Elucidating the effect of *Bacopa monnieri* on Cadmium induced toxicity in mice

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Muez Ahmed

Dedicated to my Baba

ABSTRACT

It is normal practice to divide metals according to light and heavy groups based on their density with metals over 5g/cm^3 being grouped into the heavy category and described as Heavy metals. Cadmium being a heavy metal is one of the most toxic environmental and industrial pollutants because it has the potential to impact our Central Nervous System and other vital organs. It is generally present in low levels in the environment but human activity has increased those levels beyond safe limits. According to a 2010 report by WHO Cadmium exposure is referred to as a major health concern. Hence, it is pertinent to develop low cost, effective and natural treatment modalities to address the problem. While Cadmium toxicity in various organs such as Brain, Liver, Kidney, Lungs and Reproductive organs has been investigated separately. Very few studies look at its effects on the organs collectively and its role in causing toxicity is not well investigated. *Bacopa monnieri* (BM) is a thoroughly studied medicinal plant which is famous for its neuroprotective and memory enhancing potential against metals such as Aluminium, Lead and other neurotoxins. So, *Bacopa monnieri* was selected as a treatment modality to investigate if it had the potential to counteract Cadmium's toxic effects. Molecular barcoding of the plant was done using its rbcL (Ribulose-1, 5-bisphosphate carboxylase large subunit) gene followed by testing of its ethanolic extract against Cadmium's neurotoxic, hepatotoxic and nephrotoxic effects in BALB/c mice. Behavior tests were conducted which included Y-Maze for investigation of spatial memory and Novel Object Recognition Test (NORT) for recognition memory. BM was able to mitigate Cadmium induced neurotoxicity by improving both spatial and recognition memory. Liver function test investigated levels of Alanine aminotransferase (ALT) which were higher in mice treated with Cadmium and the levels were reduced after treatment with BM. Similar results were observed in Renal function test which looked at levels of Creatinine and Urea. Histopathology of Hippocampus, Liver and Kidney validated the ameliorative effects of *Bacopa monnieri* against Cadmium toxicity that were observed in behavior and biochemical testing.

Keywords: Heavy metals, Cadmium, *Bacopa monnieri*, Ribulose-1, 5-bisphosphate carboxylase, Spatial memory, Recognition memory, Alanine aminotransferase, Creatinine, Urea

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ALT	Alanine aminotransferase
ANOVA	Analysis of Variance
Apaf1	Apoptotic protease activating factor – 1
BM	*Bacopa monnieri*
Bp	Base pair
BBB	Blood Brain Barrier
Cd	Cadmium
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
COPD	Chronic Obstructive Pulmonary Disease
PI	Preference Index
eBME	Ethanolic *Bacopa monnieri* extract
GSH	Glutathione
HSP60	Heat Shock Protein 60
MT	Metallothionein
NP	Nanoparticles
NORT	Novel Object Recognition Test
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
rbcL	Ribulose-1, 5-bisphosphate carboxylase
SEM	Standard Error Mean

CHAPTER 1
INTRODUCTION

Mother nature has blessed Pakistan with a rich flora of Medicinal plants and herbs. Amongst them, the herb, *Bacopa monnieri*, known locally as "brahmi", is of immense value to the locals. In 'Ayurveda' – the oldest concept of a holistic lifestyle in the world, the herbaceous plant is so revered that its name is synonymous with 'ultimate or transcendental knowledge' and is mostly used as a memory booster (Gohil & Patel, 2010).

It is not with unfound logic that ancient Egyptian, Chinese and Indian civilizations employed frequent use of herbaceous plants in early medicinal practices; the use of which has been documented in early medical texts (Sumner, 2000; Wu, 2005) Today, modern medicine relies heavily on pharmacological research on naturalistic and holistic methods of therapy involving use of plants and plant based products. Public and healthcare policies are keeping up, particularly, with the expansion in legalization of medicinal marijuana in much of North America. In fact, the global market for revenues generated by botanical drugs is several hundred billion dollars as of 2017 (Ahn, 2017).

Bacopa monnieri is not only a nootropic but has also been reported to be effective against other ailments such as hypercholesterolemia, anxiety and opioid toxicity (Kamesh & Sumathi, 2012; Mannan, Abir, & Rahman, 2015; Shahid et al., 2016). BM's neuroprotective ability works by promoting growth of neurons (Aguiar & Borowski, 2013). Due to of the lack of plasticity of brain cells, any damage to them is irreversible, and till regenerative medicine successfully bridges the gap between iPSC (induced pluripotent stem cell) research and its implementation in treatment of neurodegenerative diseases, medicinal plants are our refuge.

Moreover, with the increase in industrialization release of toxic metals such as Cadmium in industrial effluents has become a major health concern. Cadmium is generally present in low levels in the environment but human activity has increased those levels beyond safe limits. Like most heavy metals, it tends to stay in the body and accumulate over time. Cadmium binds to low molecular weight proteins like Metallothionein (MT) and Glutathione (GSH) which are rich in –SH groups and thus

excretes slowly which is why it has a long half-life in the body approximately 15 – 20 years. Low excretory rate results in its accumulation in tissues of the kidneys and liver, disrupting normal functioning of the renal tubular system causing calcium deposits or kidney stones. Furthermore, it deteriorates bones causing osteoporosis.

Alarmingly, sources of cadmium poisoning can be found all around us via contamination of water sources near industrial sites, factory waste and emissions, as well as via natural phenomenon such as volcanic activity and erosion. Its increased uptake poses serious risk for catching acute pneumonitis or developing COPD (chronic obstructive lung disease) (Boffetta, 1993). This is worsened by its ease of uptake via air passages where it interacts with the CNS and increases the Blood Brain Barrier's (BBB) permeability. Headaches and Parkinson-like symptoms may ensue dull vasomotor function, decreased concentration and learning disabilities (Wang & Du 2013).

Cadmium toxicity in cells results in an excess of reactive oxygen species (ROS) that can in turn lead to DNA damage. Increased levels of ROS will trigger signaling cascades that are responsible for apoptosis/cell death (Nair, DeGheselle, Smeets, Van Kerkhove, & Cuypers, 2013). It competes with Calcium , Iron and Zinc and makes use of their transport systems for uptake (Johri, Jacquillet, & Unwin, 2010). Following uptake, it gets transported to the kidney where it binds to proteins like Metallothionein's (MTs), Glutathione (GSH) and others. These MT's play a dual role: on one hand, they bind to Cd and neutralize its effect; but on the other, due to their – SH group, they can readily bind to the reactive oxygen species (ROS) produced as a result of Cd-induced oxidative stress. Intracellular Cd, in bound or unbound form, results in mitochondrial damage or cell death (Thévenod, 2009). Cd interferes with mitochondrial oxidative phosphorylation and can inhibit respiration. Moreover, it can deregulate mitochondrial genes such as HSP60 that are responsible for programmed cell death (Cannino, Ferruggia, Luparello, & Rinaldi, 2009). Various forms of cell death linked with Cd toxicity include necrosis, apoptosis and autophagy by different cell types (Templeton & Liu, 2010).

With such alarming effects it is pertinent to develop low cost, effective and natural treatment modalities to counteract harmful effects of Cadmium. *Bacopa monnieri* has been reported to be effective against metal toxicities of Aluminium and

Lead (Jyoti & Sharma, 2006; Velaga et al., 2014). Hence, the study was designed to investigate its medicinal abilities for developing a treatment modality for Cadmium toxicity.

1.1 Objectives

The study had the following objectives:

- Molecular identification of *Bacopa monnieri* indigenous to Pakistan using DNA Barcoding and Phylogenetic analysis.
- Investigation of neuroprotective potential of Ethanolic *Bacopa monnieri* extract against Cadmium using behavioral analysis.
- Elucidating the hepatoprotective and nephroprotective ability of *Bacopa monnieri* using biochemical testing of ALT, Creatinine and Urea.
- Histological examination of Hippocampus, Liver and Kidney tissues for analyzing protective effect of eBME against Cadmium toxicity.

CHAPTER 2
LITERATURE REVIEW

2.1 Heavy Metals

Used since centuries, metals are incorporated into the fabric of our everyday lives. From the electronics we use to the roads we walk and drive down. Their use in tools developed alongside human intelligence and evolution. The use of metals in antiquity started with the Copper age as it is naturally occurring and didn't require the use of fire. However, most metals found in their ore form can only be obtained by smelting. Smelting while an outstanding technical achievement and progress maker for human history causes human exposure to certain toxic fumes and environmental pollutants. For example, Hippocrates reported symptoms of Lead colic such as fatigue, irritability and nervous spasms commonly seen in lead-mine workers and these symptoms are still described the same way today. (Breitenlechner, 2013).

However, as technology has advanced; we have used many different types of metals for a plethora of purposes mainly because of their chemical properties. Metals have good heat and electric conductivity and are mostly solid at room temperature. It is normal practice to divide metals according to light and heavy groups based on their density with metals over $5g/cm^3$ being grouped into the heavy category. Metals also act as micronutrients for humans such as Manganese, Chromium and Copper.

Metals by their very nature are hard to dispose of or destroy and thus end up as environmental pollutants. In most cases, heavy metals are xenobiotic to an environment i.e substances which are foreign for metabolism and can neither be utilized or neutralized by an organism. Man-made emissions have caused the natural balance to shift and have ended up having a negative impact on human health and longevity. Metal emissions into the environment are harmful to humans as metals are absorbed into plants and animals, accumulating in the food web destroying the ecosystem. Prominent examples of this include Mercury and Lead toxicity and subsequent poisoning cases due to heavy metal contamination. Lead and Lead containing alloys were used throughout history in a variety of applications that are now inconceivable today because of Lead's toxicity. Mercury was another heavy metal used through the ages in beauty products and traditional medicine the use of which has been discontinued after realizing

about its toxic effects in living beings. Thus, the study of these heavy metal contaminants, their mechanism of disruption of normal bodily function and their possible route of entrance into organisms all have been topics of focused study in recent years.

2.1.1 Heavy metal exposure

Exposure to heavy metals in general is mostly through occupational sources. Dusts and fumes containing fine traces of metal particles at hazardous concentrations are often found in workplace atmospheres especially in factory environments. Airborne metal particles can cause inhalational exposure which is influenced by a number of factors such as breathing rate, type of metal, solubility and particle size of metal etc. Suspended dust particles to the size of 100nm to 10μm or nanoparticles (NPs, < 100nm) are dangerous as this is the thoracic fraction that can reach the alveoli in the lungs and subsequently the blood stream. Larger particulate matter is usually trapped in the upper airways. Multiple industrial processes involving metals such as welding or smelting etc can generate NPs (Antonini, 2003). In the case of heavy metal exposure to the general population due to presence of Lead in petrol, the metal left in the engine combined with the exhaust gases was released in the form of microscopic Lead oxide particles thus causing potential toxicity to people who inhaled it. Inhalation of such NPs causes them to be deposited at different sections of the airway according to their size ("Human respiratory tract model for radiological protection. A report of a Task Group of the International Commission on Radiological Protection," 1994). The size difference enables smaller particles to pass biological membranes and barriers and thus wreak potential havoc after entering our systemic circulation. Once these NPs enter our circulation, in vivo and in vitro toxicological studies have shown that even relatively inert materials become toxic in their nanoparticulate form. For example Tin oxide which has a diameter of approximately 20 nm caused severe inflammation than when it was used with a larger particle size (250nm) (Oberdorster, Oberdorster, & Oberdorster, 2005). Perhaps the most threatening aspect of NPs of heavy metals is their potential to cross the blood brain barrier once present in the systemic circulation (Kreyling, Semmler-Behnke, & Moller, 2006). Toxic metal ions such as Cadmium may weaken the blood brain barrier and thus enable NPs to aggravate the damage (Horvath et al., 2011).

Another important route of exposure to heavy metals is through ingestion. Fine airborne particles can settle on foodstuff and ultimately be ingested if not washed properly. Factors such as soil pollution and contamination from fertilizer use have to be taken into account when considering ingestion of heavy metals. Incorporation and accumulation of toxic metals from the soil is possible in the case of many plants such as rice, tobacco etc. Further consumption of Lead contaminated water due to old Lead pipes has been prevalent over the last few decades. Heavy metal ions can be absorbed from the intestines involving processes similar to the uptake of normal essential metals. Children being at a higher risk because they require calcium for bone growth hence can absorb 50% of the ingested Lead while adults absorb substantially less amounts (Chisolm & Harrison, 1956).

The mechanism of action of heavy metals primarily affects the growth, metabolism and morphology of cells. Heavy metals can disrupt the interactions of amino acids in a polypeptide chain by interacting with ionized species and breaking their secondary structure resulting in the denaturation of the proteins, ending their biocatalytic activity. This can have disastrous consequences on the function of cells and disturb the redox balance. Some metals are also involved in antioxidant defense and if this balance is disrupted, the cell can end up being destroyed.

2.2 Cadmium

Cadmium is a heavy metal that was first discovered in 1817 in Germany by Friedrich Stromeyer. It is generally found along with Zinc ores and is recovered as a byproduct of processing Zinc, Lead and Copper ores, as such it is relatively abundant. Often referred to as the metal of the 20th century and used in a wide variety of electronics such as batteries etc; it's current use is declining as it is toxic and is specifically listed in the European Restriction of Hazardous substances (Morrow, 2000). Cadmium is one of the most toxic environmental and industrial pollutants because it can impact our Central Nervous System and other vital organs (Méndez-Armenta & Ríos, 2007). While no physiologic function is known, Cd has been thought to result in toxic effects by functioning as a metallohormone (Byrne, Divekar, Storchan, Parodi, & Martin, 2009) and potentially facilitating the mechanisms involved in the onset of Cancer, Osteoporosis, Cardiovascular and Renal diseases.

2.2.1 Sources of Cadmium exposure

Cadmium exposure to the environment is primarily as a result of human activities such as fossil fuel or waste burning (Rafati Rahimzadeh, Rafati Rahimzadeh, Kazemi, & Moghadamnia, 2017). In Humans the primary cause of Cadmium exposure is through contaminated drinking water/food and inhalation of tobacco/cigarette smoke. The tobacco plant absorbs heavy metals from the surrounding soil and causes them to accumulate. It is estimated that 10% of the Cd content in tobacco is inhaled through cigarette smoke. Cadmium concentrations have been found to be higher in the blood of smokers than in non-smokers (Munisamy, Ismail, & Praveena, 2013). Moreover, a large amount of Cadmium can be ingested through contaminated seafood, however the greatest source of dietary exposure is through the ingestion of starchy and tuberous vegetables such as potatoes (Gonçalves et al., 2012). Like tobacco, such plants also absorb heavy metals from the soil. Additionally, fertilizers used for these plants such as Phosphate-based ones can contain high amounts of metal toxins thus increasing their absorption in plants and causing contamination. There have been cases where toxicity due to Cadmium resulted in bone and kidney problems such as in Japan, due to mining operations in World War 2, there were about 400 known cases of Itai-Itai disease caused by Cadmium exposure from ingesting contaminated rice (Kaji, 2012).

2.2.2 Cadmium Neurotoxicity

Cadmium has potential deleterious effects on the CNS and given that it can cause toxicity in humans, potential treatments must be stringently evaluated. Cadmium has a long half-life (approx. 15-20 years) due it's low rate of excretion from the body (T. Jin, Lu, & Nordberg, 1998). The primary method of human exposure is from employment in metal industries or through the inhalation of tobacco smoke. In humans, it can accumulate in a variety of tissues such as the Kidneys, Liver and CNS. In the CNS, evidence suggests that Cadmium can cause the Blood Brain Barrier (BBB) to become more permeable and have effects on neurotransmitters, cause oxidative damage to neurons and epigenetic modifications (B. Wang & Du, 2013). Symptoms of CNS exposure to Cadmium include movements as are observed in Parkinson's disease, decreased ability to concentrate, learning disability and olfactory dysfunction (S. D. Kim, Moon, Eun, Ryu, & Jo, 2005). Additionally there have been studies linking Cadmium presence and accumulation in children to learning difficulties and lower IQ

(Marlowe, Stellern, Moon, & Errera, 1985) .

The CNS is especially vulnerable during neonatal development, it has been shown that Cadmium can pass through the placenta to the fetus and can even be present in breast milk (Korpela, Loueniva, Yrjänheikki, & Kauppila, 1986). Cadmium can also be taken up from the nasal mucosa or nasal pathways and can start to accumulate at the Choroid plexus in much greater concentrations than in the CSF. A post mortem study revealed the concentrations of Cd at the Choroid plexus to be two to three times higher than in the Brain Cortex (Manton & Cook, 1984). Most of the work for studying Cadmium toxicity has been carried out on rat models. In rat models, it has been shown that Cadmium can increase BBB permeability and is more toxic in newborn or young rats (Shukla & Chandra, 1987). This accumulation of Cd can lead to cellular dysfunction and ultimately edema. The choroid plexus contains metal binding ligands that sequester metal ions preventing them from reaching the brain and thus providing protection. It also has its own defense system and is likely the first line of defense against neurotoxins like heavy metal ions in high concentrations. An important factor to keep in mind is that if Cadmium nanoparticles are inhaled, they likely bypass the choroid plexus and BBB and get direct access to the brain via the olfactory route (Tjälve
& Henriksson, 1999). Intranasal exposure to Cadmium has been related to olfactory dysfunction in both humans and rodent models.

In a Zebrafish model, Cadmium exposed embryos show smaller heads with unclear boundaries between Brain areas and badly affected neural progenitor cells (Chow, Hui, Lin, & Cheng, 2008). One of the mechanisms by which Cadmium causes damage is by producing free radicals in the Brain which may damage both neurons and oligodendrocytes (cells that myelinate axons in the CNS). Cd toxicity has been shown to affect White matter tracts as a study showed Cd interfering with oligodendrocyte function (Almazan et al., 2000) . In cortical neuron cell culture from fetal rats, it was found that Cd changes neuronal morphology after an exposure period of just 6-hours. However, after a 24-hour period, Cd further influences neurons resulting in a complete loss of axons. Typically in humans, we expect lower concentrations but due to prolonged exposure and accumulation Cadmium may result in inducing cell apoptosis and subsequent cell death (Yoshida, 2001). These results suggest that prolonged Cd exposure may increase chances of cell death and damage. Although experiments on the effects of Cadmium *in vivo* on human neurons are hard to demonstrate, there has been evidence of its toxic effects on cultured neurons *in vitro*. Sarchielli et al showed that

Cadmium induces apoptosis in the motor neurons from cells cultured via Human fetal spinal cords (Sarchielli et al., 2012).

The mechanism behind apoptosis induced by Cadmium is not very well understood. Different pathways have been reported but the most widely reported is the mitochondrial pathway. Decrease in intracellular ATP levels in response to high levels of Cadmium are an indicator of mitochondrial membrane damage. The damage results in an increase in reactive oxygen species and lipid peroxidation leading to cell death (Lopez, Arce, Oset-Gasque, Canadas, & Gonzalez, 2006). Cadmium exposure causes an increase in Ca^{2+} ions and free radicals which result in a change in mitochondrial membrane potential and release of Cytochrome C which binds to Apaf1 (Apoptotic protease activating factor – 1) activating Pro-caspase 9 which then triggers Caspase 9 leading to the proteolytic cascade (Earnshaw, Martins, & Kaufmann, 1999; Mehmet, 2000) as shown in Figure 2.1.

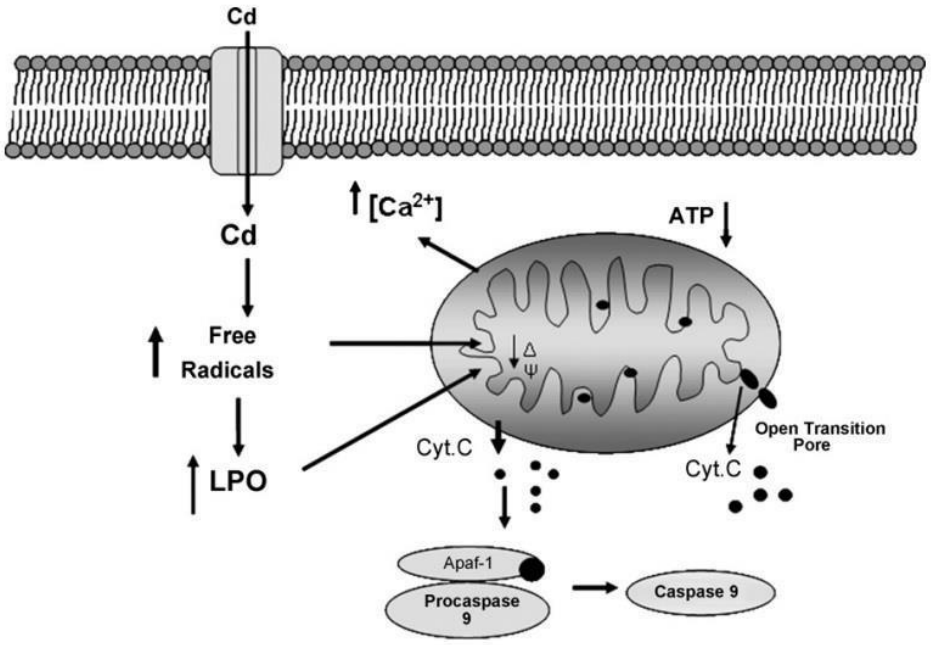


Figure 2.1 Pathway of Cadmium induced apoptosis (Méndez-Armenta & Ríos, 2007)

Cadmium's genotoxic effects are also reported which majorly occur because of disruption of DNA repair mechanisms specifically base excision repair (Beyersmann & Hechtenberg, 1997) or through interference with DNA repair proteins such as Zinc fingers (Hartwig et al., 2002).

2.2.3 Cadmium Hepatotoxicity and Nephrotoxicity

Cadmium has been reported to be responsible for causing hepatotoxicity and nephrotoxicity. During acute toxicity of Cadmium, Liver is the first to get affected. While during Chronic exposure accumulation in the Kidney has serious detrimental effects on health. The Mechanism behind Cadmium's hepatotoxicity is divided into two phases namely Primary injury and Secondary injury as summarized in Figure 2.2 (A) and (B) respectively. The vascular endothelial hypothesis forms a major part of the first phase which is the metal's interaction with the vascular endothelium. The degeneration of the hepatic endothelial lining results in ischemia which causes harm to the surrounding cells. Moreover, Cadmium has a high affinity to bind to sulfhydryl groups on low molecular weight metal binding proteins such as Metallothionein and Glutathione, which have high amounts of cysteine residues resulting in liver toxicity. The change in redox state because of such events causes oxidative stress which results in change of mitochondrial permeability which eventually leads to activation of cascades that result in apoptosis described in detail in Figure 2.1 This mechanism not only causes Hepatotoxicity and Neurotoxicity but also causes damage to the Kidney. Secondary injury is attributed to activation of immune response. The activation of Kupffer cells in the Liver tissue results in release of inflammatory molecules such as Chemokines and Cytokines. These cascades cause inflammation which causes further damage to the Liver (Rikans & Yamano, 2000).

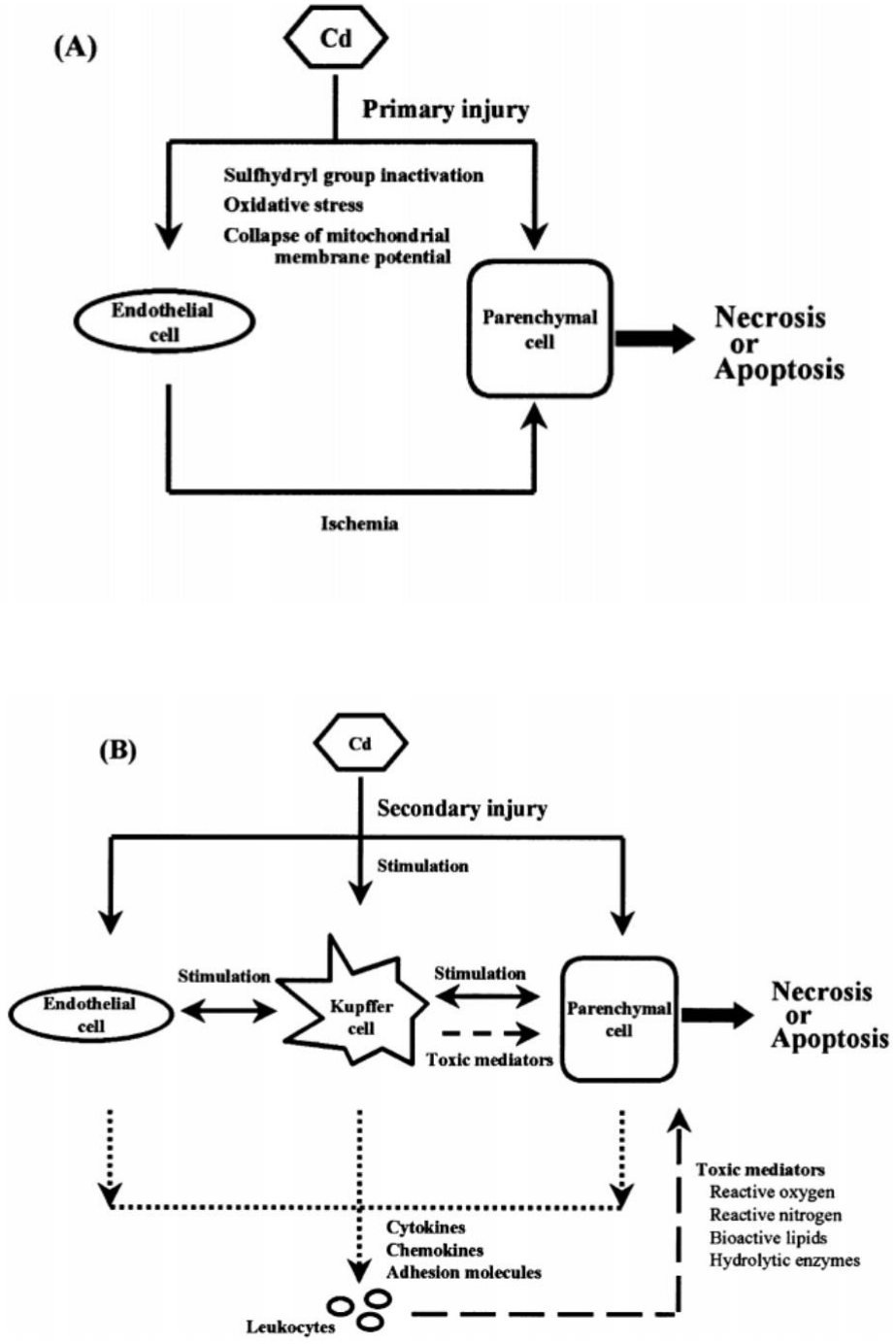


Figure 2.2 Mechanism of Cadmium induced Hepatotoxicity

(Rikans & Yamano, 2000)

2.3 Existing treatment interventions for Metal toxicity

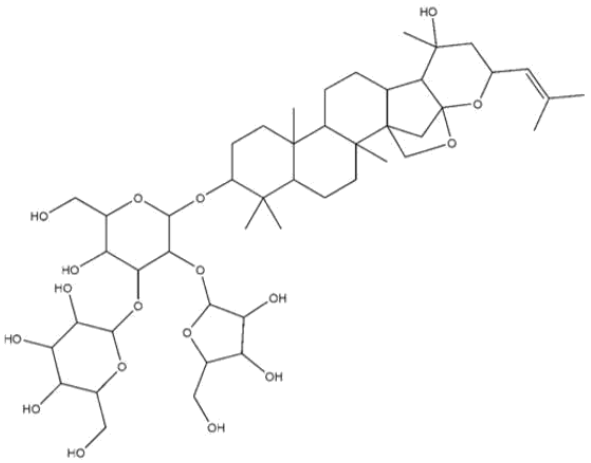
The global burden of heavy metal toxicity especially from metals such as Mercury, Lead and Cadmium represent a significant challenge. With the increasing rate of climate change and environmental pollution, the rate of such cases is only bound to increase. Hence, improved methods need to be developed for the treatment of heavy metal toxicity. Current treatment paradigms mainly focus on emergency interventions such as inducing emesis in the case of ingestion or carrying out a gastric lavage (Rafati Rahimzadeh et al., 2017). Chelating agents such as EDTA and Dimercaprol are also used as they bind to the heavy metal ions and form complex ring like structures to enhance their elimination. However, they have limitations such as dosage and schedule, further metal chelators may also cause the redistribution of some heavy metals from other tissues to the Brain thereby increasing risk in an already perilous situation. The use of potential naturally occurring compounds to ameliorate heavy metal toxicity is a safe way to counteract the harmful effects of many metal chelators.

Prominent examples of natural compounds used for the treatment of heavy metal toxicity such as caused by Lead can be evidenced using *Nigella Sativa. Nigella Sativa* is a widely used medicinal plant all over the world and has bio-active compounds known as Thymoquinones. There has been some research that shows the beneficial potential of using *Nigella* to counteract natural and chemical induced toxicities (Mabrouk & Cheikh, 2016; Tavakkoli, Ahmadi, Razavi, & Hosseinzadeh, 2017). Another example comes from a study which evaluates the use of Curcumin in animal models to counteract Aluminum toxicity (A. Kumar, Dogra, & Prakash, 2009).

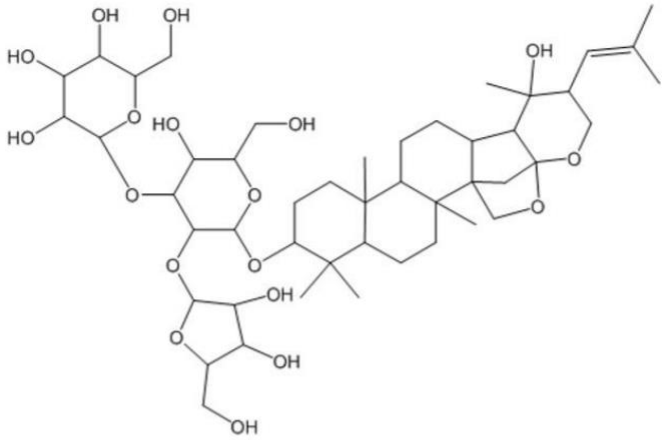
2.4 *Bacopa monnieri*

Bacopa monnieri (BM) is a nootropic herb that is primarily used as an Ayurvedic medicinal herb where it is employed for use as a neural tonic and memory enhancer. The herb belongs to the Plantaginaceae family and is commonly known as Water hyssop or Indian pennywort. It is native to the wetlands of India, Pakistan, Europe and some parts of America. BM was initially described around the 6th century A.D in manuscripts relating it as an aid to memorization of ancient Hindu Vedic texts. In the modern era, it has undergone numerous in vitro and in vivo studies evaluating its medicinal properties. Several human studies and clinical trials of its potential benefits have been carried out as well which show moderate benefits of its properties (Aguiar

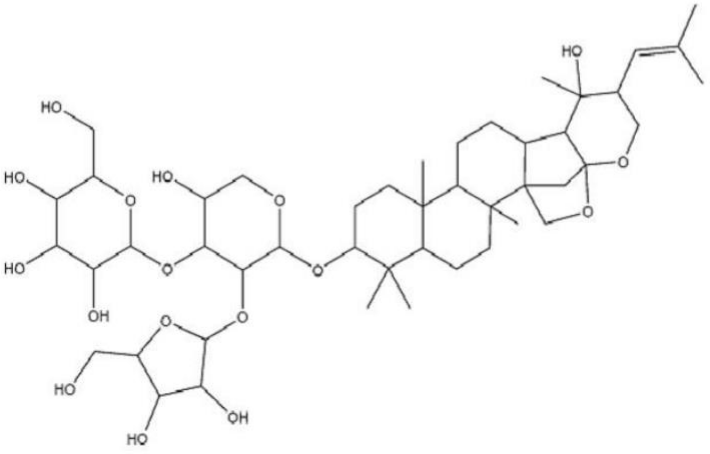
& Borowski, 2013). The primary medicinal benefit is thought to come from its bioactive constituents, Saponins known as bacosides (Sivaramakrishna, Rao, Trimurtulu, Vanisree, & Subbaraju, 2005), chemical structures of the three most therapeutically important bacosides are shown in Figure 2.3 (Rauf et al., 2012). BM shows minimal side effects at current dosages in herbal medicine and shows antioxidant and neuroprotective activities. The mechanism of action is thought to involve antioxidant neuroprotection, Acetylcholinesterase inhibition, increased Cerebral blood flow and modulation of several neurotransmitters. What has not been studied so far is the use of BM to counteract the effects of metal toxicity caused by Cadmium.



Bacoside A3



Bacopaside II



Bacosaponin C

Figure 2.3 Chemical structures of Bacosides (Rauf et al., 2012)

CHAPTER 3
MATERIALS AND METHODS

3.1 Plant Sampling

Bacopa monnieri was collected from Ramli Stream near Quaid-e-Azam university in the month of September. The Plant material was thoroughly washed, shade dried and stored at room temperature until further processing.

3.2 Herbarium Preparation

A sample of the plant with intact leaves, flowers and roots was dried by placing it on blotting paper under heavy weight. The dried plant was dipped in a solution of Mercuric Chloride to stop attack of pests and fungus. After which it was pasted on top of the herbarium sheet. The sheet was deposited at the Pakistan Museum of Natural History, Islamabad and Voucher Number was obtained for future reference.

3.3 Molecular Identification using DNA Barcoding

3.3.1 DNA Extraction

DNA from the leaves of the plant was extracted using CTAB method with slight modifications (Doyle, 1991). The leaves were grinded in liquid nitrogen using a pestle and mortar. After which, the material was added to to an autoclaved Eppendorf. CTAB buffer (CTAB + β mercaptoethanol) was pre heated and added to the tube. The tube was incubated in a water bath with a temperature of 60ºC for 30 minutes. The Eppendorf was allowed to cool down for some time and then 500µl of Chloroform-Isoamyl Alcohol (24:1) was added and shook gently. Next, the mixture was centrifuged at 13,000rpm for 10 minutes. The supernatant was collected in a new tube and was treated with Chloroform-Isoamyl alcohol once again and centrifuged. The upper layer was cautiously collected and DNA was precipitated by the addition of 600µl of ice cold Isopropanol and stored in the refrigerator at -20^{o}C overnight. The next day the pellet was collected by centrifugation of the material at 13000rpm for 10 minutes. This time the supernatant was discarded and 1ml of wash buffer (75% Ethanol) was added to the pellet. The material was centrifuged at 13,000rpm for 10 minutes, supernatant

discarded and pellet was left to dry at room temperature for 30 minutes. At the end, the DNA pellet was dissolved in 50µl of TE buffer and stored at -20^{o}C until further use.

3.3.2 Agarose gel Electrophoresis for detection of DNA

Qualitative detection of DNA was done using 1% gel through electrophoresis. DNA sample mixed with 6X loading dye was loaded into the wells. 3µl of DNA sample and 3µl of loading dye was used. A 100bp ladder (Thermo Fisher Scientific, USA) was used. The power supply was set at 80V and run for 30 minutes. Gel was visualized using a UV transilluminator (Trans Lum Solo, BioTop, China) and analysed by Dolphin-Doc gel documentation system (Wealtec).

3.3.3 Quantification of DNA using Nanodrop

NanoDrop 2000 (Thermo Fisher Scientific, USA) was used for the quantification of DNA. Before loading the sample, the lower pedestal of the instrument was cleaned with 1µl of distilled water. 1µl of TE buffer was loaded initially and used as Blank. Followed by cleaning using lint free fiber and then 1µl of DNA sample was loaded for analysis. "Nucleic acid" software coupled with the instrument was used for analysis.

3.3.4 Polymerase chain reaction for amplification of rbcL

The gene chosen for amplification was rbcL (Ribulose-1, 5-bisphosphate carboxylase Large subunit which is a highly conserved gene of the Chloroplast. The gene was amplified using the recipe in Table 3.1, the sequence of forward and reverse primers (Kress & Erickson, 2007) shown in Table 3.2 and the PCR profile shown in Figure 3.1 in a thermocycler (Applied biosystems 2720 Thermal cycler).

Component	Quantity (µl)
10X Taq Buffer	2.5 µl
2mM dNTPs	2.5 µl
50mM $MgCl_2$	1.5 µl
20mM Forward Primer	0.5 µl
20mM Reverse Primer	0.5 µl
Taq Polymerase	0.2 µl
Bovine Serum Albumin (BSA)	1 µl
Dimethylsulphoxide (DMSO)	1 µl
Nuclease Free Water	14.3 µl
DNA	1 µl
Total Volume	25 µl

Table 3.1 Recipe for PCR amplification of rbcL gene

Gene	Primer sequence 5´ to 3´	Product Size
rbcL a_f	ATGTCACCACAAACAGAGACTAAAGC	650 bp
rbcL a_r	CTTCTGCTACAAATAAGAATCGATCTC	

Table 3.2 Sequences of Forward and Reverse Pimers for rbcL gene amplification (Kress & Erickson, 2007)

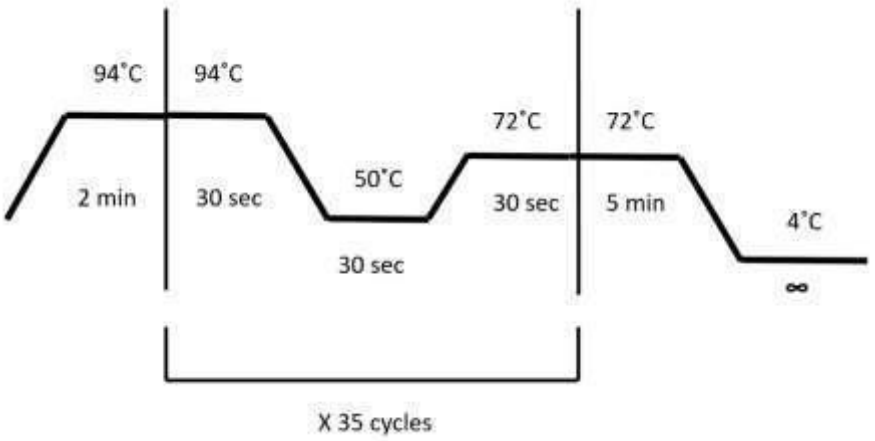


Figure 3.1 PCR profile for rbcL gene

3.3.5 Agarose gel Electrophoresis of PCR product

The Agarose gel (1%) was prepared by adding 0.5g of Agarose into 50ml of 1X TAE buffer. The solution was heated in the microwave oven for a few minutes until it started to bubble and became clear. When the gel was cool to touch, 5µl of Ethidium bromide was added and it was poured into the casting tray which already had a comb attached for making the wells. After the gel had solidified it was transferred from the casting tray into the buffer in the Electrophoresis apparatus. 3µl of loading dye mixed with same volume of amplicon was loaded into the wells along with DNA ladder of 100bp (Thermo Fisher Scientific, USA). The power supply was set at 80V and the gel was run for 30 minutes. The gel was visualized using a UV transilluminator (Trans Lum Solo, BioTop, China) and analysed by Dolphin-Doc gel documentation system (Wealtec).

3.3.6 Sanger Sequencing

The PCR product of rbcL gene was cleaned using ExoSAP-IT (Thermo Fisher Scientific, USA), 2µl of the reagent was added to 5µl of the amplicon and incubated at 37ºC for 15 minutes. Followed by another incubation at 80ºC for 15 minutes. The sample was then stored at -20ºC until shipped for Sanger sequencing to BGI, China.

3.3.7 Sequence Submission to NCBI

The sequence was deposited at the National Centre for Biotechnology Information (NCBI) and Accession Number was obtained.

3.3.8 Phylogenetic Analysis

Phylogenetic trees were constructed for molecular identification of the plant species using Maximum Likelihood, Neighbour Joining and Bayesian statistical models using the "Geneious" software.

3.4 Plant Extract Preparation

Ethanolic extract of *Bacopa monnieri* was prepared by modifications to the protocol by Kahol and his colleagues (Kahol, Singh, Tandon, Gupta, & Khanuja, 2004). Plant material was thoroughly washed with tap water and shade dried for 24 hours at room temperature. It was then placed on Aluminium foil sheets in an incubator at 40ºC for 4 hours. The dried herb was broken into smaller pieces by hand and then turned into powder using a disintegrator. The unground material from the powder was sieved out using a sieve with a mesh size of 40 to obtain a finely ground powder. Ethanolic *Bacopa monnieri* extract (eBME) was prepared using the Soxhlet extractor. 10g of the finely ground herb was added to a thimble (F5800 CHMLAB Group, Barcelona) which was loaded into the extraction chamber connected to a condenser on the upper end and a round bottomed flask on the lower end. 100ml of Ethanol was taken in the round bottomed flask and the entire assembly was fixed on top of a heating mantle set at 60ºC for 24 hours. The filtrate collected in the round bottomed flask was fed to a rotary evaporator (EVI 67/1 Inlabo, Italy) and Ethanol was removed under reduced pressure at 65ºC. The extract was collected in an autoclaved glass petri plate and covered with a piece of Aluminium foil with multiple pin sized holes. The petri was placed in an incubator at 40ºC to remove any remaining solvent overnight. The dried extract was stored at 4ºC until further use.

3.5 In-vivo Testing of Plant Extract Against Cadmium Toxicity

3.5.1 Ethics Statement

The experimental protocols were approved by Institutional Review Board at ASAB and were performed under compliance with rules of Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA (Guide for the Care and Use of Laboratory Animals: Eighth Edition, 2011).

3.5.2 Animals

Twenty male BALB/c mice, 8-10 weeks old weighing 30-35g, were procured from National Institute of Health, Islamabad. Five mice per cage were housed at ASAB Laboratory Animal House, NUST in Polypropylene cages. The mice were screened for any visible health defects and acclimatized to the new environment for 5 days before the start of experimentation. The mice were maintained on a 12:12 light/dark cycle. The temperature of the animal house was kept between 20 - 25ºC. The mice had access to standard feed and distilled water ad libitum. The standard feed comprised of: crude protein 30%, crude fat 9%, crude fiber 4% and moisture 10.4%.

3.5.3 Experimental Design and Dosage

Cadmium toxicity was induced in mice by administration of Cadmium Chloride CAS-No:10108-64-2 (Sigma Aldrich) through drinking water at a dose of 30mg/L (Chen et al., 2014). Consumption of water was monitored before start of experimentation and was found to be around 10ml per mouse per day. The plant extract was infused in mouse feed at a dose of 125mg/kg/day (Saraf, Prabhakar, Khanduja, & Anand, 2011). The consumption of feed was investigated and was found out to be around 6 - 7g per mouse per day. The experimental groups used in the study are shown in Table 3.3 and the experimental timeline in Figure 3.2.

Group (n=5)	**Treatment (15 days)**
Control	Distilled water for drinking and standard mouse feed
Ethanolic *Bacopa monnieri* extract (eBME)	Distilled water for drinking and 125 mg/kg/day eBME infused in mouse feed
Cadmium 30	30mg/L Cadmium chloride dissolved in distilled water for drinking and standard mouse feed
Cadmium 30 + Ethanolic *Bacopa monnieri* extract (eBME)	30mg/L Cadmium chloride dissolved in distilled water for drinking and 125 mg/kg/day eBME infused in mouse feed

Table 3.3 Experimental groups

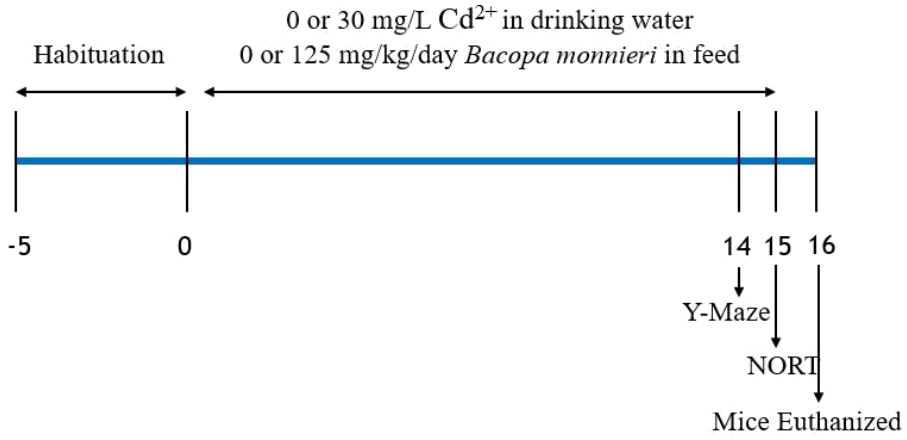


Figure 3.2 Experimental timeline for in-vivo testing of *Bacopa monnieri* against Cadmium induced toxicity

3.6 Behavioral Analysis

3.6.1 Y-Maze Spontaneous Alternation Test

The Y-Maze Spontaneous Alternation test is based on the exploratory behavior of rodents as they have a natural tendency to explore new environments. The mice don't need any dietary restrictions or prior training for conducting this test. A Y-Maze made of black acrylic sheet with equal sized arms (15cm x 7.5cm x 12.5cm) placed at 120º from each other was used as shown in Figure 3.3. The mice were placed in the testing room an hour before the start of the experiment for acclimatization. Dim lighting was used in the testing room to increase exploratory activity. Each mouse was placed in the same arm (Arm B) facing away from the center and left to freely navigate through the maze for five minutes. An overhead camera was used to record their movements in the maze. 70% Ethanol was used to clean the maze between each trial to remove any olfactory cues. An entry was counted when all four limbs of the mouse were in the arm. The protocol was adapted with slight modifications from (Hughes, 2004). Video analysis was done to calculate the Spontaneous alternation (%) using the following formula:

$$\text{Spontaneous Alternation (\%)} = \frac{\text{Number of Alternations}}{\text{Total Number of Arm Entries - 2}} \times 100$$

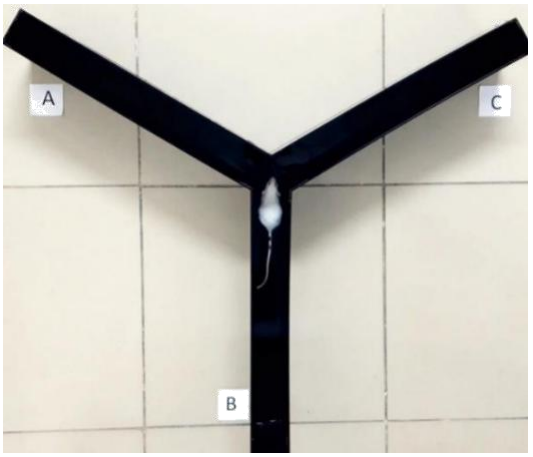


Figure 3.3 Y- Maze Spontaneous Alternation Test apparatus

3.6.2 Novel Object Recognition Test (NORT)

Novel Object Recognition Test (NORT) is used to evaluate recognition memory in rodents. The test is based on the rodent behavior of spending more time interacting with a new object as compared to a familiar one. A square box made of white acrylic sheet (40cm x 40cm x 40cm) was used to conduct the test with visual cues pasted on the walls. The box was surrounded by blinds to remove any external cues. The mice were placed in the testing room an hour before the start of the experiment for acclimatization. Dim lighting was used in the testing room to increase exploratory activity. 70% Ethanol was used to clean the maze and objects after each trial to remove any olfactory cues. Each mouse was placed in the center of the apparatus and was allowed to freely explore the apparatus in a 5 minute habituation trial which was not recorded by the video camera. Two same objects were then pasted in the box with tape on the opposite corners at an equal distance from each other and mice were allowed to interact with them for 5 minutes. Session I was recorded by an overhead video camera. The mouse was put back into its home cage for 15 minutes before the start of Session

II. In the second session, one of the objects from the first session was replaced by a novel object as shown in Figure 3.4. The mouse was put back into the center of the arena and allowed to explore the two objects for 5 minutes recorded on camera. Videos of Session II were analysed for calculating the interaction time with the objects, which was counted when mice touched the object/sniffed or had their head at a distance ≤ 1cm from the object. Chewing or sitting on top of the object was not counted as an interaction. The protocol was adapted with slight modifications from (Leger et al., 2013). The interaction time was used to calculate the Preference Index (PI) using the

following formula:

$$\text{Preference Index (\%)} = \frac{\text{Time spent with the Novel object}}{\text{Time spent with Familiar object + Novel object}} \times 100$$

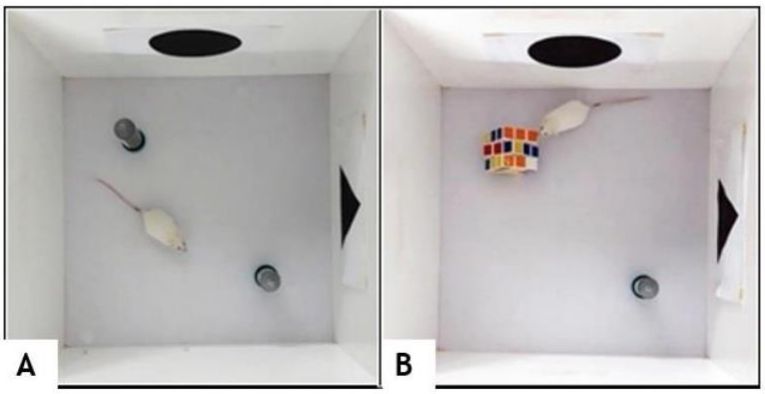


Figure 3.4 Novel Object Recognition Test apparatus

A: Mouse interacting with two same objects in Session I

B: One of the objects from Session I replaced with a novel object for Session II

3.7 Collection of Blood Sera and Organs

Mice had been fasting for at least 6 hours before they were euthanized for blood and organ collection. Chloroform was used to anesthetize the mice and blood was collected via Cardiac puncture using a 3ml syringe in a Gel and Clot Activator tube (Kantex, Germany). After which anesthetized mice were euthanized by cervical dislocation and Brain, Liver and Kidneys were collected and stored in 10% Neutral Buffered Formalin in small biopsy jars at room temperature. The collected blood was centrifuged for 15 minutes at 2500rpm at 37ºC for collection of Serum. The Serum was pipetted out into a separate labelled Eppendorf and stored at 4ºC until used for biochemical testing.

3.8 Biochemical Testing for Liver and Renal Function

3.8.1 Liver Function Test

The level of Alanine aminotransferase (ALT) in blood serum was determined to analyze Liver function in mice using standard protocols provided by manufacturers

of commercial kits (Innoline Martin Dow Marker Specialties, Pvt Limited, Lot No A190356/7).

3.8.2 Renal Function Test

The levels of Creatinine and Urea in blood serum were determined to analyze Renal function in mice using standard protocols provided by manufacturers of commercial kits (Innoline Martin Dow Marker Specialties, Pvt Limited, Lot No: A190163/64 and Lot No: A190006/7).

3.9 Histopathology

3.9.1 Trimming and Pre-Embedding

The Brain, Liver and Kidneys fixed in 10% Neutral Buffered Formalin were trimmed to an appropriate size depending on the organ being prepared. Followed by dehydration of the trimmed tissue in different concentrations of Ethanol. The following concentrations were used:

- 70% Ethanol for 1 hour
- 95% Ethanol for 1 hour (twice)
- 100% Ethanol for 1 hour (twice)

Next, the tissue was cleared with Xylene for 1 hour and then immersed in molten Paraffin.

3.9.2 Embedding and Sectioning

Molten Paraffin was poured into the mold with a cassette placed on a hot plate. A heated forceps was used to immerse and orient the Paraffin infiltrated tissue in the cassette. The mold and cassette were transferred to a cool plate for 15 minutes until Paraffin solidified. Then the mold was snapped off and a paraffin block was formed. The Paraffin block was mounted on a microtome and thin sections as thin as 5µm were cut. The sections are cut in the form of a ribbon and are floated on a water bath set at 45ºC to stretch the section. A glass slide is then used to get the ribbon out of the water and allowed to dry in an incubator at 37ºC.

3.9.3 Hematoxylin / Eosin Staining and Microscopy

The Paraffin sections were dewaxed with Xylene twice, 5 minutes each. Followed by rehydration with 95% and 100% Ethanol twice for each concentration, 5

minutes each. After which a washing with water was given for 3 minutes and was immersed in Hematoxylin for 5 minutes. Slide was given another washing with water for 3 minutes and immersed in 0.1% Eosin for 5 minutes. Another rinsing with water for 30 seconds was given and sections were dehydrated with 95% and 100% Ethanol twice for each concentration, 2 minutes each. Sections were cleared with clearing agent twice, 2 minutes each. The section was covered with a coverslip using a mounting medium. The prepared slides were visualized under a Light microscope at 40x, 100x and 400x magnifications and images for analysis were taken using "T-capture 4.03" imaging software.

3.10 Statistical Analysis

Statistical analysis was done using "GraphPad Prism 5" software and the results were expressed as mean ± SEM (Standard Error Mean). Data was analysed using One-way Analysis of Variance (ANOVA) with a confidence interval of 95 %, $p < 0.05$ was considered significant, followed by Tukey's multiple comparison post hoc test.

CHAPTER 4

RESULTS

4.1 Sampling of *Bacopa monnieri*

Bacopa monnieri was collected from Ramli Stream near Quaid-e-Azam university, Islamabad in the month of September as shown in Figure 4.1. The plant has various medicinal properties and is majorly used as a brain tonic for memory enhancement.

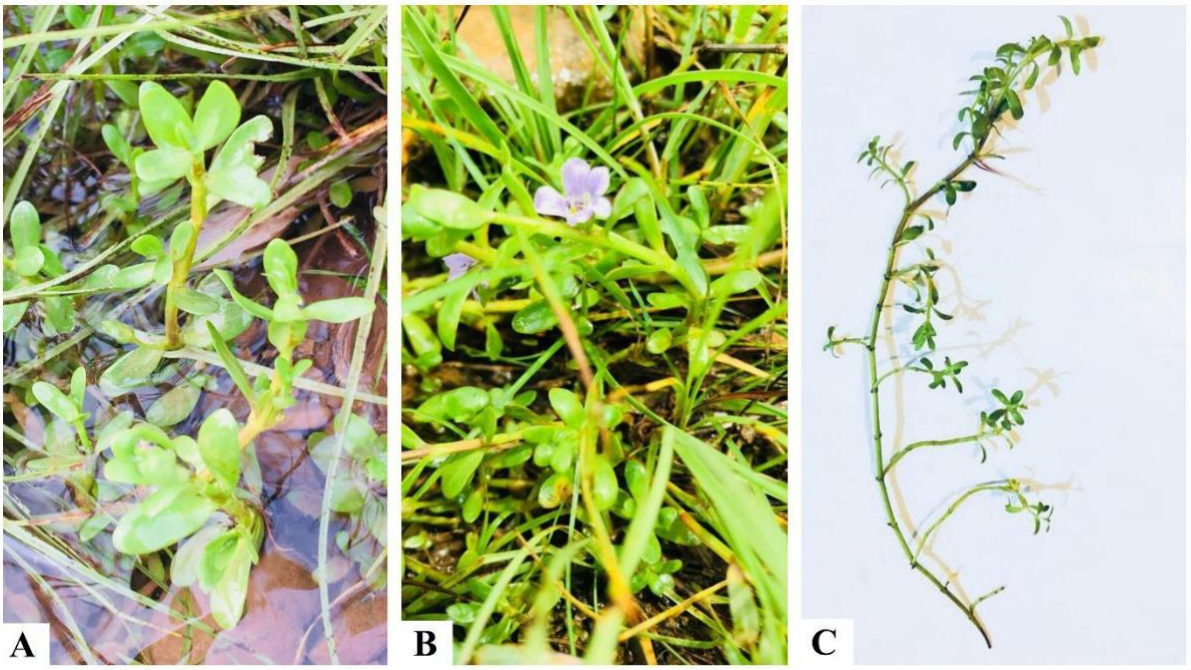


Figure 4.1 *Bacopa monnieri*

A: *Bacopa monnieri* is majorly found in marshes near streams and ponds as reflected by the site image.

B: *Bacopa monnieri* can be identified on the basis of its flowers which are white or purple in color and actinomorphic which means they have radial symmetry.

C: The herb has succulent leaves which are arranged oppositely on a stem

4.2 Herbarium Specimen

Herbarium sheet with a complete, dried plant sample of *Bacopa monnieri* was deposited at the Pakistan Museum of Natural History and Voucher Number: 043885 was acquired. Scanned image of the Herbarium sheet is shown in Figure 4.2.

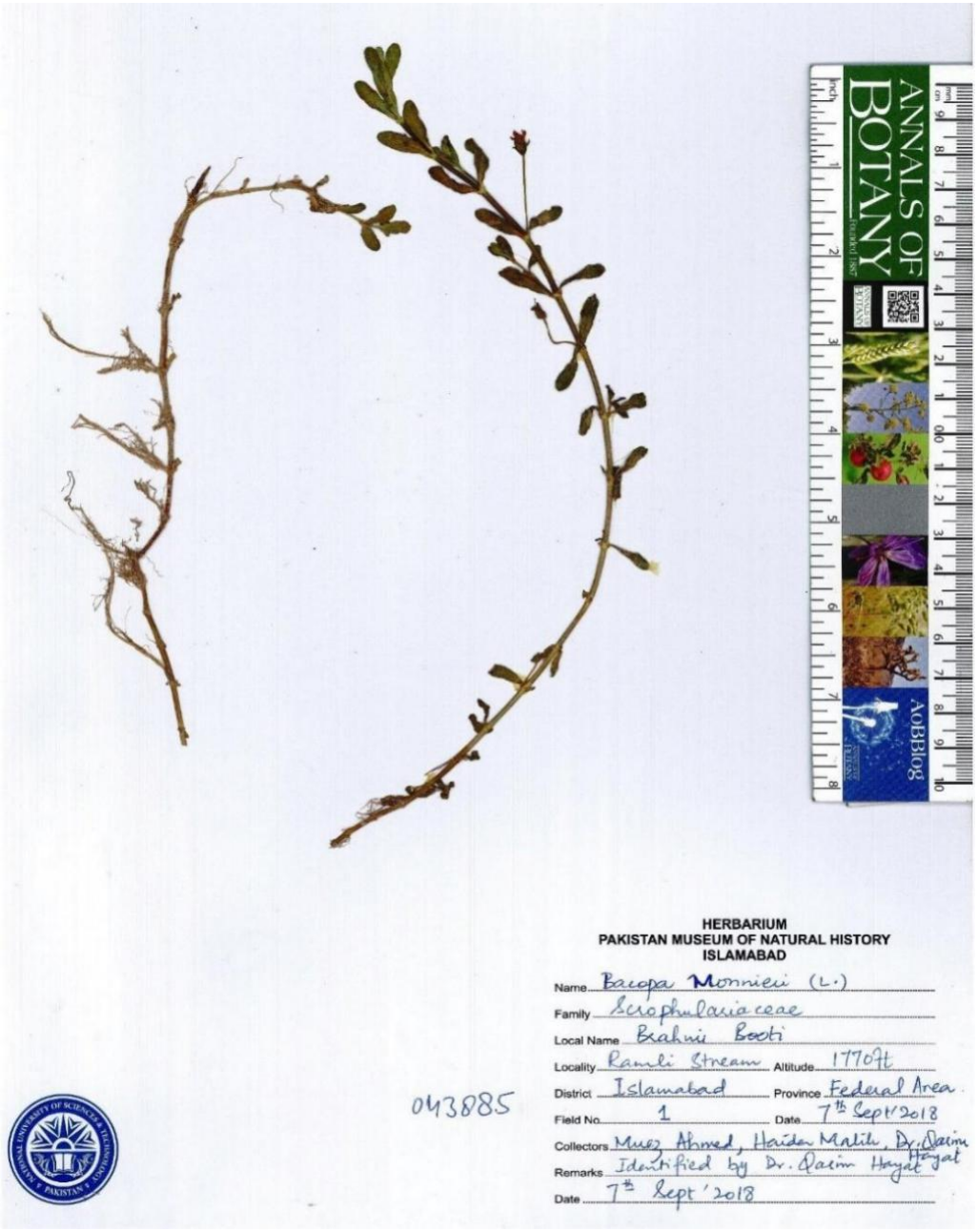


Figure 4.2 Scanned image of the Herbarium Sheet deposited at Pakistan Museum of Natural History

4.3 Molecular identification of *Bacopa monnieri*

DNA extraction from *Bacopa monnieri* was done for amplification of rbcL gene as shown by the qualitative detection of DNA on gel in Figure 4.3 (A) and quantitative detection using Nanodrop yielded a value of 140.2 ng/µl. Ribulose-1, 5-bisphosphate Carboxylase Large subunit (rbcL) gene with a size of 650 bp was amplified for molecular identification of the plant as shown in Figure 4.3 (B).

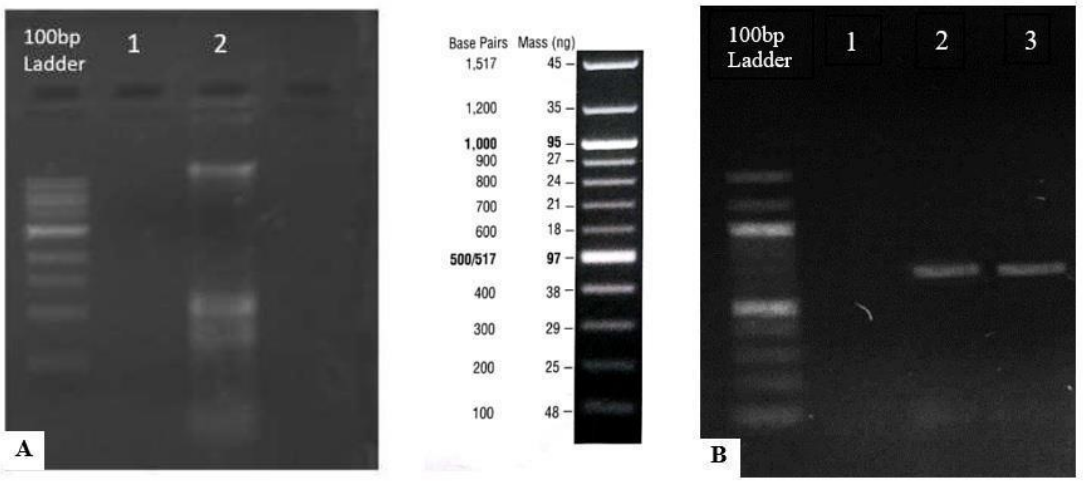


Figure 4.3 Agarose gel images from Molecular analysis of *Bacopa monnieri*

A: DNA extracted from *Bacopa monnieri*

B: rbcL gene amplified from *Bacopa monnieri*'s DNA

Lane 1: Negative Control Lane 2: Positive Control Lane 3: *Bacopa monnieri*

Sanger sequencing of the PCR product yielded a partial sequence of 613 bp as shown in Figure 4.4 which was deposited at NCBI and GenBank Accession No: MK467452.1 was acquired.

Phylogenetic analysis was done after sequence alignment of our sequence with other available sequences retrieved from NCBI after running a blast. Followed by plotting of phylogenetic trees using three different models namely Maximum Likelihood (Figure 4.5), Neighbour Joining (Figure 4.6) and Bayesian (Figure 4.7). *Wightia speciosissima* was taken as an outgroup. Our sequence of rbcL gene of *Bacopa monnieri* from Pakistan shown in green shared the same clade as *Bacopa monnieri* from India, Thailand, Mexico and USA in all three phylogenetic trees. The Maximum likelihood and Neighbour Joining trees showed bootstrap values of 68 and 76

respectively. While a posterior probability of 0.99 was shown by the Bayesian tree. All values show good significance validating the identity of our plant being *Bacopa monnieri* based on the sequence of its rbcL gene.

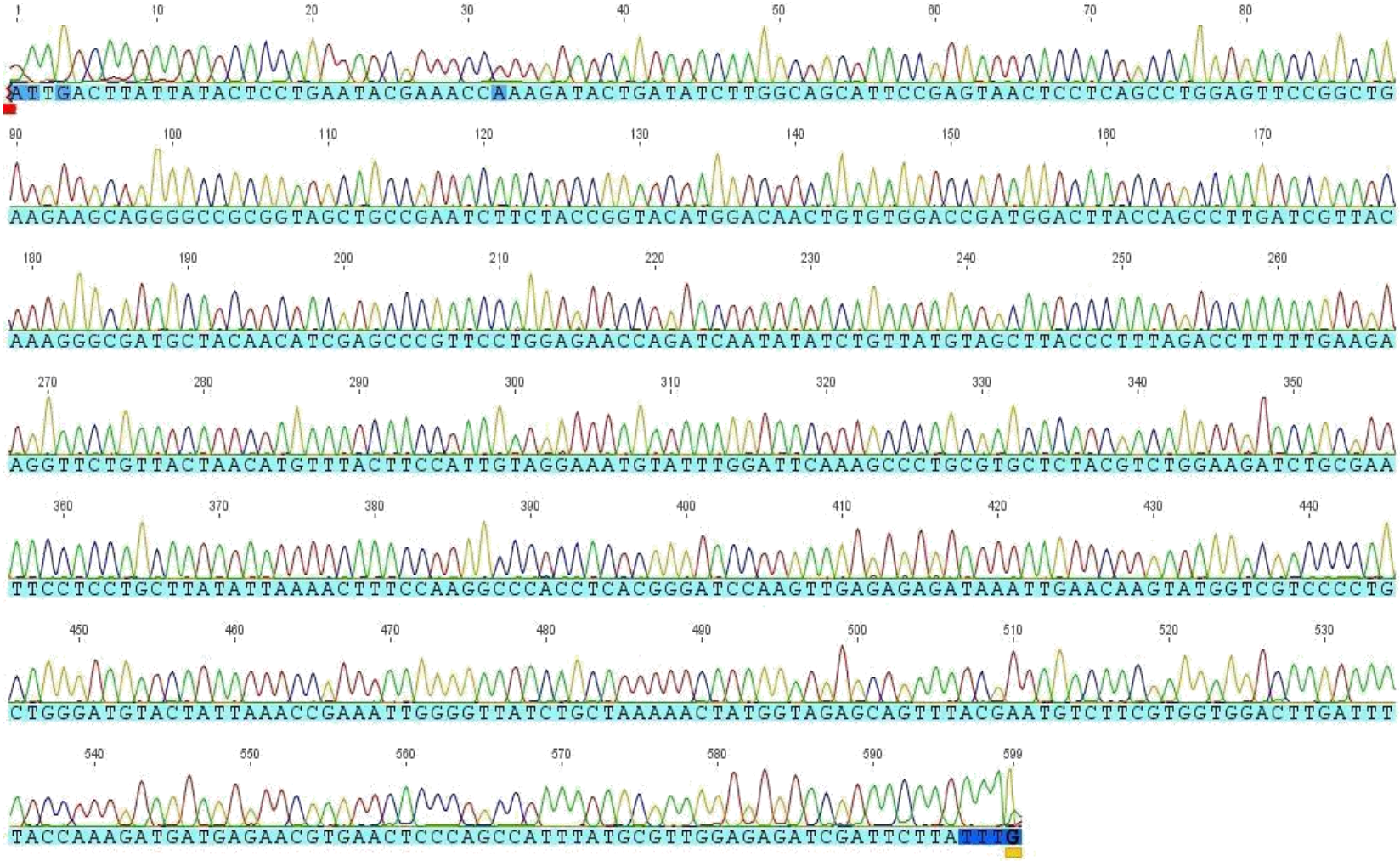


Figure 4.4 Sanger sequencing of rbcL gene

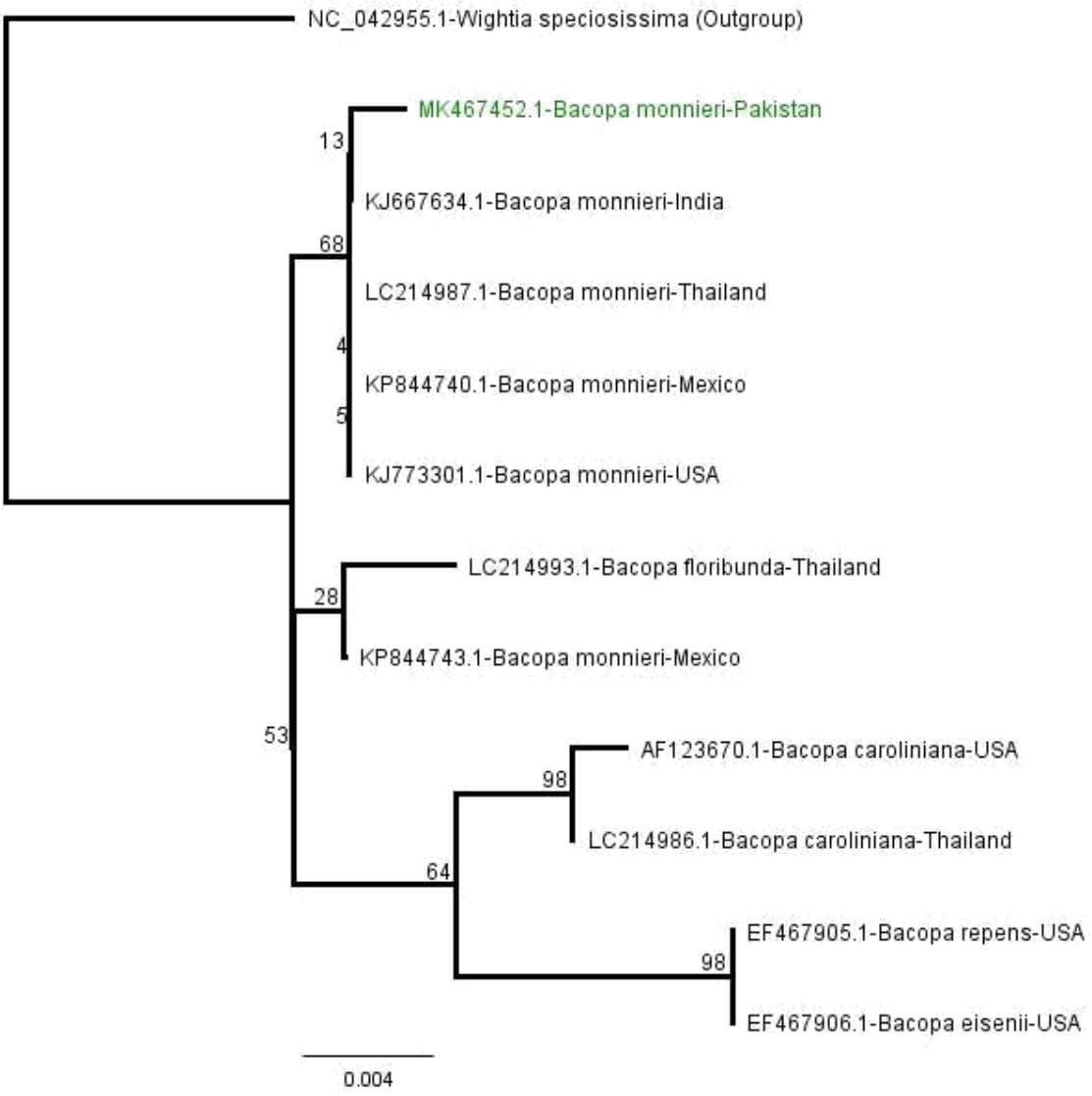


Figure 4.5 Molecular identification of *Bacopa monnieri* using rbcL gene as a DNA Barcode by plotting Maximum Likelihood Phylogenetic Tree

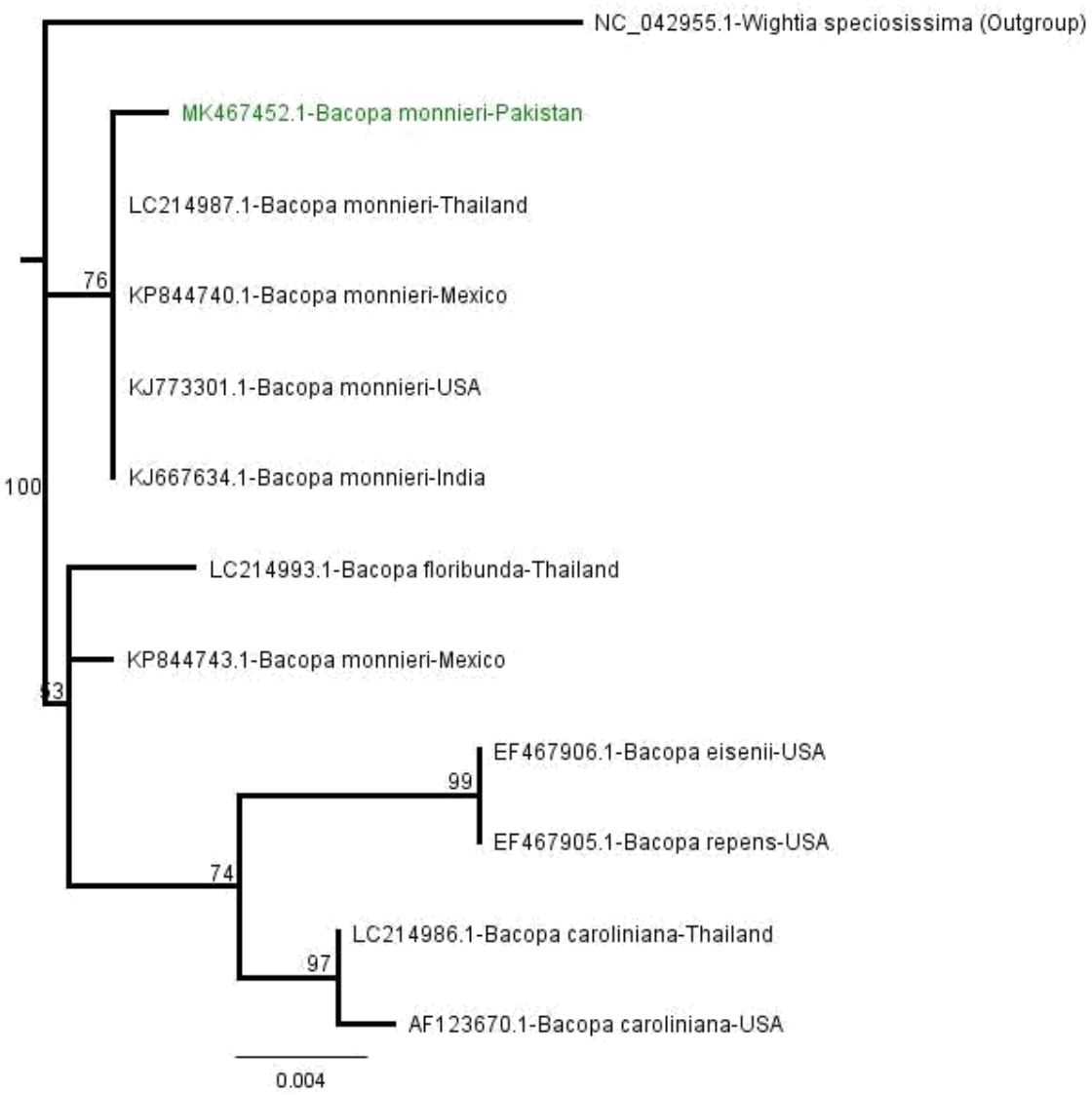


Figure 4.6 Molecular identification of *Bacopa monnieri* using rbcL gene as a DNA Barcode by plotting Neighbour Joining Phylogenetic Tree

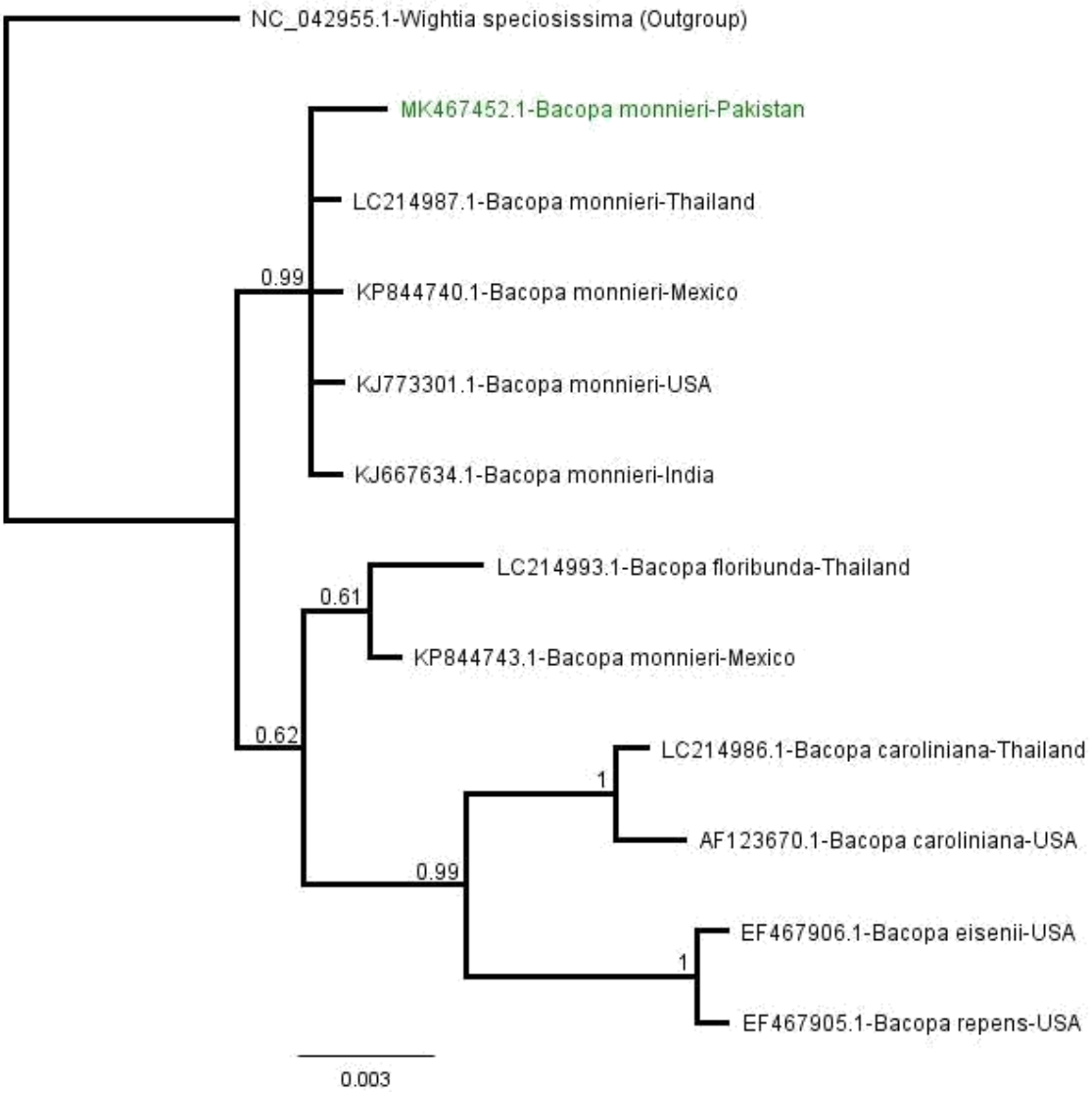


Figure 4.7 Molecular identification of *Bacopa monnieri* using rbcL gene as a DNA Barcode by plotting Bayesian Phylogenetic Tree

4.4 Plant Extract of *Bacopa monnieri*

Figure 4.8 Ethanolic *Bacopa monnieri* extract (eBME)

4.5 In vivo testing of eBME against Cadmium induced toxicity

Cadmium intoxicated distilled water was given to the mice in specialized drinking bottles with rotating metal balls to ensure all the water was being consumed by the mice without being wasted. The specialised diet with eBME infused in it was rolled into small round pellets for easy consumption of the mice as shown in Figure 4.9.

Figure 4.9 BALB/c mice being induced with Cadmium toxicity through drinking water and being treated with eBME infused diet

4.6 Behavioral Analysis

4.6.1 Y-Maze Spontaneous Alternation Test for Spatial Working Memory

Spatial working memory was assessed using Y-Maze Spontaneous Alternation test. The mean values of Spontaneous Alternation for Control, eBME, Cd 30 and Cd 30 + eBME were calculated to be 65.12%, 77.5%, 55.8% and 62.78% respectively. One way ANOVA followed by Tukey's multiple comparison post hoc test yielded a *p* value of < 0.0001 validating the significance of the results as shown by the bar graph in Figure 4.10 below.

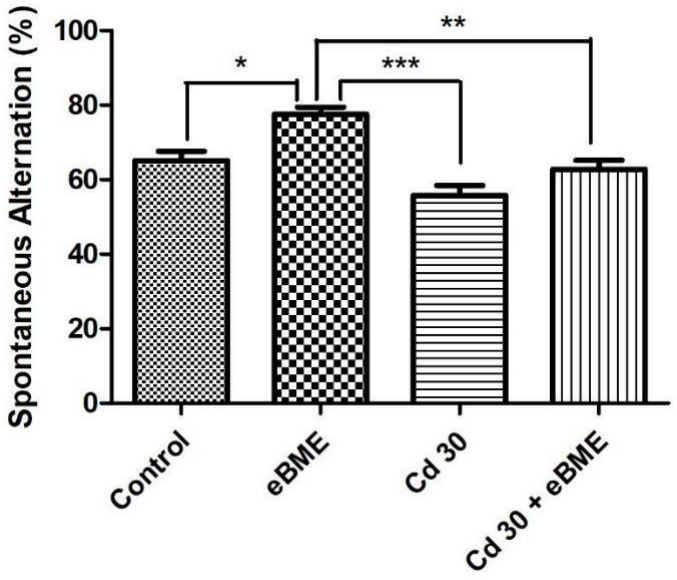


Figure 4.10 Bar graph for Spontaneous Alternation (%)

Data is expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ was considered significant, One way ANOVA followed by Tukey's multiple comparison post hoc test. n = 5 mice per group

4.6.2 Novel Object Recognition Test for Recognition Memory

Recognition memory was assessed using Novel Object Recognition Test (NORT). The mean values of PI for Control, eBME, Cd 30 and Cd 30 + eBME were calculated to be 76.04%, 77.82%, 48.64% and 74.95% respectively. One way ANOVA followed by Tukey's multiple comparison post hoc test yielded a *p* value 0.0001 validating the significance of the results as shown by the bar graph in Figure 4.11.

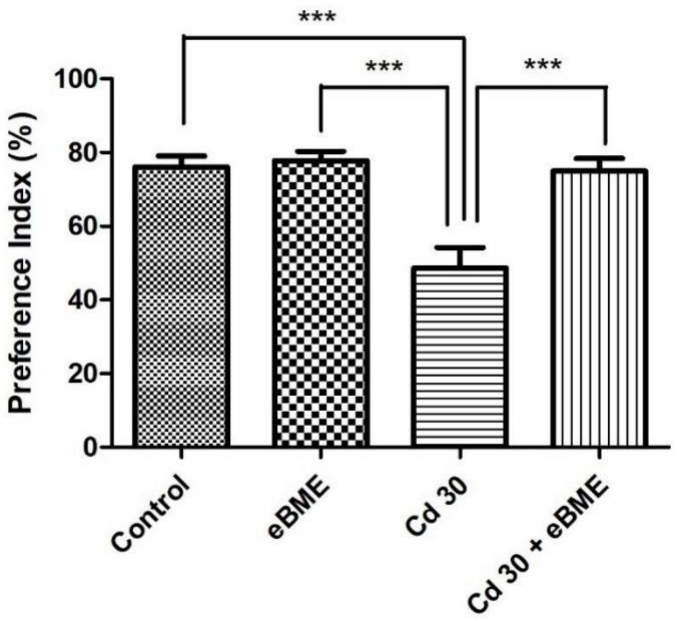


Figure 4.11 Bar graph for Preference Index (%)

Data is expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ was considered significant, One way ANOVA followed by Tukey's multiple comparison post hoc test. n = 5 mice per group

4.7 Liver Function Test – Alanine aminotransferase activity

Liver function was assessed using biochemical testing for Alanine aminotransferase (ALT) activity. The mean values of enzyme activity for ALT (U/L) for Control, eBME, Cd 30 and Cd 30 + eBME were calculated to be 71, 61.2, 88.6 and 69 respectively. One way ANOVA followed by Tukey's multiple comparison post hoc test yielded a *p* value of 0.0679 indicating the results were non-significant as is shown in Figure 4.12.

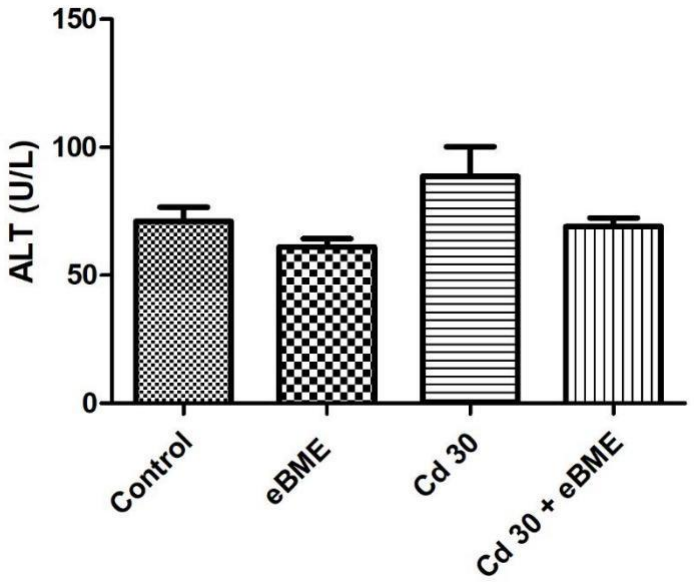


Figure 4.12 Bar graph for Alanine aminotransferase activity (U/L).

Data is expressed as mean ± SEM. One way ANOVA followed by Tukey's multiple comparison post hoc test. n = 5 mice per group

4.8 Renal Function Test – Urea and Creatinine Levels

Renal function was assessed using biochemical testing for Urea and Creatinine. The mean values for Urea (mg/dl) for Control, eBME, Cd 30 and Cd 30 + eBME were calculated to be 23.3, 22.3, 26 and 23.3 respectively. One way ANOVA followed by Tukey's multiple comparison post hoc test yielded a *p* value of 0.2157 indicating the results were non-significant for Urea levels as shown in Figure 4.13.

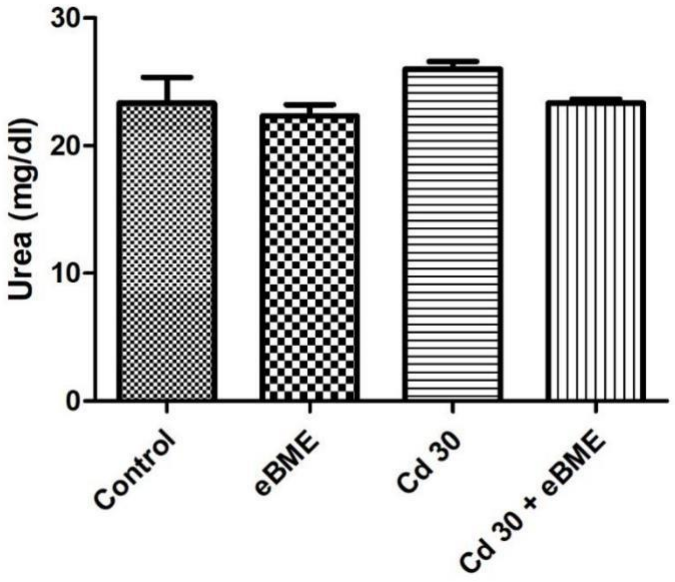


Figure 4.13 Bar graph for Urea levels (mg/dl).

Data is expressed as mean ± SEM. One way ANOVA followed by Tukey's multiple comparison post hoc test. n = 5 mice per group

The mean values for Creatinine (mg/dl) for Control, eBME, Cd 30 and Cd 30 + eBME were calculated to be 0.27, 0.32, 0.48 and 0.25 respectively. One way ANOVA followed by Tukey's multiple comparison post hoc test yielded a p value < 0.0001 indicating the results were highly significant for Creatinine levels. Control Vs Cd 30, eBME Vs Cd 30, Cd 30 Vs Cd 30 + eBME groups showed high significance as compared to one another having a p value < 0.001. eBME Vs Cd 30 + eBME showed significance having a p value < 0.01. While Control Vs eBME had a p value < 0.05 indicating significance amongst the two groups as is reflected by the bar graph in Figure 4.14.

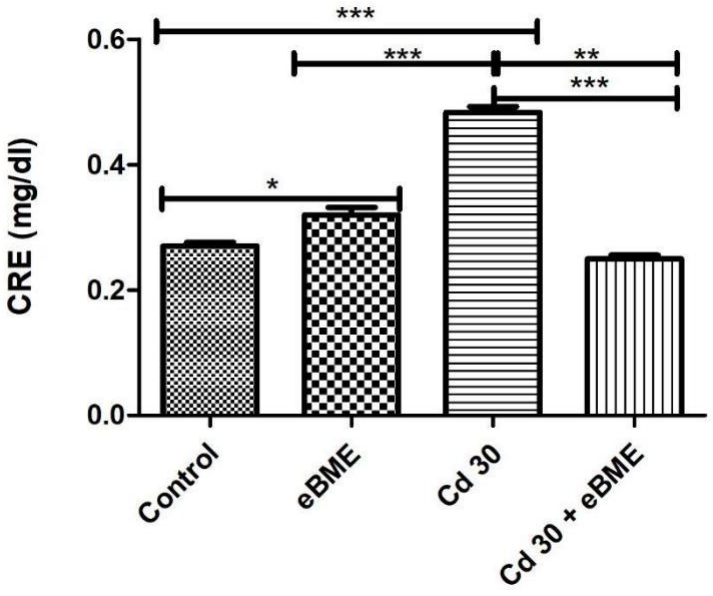


Figure 4.14 Bar graph for Creatinine levels (mg/dl).

Data is expressed as mean ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001 was considered significant, One way ANOVA followed by Tukey's multiple comparison post hoc test. n = 5 mice per group

4.9 Histopathology of Hippocampus

Hippocampal tissue from mice brains were stained with Hematoxylin and Eosin for Histopathology. The Control group showed normal morphology of the Hippocampus with intact neurons in the CA1, CA3 and Dentate gyrus as shown in A1, A2, A3 and A4 plates in Figure 4.15. eBME group showed similar morphology as the Control group. While number of neurons were visibly reduced in both the CA1 (Plate C2) and CA3 region (Plate C3) in the Hippocampus of mice exposed to Cadmium indicating neurodegeneration. Moreover, Cd 30 + eBME group showed mitigation of Cadmium's neurodegenerative effects by *Bacopa monnieri* as reflected by restoration of neurons in CA1 (Plate D2) and CA3 regions (Plate D3).

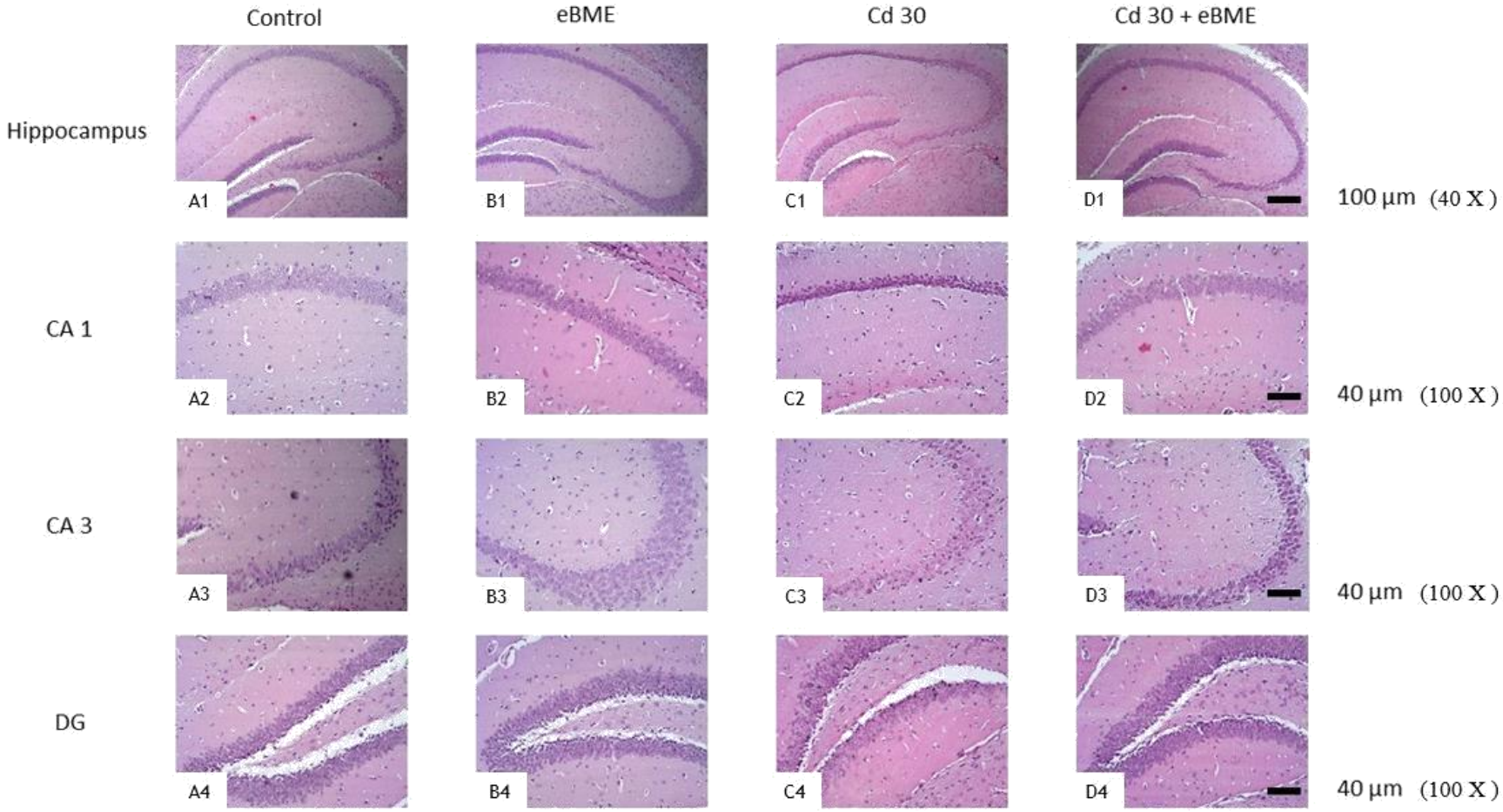


Figure 4.15 Representative images for Histology of Hippocampus

4.10 Histopathology of Kidney

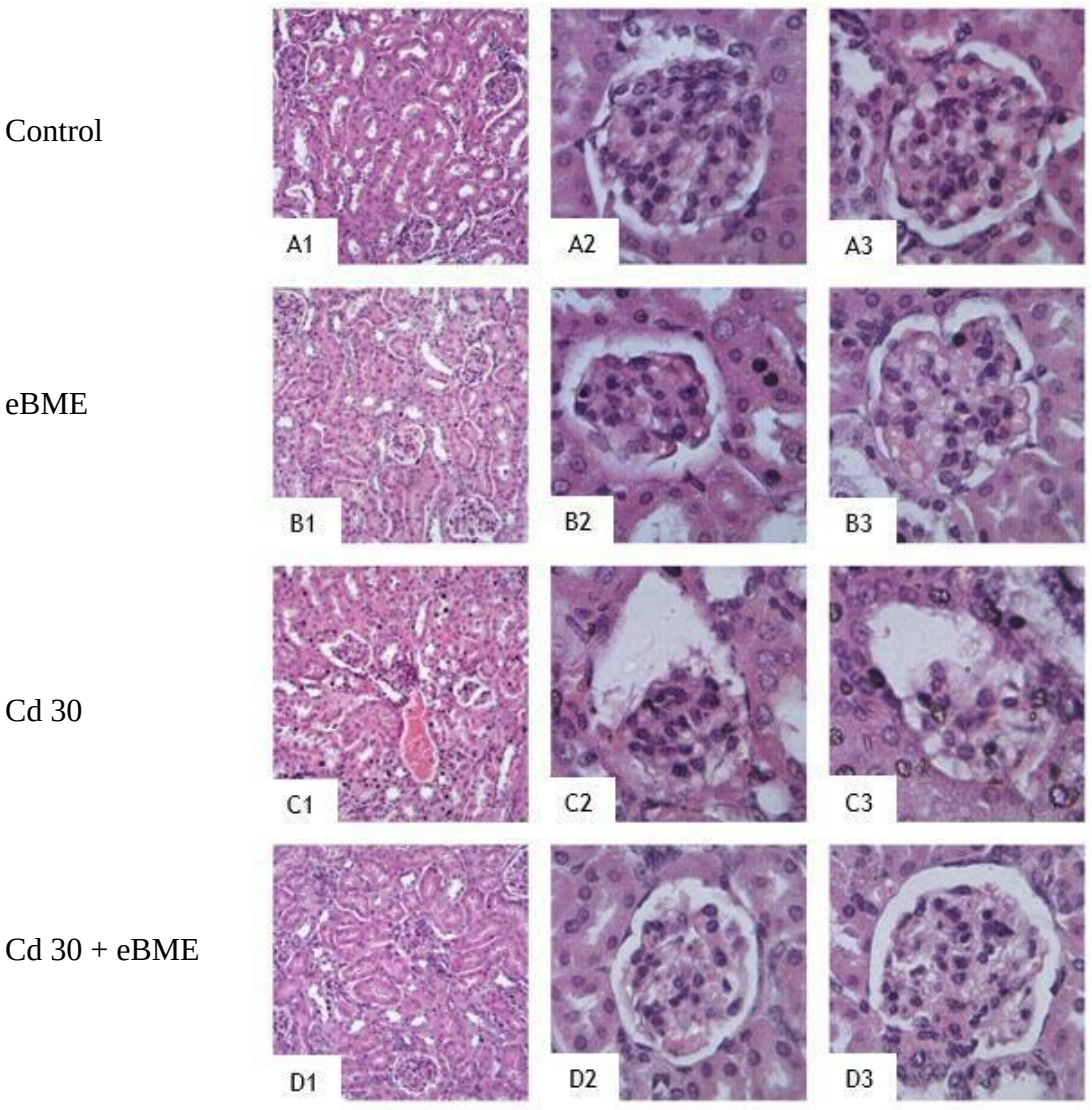


Figure 4.16 Representative images for Histology of Kidney tissue

A1: Normal appearance of Proximal and Distal tubules (100x)

A2-A3: Normal morphology of Renal Corpuscle (400x)

B1: Normal appearance of Proximal and Distal tubules (100x)

B2-B3: Normal morphology of Renal Corpuscle was retained (400x)

C1: Dilation of tubules was observed (100x)

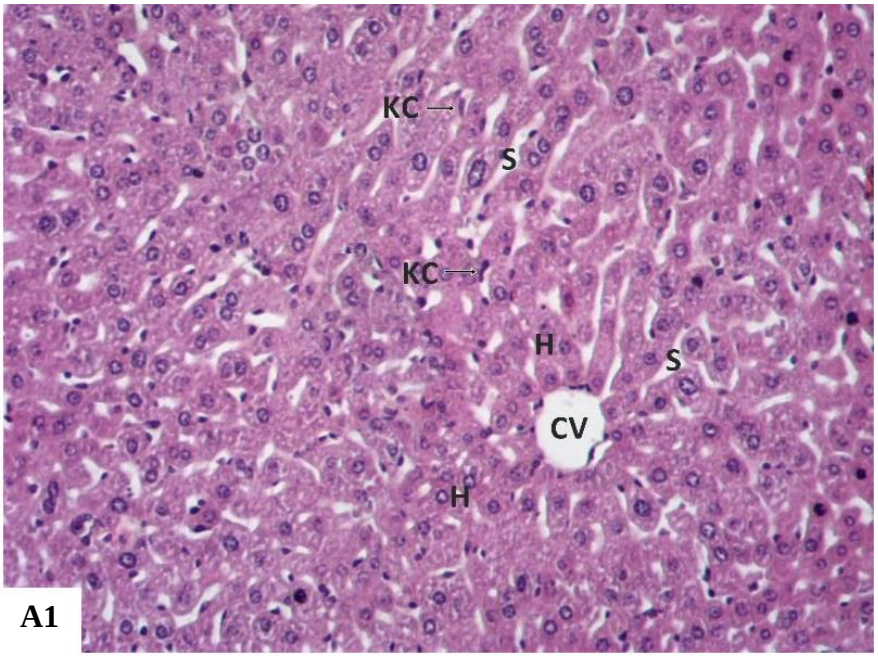
C2-C3: Enlarged subcapsular space with Glomerular atrophy (400x)

D1: It was observed that most of the tubules had returned back to their normal appearance (100x)

D2-D3: Most of the Renal Corpuscle's had normal morphology (400x)

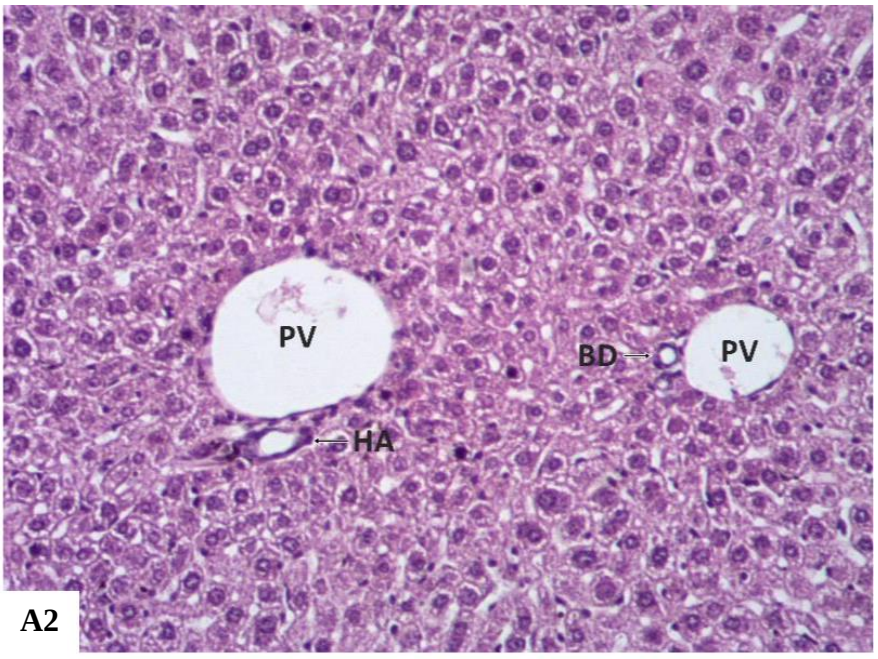
4.11 Histopathology of Liver

4.11.1 Histology of Control group



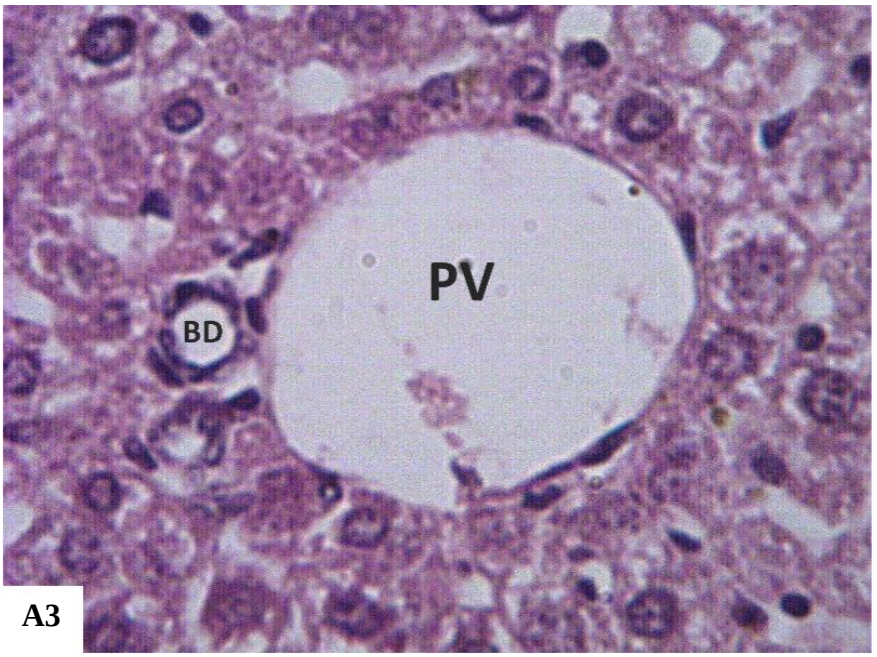
A1

A1: Normal histological structure of Liver with Central vein (CV) surrounded by cords of Hepatocytes (H), Blood Sinusoids (S) and Kupffer cells (100x)



A2

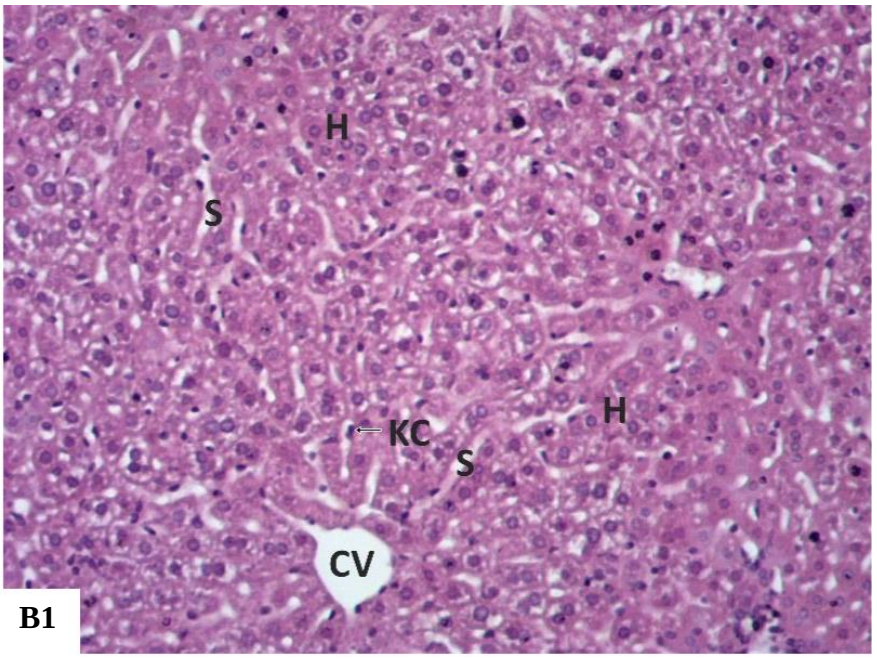
A2: Portal triad with Portal Vein (PV), Hepatic Artery (HA) and Bile Ductule (BD) (100x)



A3: Portal triad with Portal Vein (PV), Hepatic Artery (HA) and Bile Ductule (BD) (400X)

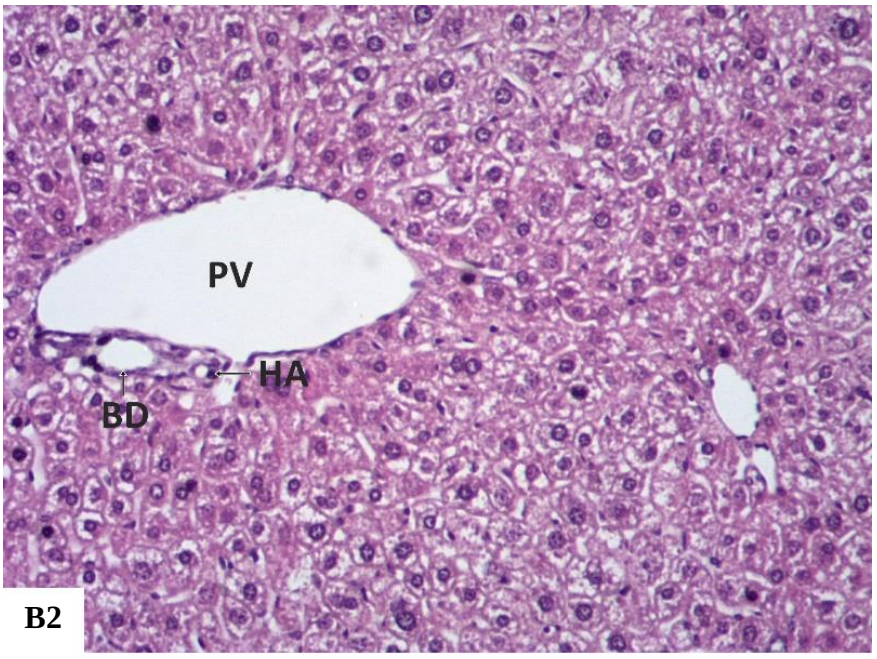
Figure 4.17 Representative images for Histology of Liver tissue from Control group

4.11.2 Histology of eBME group



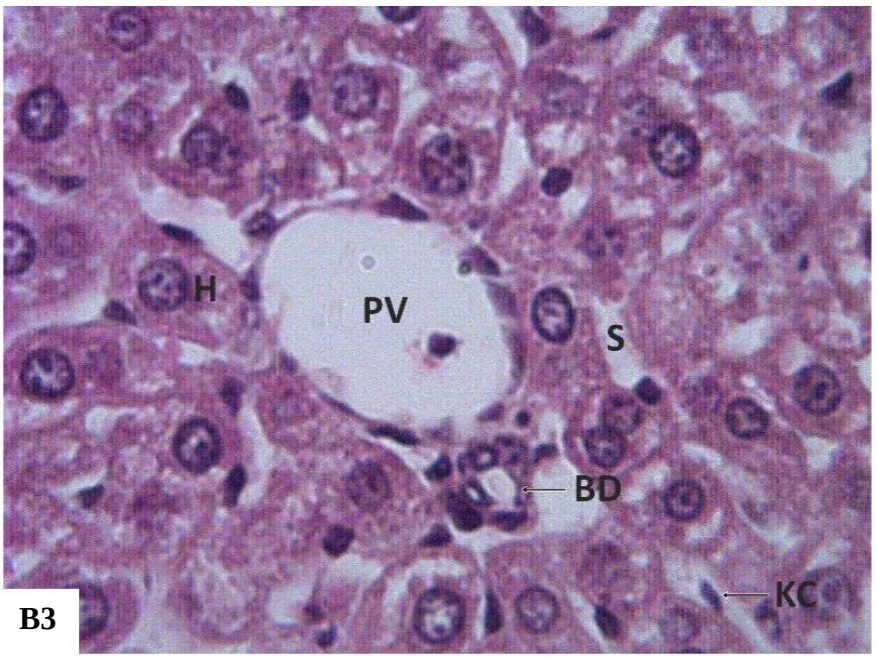
B1

B1: Normal histological structure of Liver with Central vein (CV) surrounded by cords of Hepatocytes (H), Blood Sinusoids (S) and Kupffer cells (KC) was retained (100x)



B2

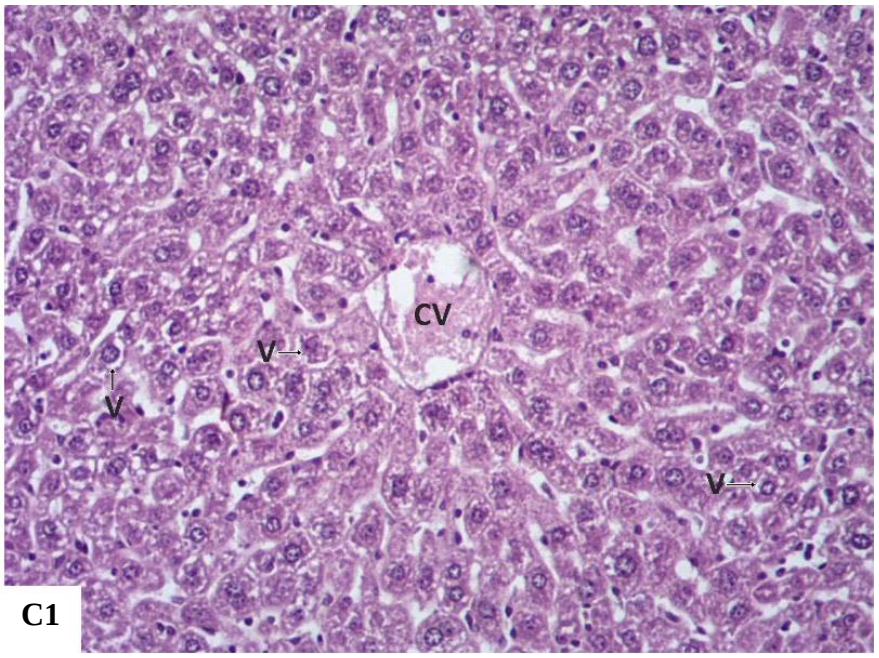
B2: Portal triad with Portal Vein (PV), Hepatic Artery (HA) and Bile Ductule (BD) (100x)



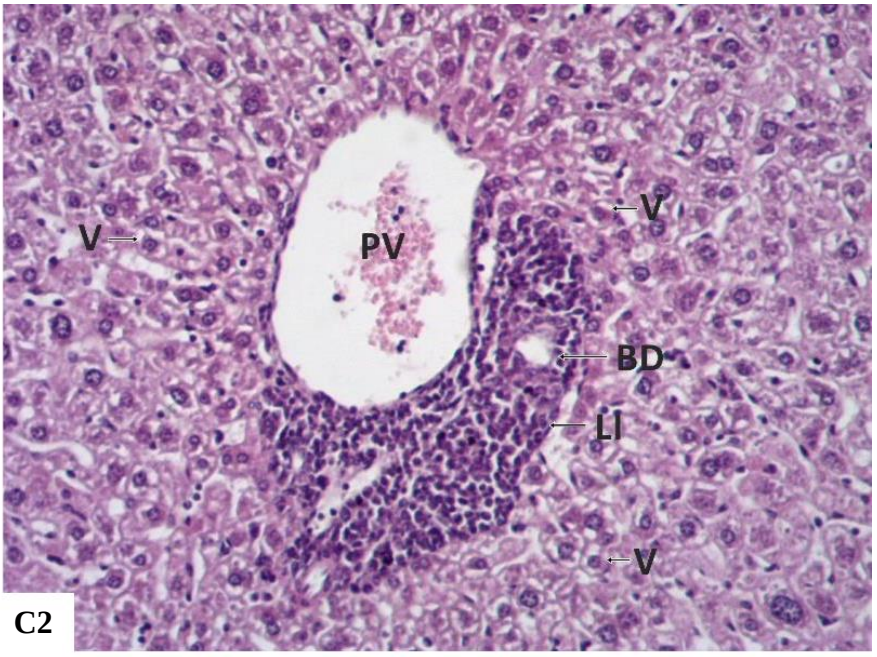
B3: Portal Vein (PV) with Bile Ductule (BD) (400X)

Figure 4.18 Representative images for Histology of Liver tissue from eBME group

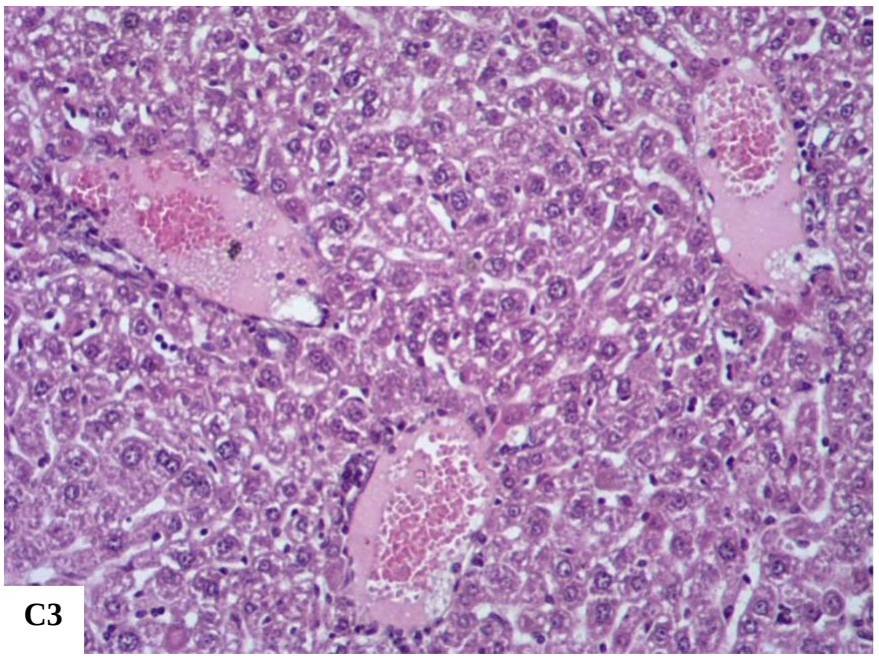
4.11.3 Histology of Cd 30 group



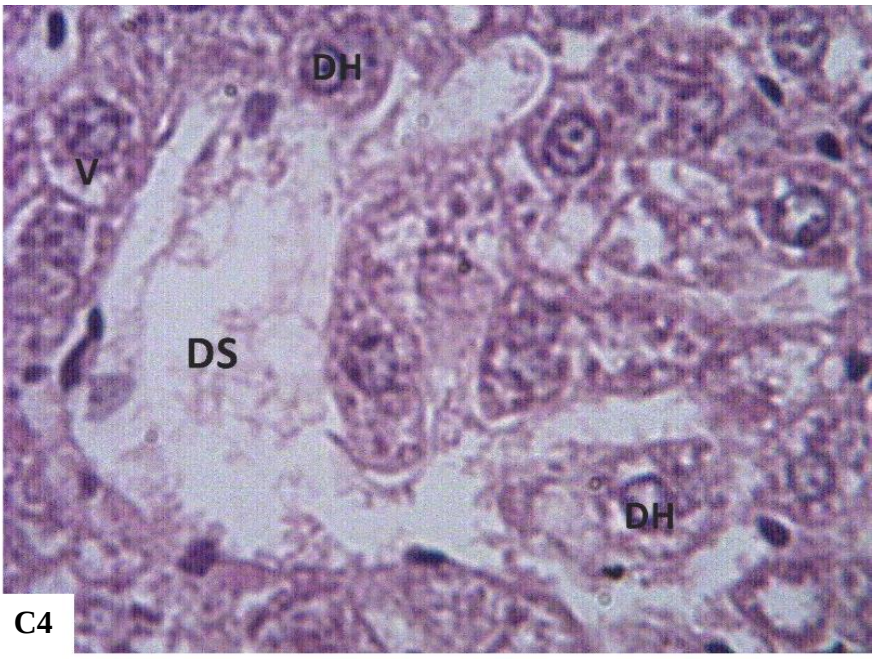
C1: Enlarged and congested Central vein (CV) surrounded by Hepatocytes with vacuolated cytoplasm (V) (100x)



C2: Portal Vein (PV) and Bile Ductule (BD) engulfed by Perivascular Leukocytic infiltration (LI) (100x)



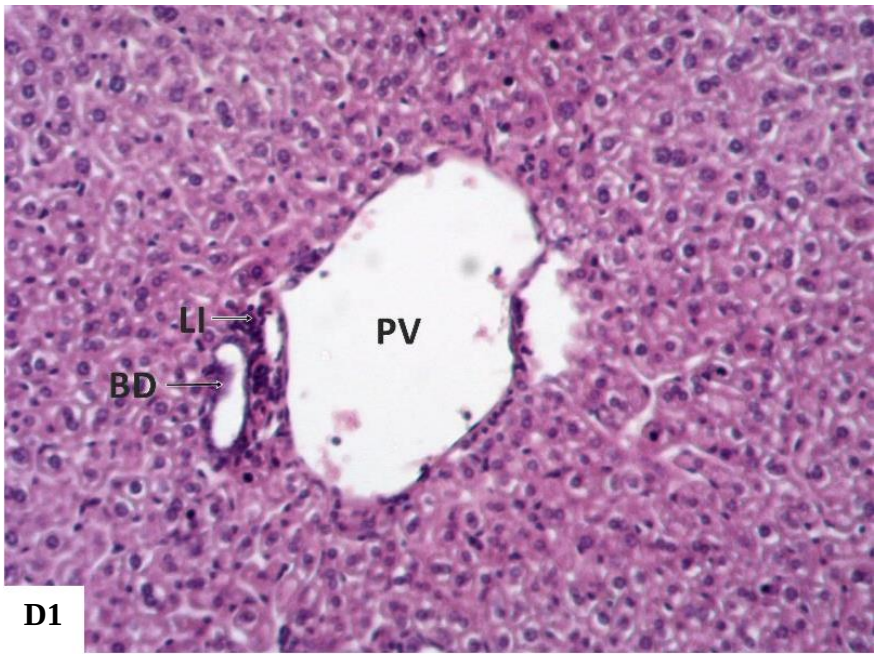
C3: Cystic blood filled cavities which are responsible for Peliosis hepatis (100X)



C4: Dilated Sinusoidal space (DS) with degenerated Hepatocytes (DH) (400X)

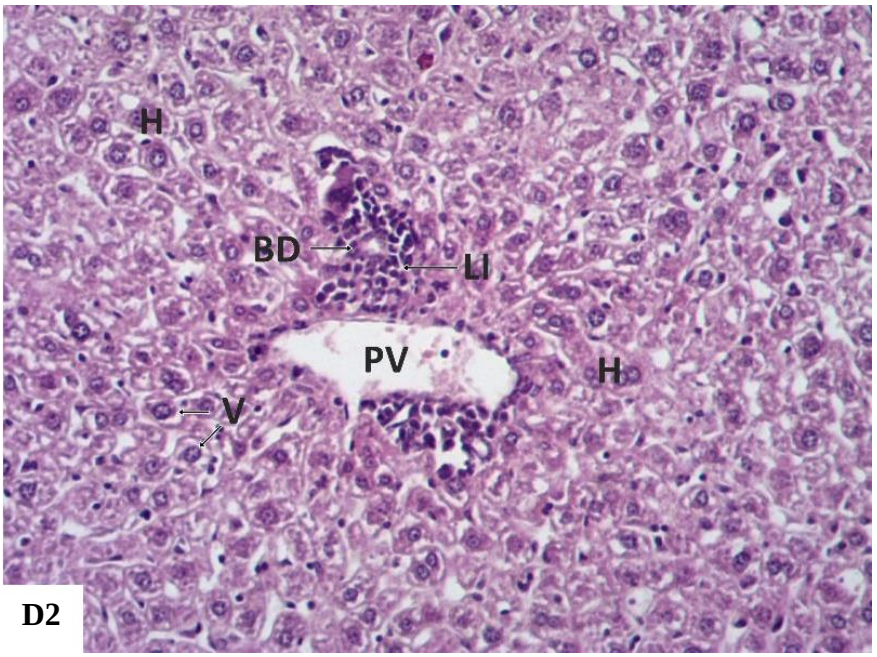
Figure 4.19 Representative images for Histology of Liver tissue from Cd 30 group

4.11.4 Histology of Cd 30 + eBME group



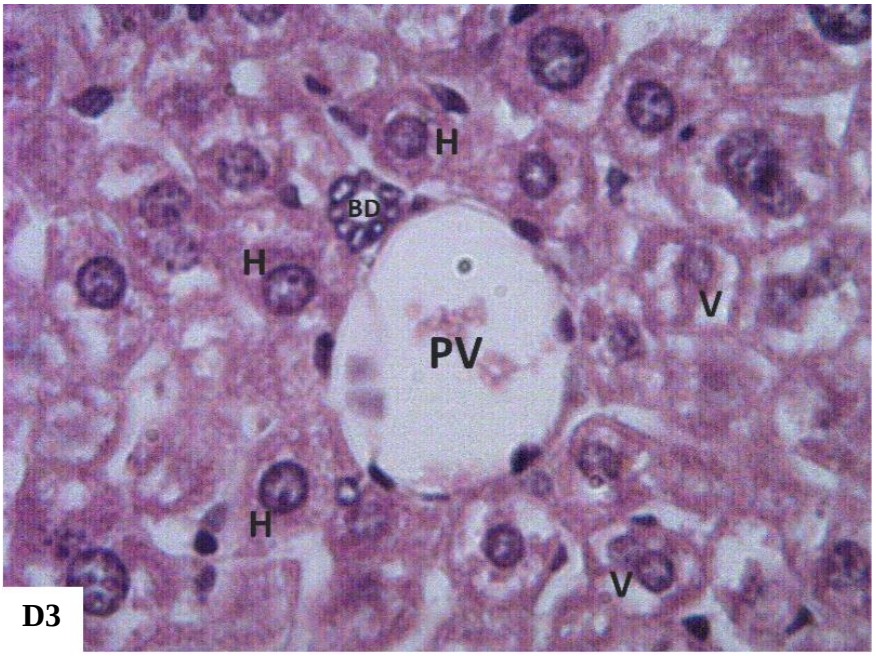
D1

D1: Portal Vein (PV) and Bile Ductule (BD) surrounded by barely noticeable Leukocytic infiltration (LI) with most of the Hepatocytes having normal morphology (100x)



D2

D2: Portal Vein (PV) and Bile Ductule (BD) with some perivascular Leukocytic infiltration (LI) (100x)



D3: Portal Vein (PV) and Bile Ductule (BD) with some normal Hepatocytes and a few with vacuolated cytoplasm (400X)

Figure 4.20 Representative images for Histology of Liver tissue from Cd 30 + eBME group

CHAPTER 5
DISCUSSION

Bacopa monnieri being an important medicinal plant from South Asia has been investigated for its beneficial properties time and again. It was sampled from Ramli stream near Quaid-e-Azam university, Islamabad. It has previously been sampled from the same location by other researchers from Pakistan but molecular identification was never done to confirm the identity of the plant (Rauf et al., 2012; Shahid et al., 2016). Plants from the Bacopa genus have a lot of similarity in their structures, especially in the size of their leaves and other traits, which makes it extremely difficult to differentiate *Bacopa monnieri* from other species such as *Bacopa caroliniana* on the basis of physical appearance (M. Kumar, Gopi, Lakshmanan, Panneerselvam, & Raj, 2013). Hence, it was proposed to identify the plant on molecular basis using phylogenetic analysis based on rbcL as a DNA barcode. rbcL has been used for phylogenetic analysis for a really long time and is amongst the two plastid loci the other one being matK that are proposed as core barcodes. However, rbcL sequences of only less than one-tenth of the plant species are available at the GenBank. DNA sequence of rbcL from *Bacopa monnieri* indigenous to Pakistan wasn't available so it was deposited to the GenBank at NCBI. The 613bp sequence yielded a homology of 99.84% using the Basic Local Alignment Tool (BLAST). Furthermore, the Phylogenetic analysis using three different statistical models confirmed the plant to be *Bacopa monnieri*. Bootstrap values > 50 are considered to be significant. Hence, both Maximum Likelihood and Neighbour joining trees with bootstrap values of 68 and 76 respectively yielded results that confirmed the sampled plant to be *Bacopa monnieri*. A posterior probability value of 0.99 from the Bayesian tree further validated the result. This confirms that rbcL can be used as a DNA barcode for identification of BM. Similar results have been reported with rbcL showing the least sequence variation amongst six barcoding regions i.e. ITS, matK, rbcL, ycf1, psbA-trnH and trnL184 F, for identification of Bacopa species from Thailand (Tungphatthong et al.).

Y-Maze Spontaneous Alternation Test is used for the assessment of Spatial working memory and Hippocampal integrity in mice. Alternation was counted when the arm choice differed from the previous two choices. Over the course of time

normal mice tend to visit the least recently visited arm hence alternate between the three arms. Mice utilize their working memory for successful alternation as they need to remember recently visited arms and continuously update that memory. The results reflected that Cadmium exposure reduced the spontaneous alternation (%) from 65.12% in Control group to 55.8% in Cd 30 group signifying spatial memory loss empirically. Our results are in congruence with previous studies in rodents (Adeniyi, Olatunji, Ishola, Ajonijebu, & Ogundele, 2014; Łukawski, Nieradko, & Sieklucka-Dziuba, 2005; H. Wang, Zhang, Abel, Storm, & Xia, 2017). eBME group had a significantly higher spontaneous alternation as compared to Control and Cd 30 group validating the memory enhancing potential of BM. *Bacopa monnieri* has been reported to improve spatial memory assessed through T - Maze in neonatal rats (Vollala, Upadhya, & Nayak, 2011). Treatment of Cadmium toxicity with eBME improved the value of alternation (%) empirically from Cd 30 group 55.8% to 62.78% in Cd 30 + eBME group but the difference wasn't statistically significant.

Recognition memory was evaluated by Novel Object Recognition Memory Test, by calculating Preference Index (D. Wang et al., 2007). Preference Index > 50 indicates a preference towards the novel object and < 50 towards the familiar object representing loss of recognition memory (Hammond, Tull, & Stackman, 2004). A Preference Index (%) of 76 and 48.6 represent Control and Cadmium treated group respectively. The result shows a statistically significant loss of recognition memory. The result is consistent with previous studies in rodents (Adeniyi et al., 2014; Batool et al., 2019; W. Kim et al., 2016). The eBME group had a slightly higher PI empirically as compared to Control group as shown in Fig 4.11. The Preference Index for Cd 30 and Cd 30 + eBME group are statistically significant to one another which validates the improvement of recognition memory by treatment with *Bacopa monnieri* as reported in previous studies (Kwon et al., 2018; Le et al., 2013; Le et al., 2015).

Alanine aminotransferase activity (U/L) was evaluated for analysis of Liver function. The results were non-significant but showed empirical difference between the values. The ALT levels were higher in Cd 30 group as compared to Control group signifying liver injury. Cadmium has been previously reported to increase ALT levels (Ivanova, Gluhcheva, Kamenova, Arpadjan, & Mitewa, 2014). eBME showed lower levels as compared to Control and Cd 30 group. Moreover, Cd 30 + eBME group had an ALT level of 69 in comparison to Cd 30 with 88.6. These findings prove that BM possesses hepatoprotective ability (Shahid et al., 2016; Sireeratawong, Jaijoy,

Khonsung, Lertprasertsuk, & Ingkaninan, 2016).

The Renal function was evaluated on the basis of Urea and Creatinine levels (mg/dl). The results for Urea levels were non-significant but showed empirical difference between the values. The Urea levels of Cd 30 group were slightly higher as compared to Control group which represents Cadmium's nephrotoxic effect (Gabr, Alghadir, & Ghoniem, 2017; Ibraheem, Seleem, El-Sayed, & Hamad, 2016; Kara, Karatas, Canatan, & Servi, 2005). Although, the value was in the safe range for Urea levels, majorly because an acute model of Cadmium toxicity was established in this study and detrimental effects on Kidney are usually visible in chronic toxicity of the metal (Rikans & Yamano, 2000). BM was able to return the raised levels back to normal showcasing its nephroprotective effect (Kamesh & Sumathi, 2014; Sumathi & Devaraj, 2009).

Creatinine levels in Control group and eBME group were statistically significant as compared to eachother. eBME showed a higher value for CRE as compared to Control which is surprising as eBME has majorly been reported to reduce Creatinine (Shahid et al., 2016; Sumathi & Devaraj, 2009). The results are closely related with results from the study by Sireeratawong and his colleagues, which demonstrated increase in CRE levels as compared to Control at 300 mg of BM (Sireeratawong et al., 2016). However, the underlying mechanism needs further investigation. CRE levels were significantly higher in Cd treated group as compared to Control which indicates renal damage as supported by other studies (Gabr et al., 2017; Hernayanti, Lestari, & Taufiq, 2019; Kara et al., 2005). Furthermore, there was a significant difference between Cd 30 and Cd 30 + eBME group that implies *Bacopa monnieri* was able to ameliorate nephrotoxic effects of Cadmium.

Hippocampus is the learning and memory center of the Brain and plays a key role in formation of new memories. Cadmium's ability to cross the Blood Brain Barrier has been attributed to neurodegeneration. Reduction in neuronal density in CA1 and CA3 region and pyknosis was clearly visible in the Hippocampal tissue. The results support studies done in the past (Kanter, Unsal, Aktas, & Erboga, 2016; Omotoso et al.). The group treated with eBME showed normal neuronal density and no abnormal changes were observed. eBME was able to restore neurotoxic effects in the Hippocampus caused by Cd, restoration of cellular density with reduction in pyknosis was visible in treated group which proves the therapeutic potential of BM in mitigating heavy metal toxicity (Khan et al., 2015; Ramesh Kumar, Kathiravan, & Muthusamy,

2012; Tembhre, Ahirwar, Gour, & Namdeo, 2015).

Histopathology of the Liver tissue represents normal tissue morphology in the Control group with Central vein, Portal triads, cords of Hepatocytes, blood Sinusoids and Kupffer cells. On the contrary Cd treated group showed congested Central veins, degenerated Hepatocytes with vacuolated cytoplasm, dilated Sinusoids and Leukocytic infiltration. These observations are in congruence with previous studies (Alkushi, Sinna, Mohammed, & ElSawy, 2018; El-Refaiy & Eissa, 2013; Ibraheem et al., 2016; H. Jin et al., 2013). The presence of Peliosis hepatis or "Blood lakes" were also visible in the tissue as previously described by Habeebu and his colleagues (Habeebu, Liu, & Klaassen, 1998). No toxic effects were seen in group treated with just eBME (Sireeratawong et al., 2016). BM ameliorated the toxic effects restoring the normal structure of the Liver tissue and Leukocytic infiltration was widely reduced. The findings stand true to previous research on therapeutic potential of *Bacopa monnieri* in treating hepatotoxicity (Shahid et al., 2016; Sumathi & Devaraj, 2009).

Kidney histology for Control group showed normal structure of Renal corpuscle, normal sized Glomerulus and capsular spacing. The Distal and Proximal tubules also showed normal morphology. Cd 30 group showed distorted Renal corpuscles with atrophied Glomerulus and enlarged subcapsular spaces. The Distal and Proximal tubules were also dilated. The results are in support of previous studies (Brzoska, Moniuszko-Jakoniuk, Piłat-Marcinkiewicz, & Sawicki, 2003; El-Refaiy & Eissa, 2013; Hu, Zhang, Du, Chen, & Lu, 2017; Ibraheem et al., 2016). Similar to histology of Liver no toxic effects were seen in group treated with just eBME (Sireeratawong et al., 2016). Moreover, BM mitigated the toxic effects of Cadmium exposure and restored the normal structure of nephrons administering its nephroprotective effect as has been reported in other studies (Kamesh & Sumathi, 2014; Shahid et al., 2016; Sumathi & Devaraj, 2009).

5.1 Conclusion

The present study was able to use rbcL as a DNA barcode for *Bacopa monnieri* indigenous to Pakistan. The prepared ethanolic Bacopa monnieri extract (eBME) proved its therapeutic potential in combating Spatial and Recognition memory impairment caused by exposure of Cadmium chloride in drinking water. It was also able to overcome the hepatotoxic and nephrotoxic effects of Cd by altering levels of

vital biomarkers such as Alanine aminotransferase, Creatinine and Urea. The Histopathology of Hippocampus, Liver and Kidney tissues validated the ameliorative effect of *Bacopa monnieri* against Cadmium induced toxicity. The study is important in establishing *Bacopa monnieri* as a therapeutic intervention for overcoming deleterious effects of Cadmium exposure.

5.2 Future Prospects

The beneficial phytochemicals present in ethanolic *Bacopa monnieri* extract used in this study need to characterized using HPLC. The identified phytochemicals can be singularly tested for their ability to counteract heavy metal toxicity. Real time PCR of vital neuronal markers needs to be carried out to elucidate the mechanism behind *Bacopa monnieri's* therapeutic potential against Cadmium toxicity.

CHAPTER 6

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