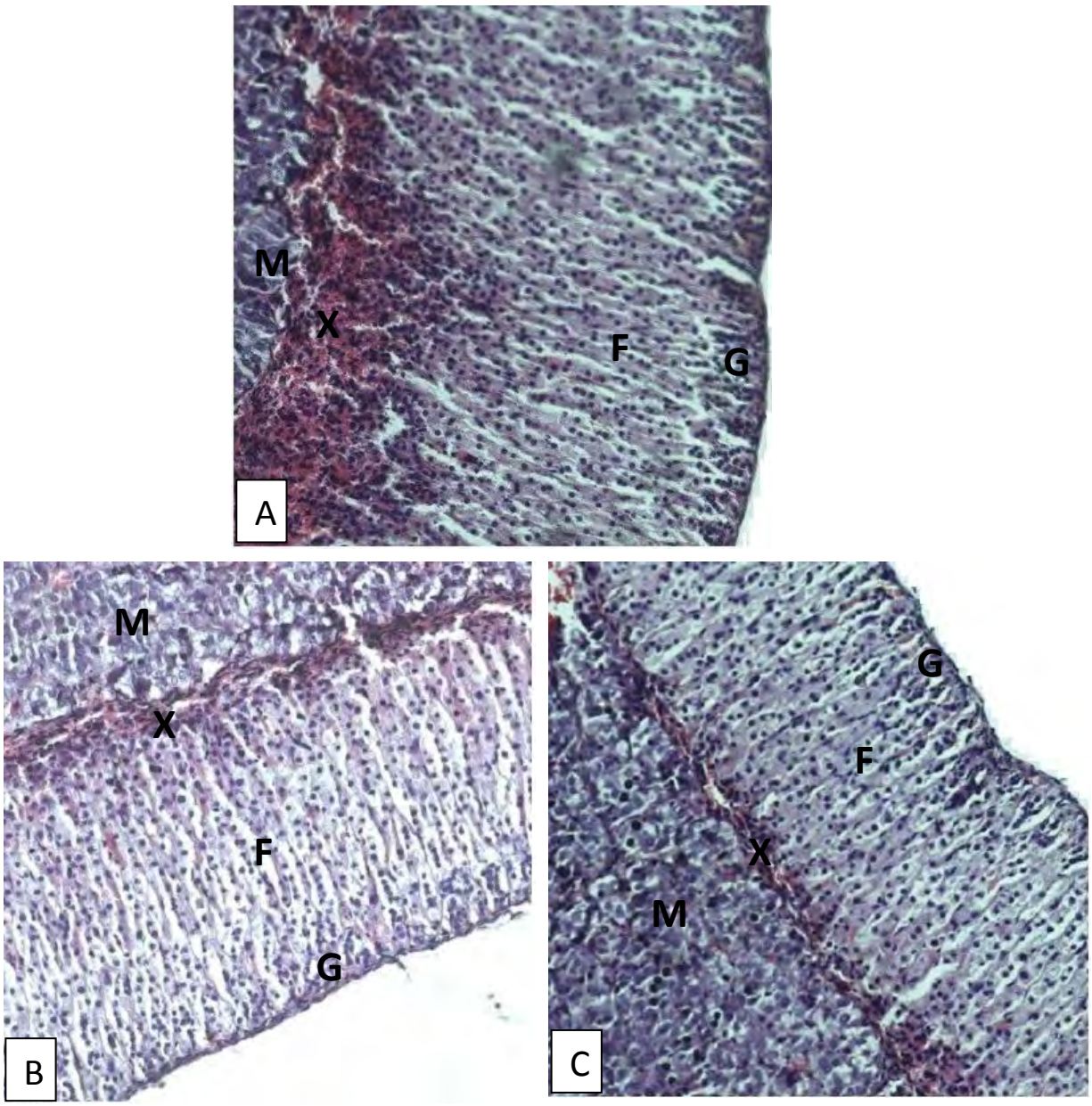

Figure 6 Photomicrographs of sections of suprarenal glands of female mice control (A) as compared to mice treated with 600mg/kg body weight/day (B) and 1800mg/kg of body weight/day (C) of aqueous extract of *A.afra.* G=zona golerulosa, F= zona fasciculate, X= x zone and M= adrenal medulla (Stained with H&E, X200).

7. DISCUSSION

In reference to medications, safety is the likelihood of not causing harm under the proposed conditions of use, while efficacy is the capacity to induce a clinical benefit. Both safety and efficacy depend on the drug's therapeutic indication; in principle, a substance has no clinical usefulness if it is "safe" but lacks efficacy or if it is active on a relevant therapeutic target but its use is unsafe. Although these are recognized as equally essential attributes of any medicine, safety has taken precedence over proof of efficacy in drug regulation history. The idea that safety should come first in therapeutic interventions is conveyed by the famous Latin expression "primum non nocere" ("first, not do harm"), the origin of which is uncertain (Smith, 2005). Evaluation of the pathological alterations induced in laboratory animal by novel drugs represents the cornerstone of their safety assessment before they can be first tried in patients. This preliminary assessment represents major contributions to the development of new treatments for human and animals.

Acute toxicity tests are generally the first tests conducted. They provide data on the relative toxicity likely to arise from a single or brief exposure. It is an initial assessment of toxic manifestations and is one of the initial screening experiments performed with all compounds (Shetty *et al*., 2007). The acute study of this research confirms that the LD_{50} of aqueous extract of *A.afra* is above 5000mg/kg which is grouped under category 5 or unclassified according to Globally Harmonized Classification System (OECD, 2001). This is in line with previous research done on aqueous extract of *A.afra* on mice, i.e. LD_{50} was 8960 mg/kg (James, 2012). In present study, oral administrations of the extract up to dose of 2200 mg/kg did not produce any mortality or significant alteration in the behavioral pattern of mice as compared to the control group. However mild toxicity sign was observed on the higher three dose. There was no significant weight gain difference between the treated group and control. On gross examination of organ of interest (heart, suprarenal glands and brain), no treatment-related gross findings were observed at necropsy. According to the OECD guideline for testing of chemicals, the results of acute toxicity test in this study indicate that aqueous extract of *A.afra* is fairly non-toxic.

Subacute toxicity study provides information on the possible health hazards likely to arise on the nervous system, immune and endocrine systems from repeated exposure over a relatively limited period of time (OECD, 2008). In the subacute phase of this research, effect of oral administration of aqueous extract of *A.afra* on body weight, brain, heart and suprarenal glands was studied.

One common topic of debate and confusion in a neurotoxicity assessment approach has been in choosing between pathological or behavioral endpoints as the method to use in a safety study. However both should be considered as complementary approaches in neurotoxicity study (Brad and Mark T, 2011). On behavioral observation, no significant behavioral change was observed between the treated and control group in both sex.

Increases or decreases in the body weights of animals can be used as an indicator of adverse effects of drugs and chemicals (Teo *et al*., 2002). However, body weight increases of animals are more closely related to body fat accumulation rather than to the toxic effects of drugs or chemicals (Harizal *et al*., 2010). On the other hand reductions in the body weights of animals in toxicity studies may be associated with normal physiological adaptation responses to the plant extracts or compounds, which lead to low appetite and lower caloric intake by the animal (Rhiouani *et al*., 2008). In the present studies, all mice showed significant increase in body weight compared to their initial values. However there was no significant difference between the different treatment groups and the control, indicating that it did not have any adverse effects on the body weight.

Evaluation of organ weight changes in the presence of body weight differences has resulted in the use of additional tools such as organ-to-body weight and organ-to-brain weight ratios to assess treatment effects in toxicology studies (Stevena *et al*., 2004). For suprarenal glands organ to brain ratio is more predictive of suprarenal glands weight change than organ to body weight ratio (Stevena *et al*., 2004). In this research, there were no significant changes in absolute and relative organ weights of all groups. No significant difference was observed between treated and control groups. Such result is in line with research done on other species of the genus Artemisia (Sung - Ha *et al*., 2011).

Because of the heterogeneous nature of the nervous system, different neurotoxic chemicals can affect neurologic and behavioral functioning in different ways. There are certain expressions of neurotoxicity that are most easily appreciated in the course of behavioral testing. Other effects do not lead to observable differences in behavior, but may have observable temporary or permanent changes in the morphology of the brain (Brad and Mark T, 2011).

In response to injury, a number of changes occur in neurons and their processes (axons and dendrites). There is shrinkage of the cell body, pyknosis of the nucleus, disappearance of the nucleolus, and loss of Nissl substance, with intense eosinophilia of the cytoplasm (red neurons). Often, the nucleus assumes angulated shape of the shrunken cell body. Injured axons undergo swelling and show disruption of axonal transport. The swellings can be recognized on H&E stains. Axonal injury also leads to cell body enlargement and rounding, peripheral displacement of the nucleus, enlargement of the nucleolus, and peripheral dispersion of Nissl substance (central chromatolysis) (Vinay *et al*., 2013). On the histopathological examination of cerebral cortex none of this morphological change was observed which is supported by absence of abnormal behavior and motor activity on cage side observation. Therefore, the herb *A.afra* may not cause toxicity to cerebral cortex of the mice.

Because of its high oxidative metabolic need, the heart can be injured by any compounds that interfere with its oxygen supply. Although the myocardium can be damaged by a variety of insults such as anoxia, ischemia, infectious agents, physical and chemical agents, its pattern of response is limited. Myocardial fiber damage can take the form of a cytoplasmic alteration such as vacuolation but the sever consequence of myocyte damage is necrosis. Necrosis is accompanied by a variable degree of inflammation that depends to some extent on the injurious agent (Peter, G. 2004).

The ultimate response of the heart to toxic exposure is myocardial degeneration, which can be measured by both morphological and functional changes. However, myocardial degeneration should not be considered an irreversible toxic response. In the past, the heart has been considered incapable of regenerating, so that cardiac injury in the form of cell loss or scar tissue formation was considered permanent damage to the heart. However, evidence now indicates that heart is capable of regeneration and recovery from injuries (Anversa *et al*., 2006). This is because of cardiac progenitor cells also called cardiac stem cells that can develop into new myocytes and

vascular structure. So, myocardial damage from toxic agent can be divided into reversible and irreversible.

Myocardial cell death, fibrosis (scar tissue formation), and contractile dysfunction are considered as degenerative responses, which can result in cardiac arrhythmia, hypertrophy, and heart failure (Daniel, A. 2008). If acute cardiac toxicity does not affect the capacity of myocardial regeneration, the degenerative phenotype is reversible, otherwise irreversible. Both acute and chronic toxic stresses can lead to irreversible degeneration, depending on whether or not the cardiac repair mechanisms are damaged. Cell death is the most common phenotype of myocardial degeneration. Both apoptosis and necrosis occur along with hypertrophy of the remaining cardiac myocytes so that in the hypertrophic heart, the total number of cardiac myocytes is often reduced but the size or volume of individual cells is increased.

Myocardial adaptation refers to the general process by which the ventricular myocardium changes in structure and function in response to endogenous and exogenous stressors. This process is often referred to as "remodeling." The central feature of myocardial remodeling is an increase in myocardial mass associated with a change in the shape of the ventricle (Frey and Olson, 2003). The increase in myocardial mass is reflected by cardiac myocyte hypertrophy, which is characterized by enhanced protein synthesis, heightened organization of the sarcomere, and the eventual increase in cell size.

In the present study involving microscopic examination of the heart, none of these lesions were observed which is in line with other research done on the same plant (James, 2012). Furthermore, other research done to assess the effect of *A.afra* on isoproterenol (ISO)-induced myocardial injury in rats showed cardioprotective property of this herb (Anthony and Taofik, 2010). Therefore instead of causing toxicity, *A.afra* may even have active component that can activate cardiac stem cell to divide and replace dead myocytes.

Suprarenal glands are reported to be the most common endocrine organ associated with chemically induced lesions (Thomas *et al*., 2001). In the adrenal cortex, lesions are more frequent in the zona fasciculata than in the zona glomerulosa. The adrenal cortex produces steroid hormones with a 17-carbon nucleus following a series of hydroxylation reactions that occur in the mitochondria and endoplasmic reticulum. Toxic agents for the adrenal cortex include short-chain aliphatic compounds, lipidosis inducers, amphiphilic compounds, natural and synthetic steroids, and chemicals that affect hydroxylation (Curtis, 2001). Morphologic

evaluation of cortical lesions provides insight into the sites of inhibition of steroidogenesis. In general, the adrenal medulla is a less common site of chemically induced degenerative lesions. However, in this research no histhological degenerative or prolifertative lesions of cortex or medulla were observed suggesting non-toxicity of the plant on suprarenal glands. The result is in line with research done in other species of the same genus (Sung - Ha *et al*., 2011).

8. Conclusion

Treatment of mice with a single dose of aqueous extracts of A.afra does not induce any mortality up to 5000mg/kg. This indicate that the LD_{50} of the plant is above 5000mg/kg. In the sub acute study both the lower and higher dosage of aqueous extract of the herb *A.afra* does not induce any significant gross or microscopic morphological change of brain, heart and suprarenal glands. In addition, no significant general behavioral and motor activity change was observed during subacute study.

In conclusion, the finding of the present study support that aqueous extract of *A.afra* is nontoxic when administered with dose of 600mg/kg and 1800mg/kg of body weight in mice for a month.

9. Recommendation

- Further toxicological investigation is recommended on other parts of the brain like hippocampus and cerebellum
- Further toxicological investigation is recommended on other endocrine gland like thyroid gland.
- Further sub chronic and chronic toxicity study in other animal model should be carried out.
- Further toxicological investigation is recommended on fetus and pregnant animals.

Appendix I: Preparation of working solutions

10% Neutral Buffered Formalin

40% formaldehyde	100 ml
Distilled water	900 ml
Sodium dihydrogen phosphate monohydrate	4 gm
Disodium hydrogen phosphate anhydrous	6.5 gm

Harris's Hematoxylin (H)

Hematoxylin crystals	2.5 gm
Absolute alcohol	25 ml
Potassium alum	50 gm
Distilled water	500 ml
Sodium iodate	0.5 gm
Glacial acetic acid	20 ml

1% Alcoholic Eosin (E)

Eosin Y, water soluble (CI 45380)	1 gm
95% Ethanol	100 ml
Glacial acetic acid	0.5 ml

1% Acidic alcohol

70% alcohol	500 ml
Hydochloric acid, concentrated	5 ml

Bluing solution

Sodium bicarbonate	2.5 gm
Distilled water	1000 ml

Appendix II: Tissue processing procedures

Fixation

10% Neutral Buffered Formalin	24 hrs

Washing

Tap water	several changes

Dehydration

70% Ethanol	2 hrs
80% Ethanol	2 hrs
90% Ethanol	2 hrs
Absolute alcohol I	$1^1/_2$ hrs
Absolute alcohol II	$1^1/_2$ hrs
Absolute alcohol III	$1^1/_2$ hrs
Absolute alcohol IV	overnight

Clearing

Xylene I	$1^1/_2$ hrs
Xylene II	$2^1/_2$ hrs

Infiltration

Paraffin wax I	$1^1/_2$ hrs
Paraffin wax II	$2^1/_2$ hrs
Paraffin wax III	overnight

Appendix III: Heamatoxylin and Eosin (H & E) Staining Protocol

Deparaffinization

Xylene I	5 min
Xylene II	5 min

Rehydration

Absolute alcohol I	3 min
Absolute alcohol II	3 min
95% Ethanol	3 min
70% Ethanol	3 min
Rinse in distilled water	5 min
Stain in Hematoxylin	15 min
Rinse in running tap water	5 min
Decolorize in acid alcohol	1-3 sec
Rinse in running tap water	5 min
Immerse in Sodium bicarbonate solution	1 min
Rinse in running tap water	5 min
Counter stain in Eosin	1 min

Dehydration

70% Ethanol	3 min
95% Ethanol	3 min
Absolute alcohol II	3 min
Absolute alcohol I	3 min

Clearing

Xylene II	5 min
Xylene I	5 min

Annex IV: Functional Observational Batteries scale for neurobehavioral evaluation of selected sign.

In- cage observation	
Sign	**Scoring**
Posture	1 = Sitting or standing normally
	2 = Rearing
	3 = Curled up
	4 = Hunched
	5 = Flattened
	6 = Lying on side
Abnormal motor activity	1 = No abnormal movements
	2 = Tremors
	3 = Fasciculation
	4 = Convulsions
	5 = Stereotypy (circling, sniffing, Licking & grooming)
	6=other
Handling evaluation	
Ease of removal	1 = Atypically docile, with minimal awareness
	2 = Easy; shows awareness but no avoidance or resistance
	3 = Moderately difficult; some resistance or avoidance but shows no aggression
	4 = Difficult; considerable resistance or avoidance, minimal signs of fear or aggression
	5 = Very difficult; runs around cage, is hard to pick up, attempts to bite or attack
Lacrimation	1 = No lacrimation
	2 = Slight discharge (fluid on fur at the corner of the eye)
	3 = Moderate discharge (fluid on fur just under eye)
	4 = Marked discharge (fluid on facial fur)
Anorexia	1= None(absent)

	2=Present
Salivation	1 = No salivation
	2 = Slight salivation (damp around mouth)
	3 = Moderate salivation (noticeably wet around mouth)
	4 = Marked salivation (wet on the chin and extending to the throat)
Piloerection	1 = Absent (normal)
	2 = Present (coat erect on dorsal surface)
Irritability/ aggressiveness	1= None (absent)
	2=Present
	Open field observation
Gait	U = Unable to assess (insufficient locomotion)
	1 = Normal
	2 = Slightly abnormal
	3 = Moderately abnormal
	4 = Markedly abnormal
Locomotion	1 = Normal (animal moves easily around open field)
	2 = Increased movement (animal moves mostly continuously, rarely stopping to sniff or groom)
	3 = Decreased movement (reduced movement around the field; movements may be sluggish)
	4 = None (animal does not move around the field)
Arousal	1 = Normal; alert with sniffing and exploratory movements
	2 = Low; slight stupor, some head or body movements
	3 = Very low; stupor, little or no responsiveness to the environment
	4 = High; slight excitement, tense, sudden darting, or freezing
	5 = Very high; hyper alert, sudden boost of running or movement

Annex V: Score of neurological sign of individual mice treated with 600mg/kg and 1800mg/kg of aqueous extract of *A.afra* as compared to control.

Sign	Control										600mg/kg										1800mg/kg									
	Male					Female					Male					Female					Male					Female				
	M1	M2	M3	M4	M5	M1	M2	M3	M4	M5	M1	M2	M3	M4	M5	M1	M2	M3	M4	M5	M1	M2	M3	M4	M5	M1	M2	M3	M4	M5
Posture	1	2	1	1	2	2	1	1	1	2	1	1	1	1	1	2	1	1	1	2	1	1	2	1	1	2	1	1	2	1
Abnormal motor activity	5	5	1	1	1	5	1	5	1	5	1	1	1	5	1	1	5	5	1	1	1	5	1	1	1	1	5	1	1	1
Ease of removal	4	3	3	4	4	3	4	3	3	3	4	4	4	3	5	3	3	4	3	3	4	4	3	4	4	4	3	4	3	4
Lacrimation	2	1	2	3	2	2	3	3	2	2	3	2	1	3	2	2	3	3	2	2	3	2	2	2	2	2	2	2	2	2
Anorexia	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1
Salivation	2	2	3	1	2	2	3	2	3	1	2	2	3	2	1	2	1	3	2	3	2	2	1	3	1	2	1	3	3	1

Piloerection	2	2	1	2	1	1	2	1	1	1	1	1	2	2	1	1	1	1	2	1	1	2	1	2	1	1	1	2	1	1
Agressivenes	2	2	1	2	1	1	2	1	1	1	1	2	2	2	1	1	1	1	1	1	2	2	1	2	1	1	1	1	1	1
Gait	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Locomotion	2	1	1	1	2	1	1	1	1	2	2	1	2	1	2	1	1	1	1	1	2	2	1	1	1	1	1	2	1	1
Arousal	5	4	1	1	1	1	1	1	1	1	4	1	4	1	5	1	1	1	1	1	1	4	1	5	1	1	1	1	1	1

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