ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

THE TOXIC EFFECTS OF *Vernonia bipontini* ON SOME BLOOD PARAMETERS AND ON LIVER AND KIDNEY TISSUES

MEBRATU ALEBACHEW

May 2009

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BY: MEBRATU ALEBACHEW

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MEBRATU ALEBACHEW

DEPARTMENT OF ANATOMY, FACULTY OF MEDICINE, AAU

APPROVED BY: THE EXAMINING BOARD:

CHAIRPERSON, DEPARTMENT OF GRADUATE COMMITTEE:

NAME	SIGNATURE	DATE
_____	_____	_____

ADVISORS:

NAME	SIGNATURE	DATE
1. Dr. Yamrot Kinfu	_____	_____
2. Prof. Eyasu Makonnen	_____	_____
3. Dr. Yonas Bekuretsion	_____	_____
4. Ato. Kelbessa Urga	_____	_____

EXAMINER:

NAME	SIGNATURE	DATE
Prof. Talat Ahmed	_____	_____

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†ናቶሚ ት/ት ክፍል: ሜዲካል ተkም: †.†.¤

¦Ðደቀው: ፈታ„ ቦርድ

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TABLE OF CONTENTS

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LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
DDR	Drug Research Department
E & H	Eosin and hematoxylin
DPX	Dibutyl phthalate in xylene
^{0}C	Degree celicius
EDTA	Ethylene-Diamine-Tetra-Acetic acid
EHNRI	Ethiopian Health and Nutrition Research Institute
EPA	Ethiopian Pharmaceutical Association
g	Gram
GGT	Gamma-glutamyl transferase
Hct	Hematocrit
Hgb	Hemoglobin
JOEP	Journal of Ethno pharmacology
L	Lymphocytes
LD_{50}	Lethal dose for 50 percent of the population
mbr	Millibar
Mch	Mean corpuscular hemoglobin
Mchc	Mean corpuscular hemoglobin concentration
Mcv	Mean cell volume
mg/kg	Milligram per kilogram
mg/kg/bw	Milligram per kilogram per body weight
ml	Milliliter
P	*Plasmodium*
Pcv	Packed cell volume
RBC	Red blood cells
SDH	Sorbital dehydrogenase
SEM	Standard error of the mean
SPSS	Statistical package for social sciences
V	*Vernonia*
WBC	White blood cells
WHO	World Health Organization

ABSTRACT

Toxicological studies are sources of useful information for evaluating the therapeutic benefits of locally used medicinal plants. *Vernonia bipontini* (*V. bipontini)* is a herb used for treatment of malaria and malaria related symptoms in Ethiopia. However, its side effects have not been studied. In this study, the toxic effects of aqueous and methanolic extracts of *V. bipontini* leaves on some blood parameters and on liver and kidney, tissues in mice were investigated.

Lethal doses at which 50% of experimental mice died (LD_{50}s) in both aqueous and methanolic leaf extracts of *V. bipontini* were determined by administering different doses (1250-3250mg/kg for aqueous leaf extract and 1250-2750mg/kg for methanolic leaf extract) to experimental animals using intragastric catheter. For long-term toxicity study, sixty male and female Swiss albino mice were equally divided into six groups of 10 animals each. Groups1 and 2 were set as the control and received 0.5ml of distilled water and 0.5ml of 4% tween in distilled water, respectively, at 24 hrs intervals for 45 days. Group 3 and 4 were subjected to oral administration of the aqueous leaf extract at 400 and 800mg/kg, respectively, while group 5 and 6 were treated at 400 and 800mg/kg of methanolic leaf extract, respectively in 24 hrs intervals for 45 days. All groups were closely observed for any physical and behavioral alterations. Body weights of the mice were recorded on the first day and the last day of administration. Each animal was sacrified under diethyl ether anesthesia on the 46^{th} day. Following sacrifice, blood sample was collected by cardiac puncture using sterile needle and 5ml syringe into heparinized test tubes for hematological studies and into non-heparinized tubes for biochemical analysis. Hematological parameters (total RBC, WBC, platelets, lymphocytes, Hgb, Hct, Mcv, Mch, and Mchc) and biochemical parameters (AST, ALT, ALP, and urea) were

evaluated. Through a vertical, midline incision, the liver and kidney of each animal were removed and cleaned of the surrounding tissues. Each sample was fixed in 10% buffered formalin overnight. The tissues were processed for light microscopy.

The LD_{50s} of the aqueous and methanolic leaf extracts of *V. bipontini* were 2500.62±5.24 mg/kg and 2130.6±1.5mg/kg, respectively. No deaths were recorded among the control groups. The aqueous leaf extract has no significant ($P>0.05$) effect on liver and kidney weights, and hematological and biochemical parameters at all doses when compared with control group. Treatment with 800mg/kg body weight of methanolic leaf extract significantly ($P<0.05$) decreased body, liver and kidney weights, RBC, Hgb, Mch, Mchc, platelets and significantly increased serum AST, ALT and ALP levels while 400mg/kg dose had no effect on these parameters. The reduced organ weights did not correlate with loss of body weight at 800mg/kg bw of methanolic leaf extract of the plant.

Light microscope observations of liver tissue of mice treated with 800mg/kg of the methanolic leaf extract revealed dilated sinusoids, nuclear enlargement, bi-nucleation of hepatocytes, peripheral cramped chromatin, shrinkages (single cell death) of hepatocytes, fragmentation of hepatocytes (apoptosis) while no histopathological changes were observed in liver and kidney of mice treated at 400mg/kg of the methanolic leaf extract and at all doses of aqueous leaf extract. Kidney tissue sections of mice did not show significant histopathological changes at 400mg/kg of the same methanolic leaf extract of *V. bipontini*. However, at 800mg/kg kidney sections showed that increase cellularity of glomerulus and urinary space obliteration.

In conclusion, this study suggests that the aqueous leaf extract of this plant may be safe, even when taken for 45 days at higher dose (800mg/kg). This is in agreement with traditional claim of the water preparation of *V. bipontini* leaves. However, methanolic leaf extract may be phytotoxic to liver that resulted in a rise in serum AST, ALT and ALP levels after 45 days herbal administration at high dose. Further studies would be needed to identify the active ingredients responsible for such toxicities.

Key words: *V. bipontini*, Swiss albino mice, Liver, Kidney, Hematological and Biochemical parameters

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የሥነ መርዝ ጥናቶች በሀገር ውስጥ ለሕክምና የሚውሉ ተክሎችን የፈዋሽነት ጠቀሜታ ለመገምገም ጠቃሚ የሆነ መረጃ ምንጮች ናቸው። ቬርኖኒያ ባይፖ᎓ንቲኒ (*Vernonia bipontini*) ኢትዮጵያ ውስጥ ወባና ከወባ ጋር የተያያዙ በሽታዎችን ለማከም የሚውል ተክል ነው። ይሁን እንጂ ተጓዳኝ ውጤቶቹ አልተጠኑም። በዚህ ጥናት ውስጥ የቬ.ባይፖ᎓ንቲኒ ውሃ እና ሜታኖሊክ ጭማቂዎች መርዛማ ውጤት በአንዳንድ የደም መለኪያዎች እና እንደዚሁም በአይጦች ጉበትና ኩላሊት ሕብረ ሕዋስ ላይ ተመርምሯል።

በቬ.ባይፖ᎓ንቲኒ ውሃና ሜታኖሊክ የቅጠል ቅሪቶች ላይ 50 በመቶ ለምርምር የሚያገለግሉ አይጦች የሞቱበት ገዳይ የሆነ መጠን የተለያዩ የመድኃኒቱን መጠኖች በማየት (1250-3250 ሚግ/ኪግ ለውሃማ የቅጠል ቅሪት እና ከ1250-2750 ሚግ/ኪግ ለሜታኖሊክ የቅጠል ቅሪት) ታይቶ በምግብ ቧንቧ በኩል የሚያልፍ ካቲተር በመጠቀም የሙከራ እንስሳት ላይ ተግባራዊ ሆኗል። ለረዥም ጊዜ የሥነ መርዛማነት ጥናት 60 ሴትና ወንድ የስዊዝ አልቢኖ አይጦች እያንዳንዳቸው 10 እንስሳትን በሚይዝ 6 ቡድኖች በእኩል ተከፋፍሎ ነበር። ቡድን 1 እና 2 እንደመቆጣጠሪያ ሆነው 0.5 ሚሊ ሊትር የተጣራ ውሃ እና የ4 በመቶ ትዊን 0.5 ሚሊ ሊትር በተጣራ ውሃ ውስጥ በቅደም ተከተላቸው ለ45 ቀናት ያህል በየ24 ሰዓቱ ተሰጥቷቸው ነበር። ቡድን 3 እና 4 ደግሞ በቅደም ተከተላቸው በ400 እና 800 ሚግ/ኪግ በአፍ በኩል የሚሰጥ የፈሳሽ ቅጠል

ቅሪት የተደረገባቸው ሲሆን ቡድን 5 እና 6 ደግሞ በቅደም ተከተላቸው በ400 እና 800 ሚግ/ኪግ ሜታኖሊክ የቅጠል ቅሪት ለ45 ቀናት ያህል በ24 ሰዓቱ እንዲያገኙ ተደርጓል። ሁሉም ቡድኖች አካላዊና ባህሪያዊ ለውጦች የሚያሳዩ መሆኑን ለማየት የቅርብ ክትትል ተደርጉባቸዋል። የአይጦቹ ክብደት ምርምሩ በተጀመረበት እና በተጠናቀቀበት እለት ላይ ተመዝግቧል። እያንዳንዱ እንስሳ በ46ኛው ቀን ላይ በዳይኢታይል ኢተር ማደንዘዣ እንዲቆዩ የተደረገ ሲሆን ማደንዘዣውን ከወሰዱ በኋላ ባልተበከለ መርፌ እና 5 ሚሊ ሊትር ሲሪንጅ አማካይነት በልብ ውስጥ በመግባት የደም ናሙናቸው ተሰብስቦ ለደም ጥናት እንዲሆን ሄፓሪናይዝድ በሆኑ የመመርመሪያ ቱቦዎች ውስጥ የተቀመጠ ሲሆን ለባዮኬሚካል ትንተና ደግሞ ሄፓሪናይዝድ ወዳልሆኑ ቱቦዎች ገብቷል። ሳይንሳዊ የደም ጥናት መለኪያዎች (አጠቃላይ ቀይ የደም ሴል፣ ነጭ የደም ሴል፣ አነስተኛ የደም ሴሎች፣ የነጭ የደም ሴል ፈሳሽ፣ ሔሞግሎቢን፣ ሔማቶክሪት፣ ኤምሲቪ፣ ኤምሲኤች እና ኤምሲኤችሲ) እና የባዮኬሚካል መለኪያዎች (ኤኤስቲ፣ ኤኤልቲ፣ ኤኤልፒ እና ዩሪያ) ተመርምረው ነበር። በቀጥታማ መንገድ በስለት በመክፈት የእያንዳንዱ እንስሳ ጉበት እና ኩላሊት እንዲወጣ ተደርጐ በዙሪያው ካሉ ስስ ሕብረ ሕዋሶች እንዲፀዳ ተደርጓል። እያንዳንዱ ናሙና በአንድ ምሽት በ10 በመቶ ፎርማሊን እንዲያዝ ተደርጐ ስስ ሕብረ ሕዋሶቹን ብርሃን ባለው ማይክሮስኮፒ እንዲሆኑ ተዘጋጁ።

የቬ.ባይፖንቲኒ ውሃ እና ሜታኖሊክ ቅጠል ቅሪቶች ኤልዲ 50 በቅደም ተከተላቸው 2500.62±5.24 ሚግ/ኪግ እና 2130.6±1.5 ሚግ/ኪግ ነበሩ። ለቁጥጥር ከተያዙት ቡድኖች ውስጥ ምንም ዓይነት ሞት አልተመዘገበም። ፈሳሻማው የቅጠል ቅሪቱ በጉበት እና በኩላሊት ክብደት ላይ እንደዚሁም በቁጥጥር ቡድኑ ጋር ሲነፃፀር በሳይንሳዊ የደም ጥናት እና ባዮኬሚካል መለኪያዎች ረገድ በሁሉም መጠን ላይ ከፍተኛ የሚባል ውጤት አላሳየም (ፒ>0.05) 800 ሚግ/ኪግ የሰውነት ክብደት ባለው ሜታኖሊክ የቅጠል ቅሪት ማከም የሰውነት፣ የጉበት እና የኩላሊት ክብደትን እንደዚሁም የቀይ የደም ሴል፣ ሔሞግሎቢን፣ ኤምሲኤች፣ ኤምሲኤችሲ፣ አነስተኛ የደም ሕዋሶችን በመቀነስ የኤኤስቲ፣ ኤኤልቲ እና ኤኤልፒ መጠኖችን በከፍተኛ ሁኔታ የሚያሳድግ ሲሆን የ400 ሚግ/ኪግ መጠን ደግሞ በእነዚህ መለኪያዎች ላይ ውጤት አልነበረውም።

የተቀነሰው የሰውነት ክፍል ክብደት ከተክሉ ሜታኖሊክ የቅጠል ቅሪት 800 ሚግ/ኪግ የሰውነት ክብደት መቀነስ ጋር ግንኙነት አልነበረውም።

በ800 ሚግ/ኪግ ሜታኖሊክ የቅጠል ቅሪት ላይ የታከሙ አይጦች የጉበት ሕብረ ሕዋስ ላይ የተደረጉ የማይክሮስኮፕ ምርመራዎች ያሳዩት የተለጠጡ አነስተኛ የጉበት ውስጥ የደም ቧንቧዎች፣ የኒኩሌስ መለጠጥ፣ በብዛት የጉበት ሕዋሳት ላይ ሁለት ኒኩለስ መኖር፣ የክሮማቲን ወደ ኒኩለሱ የዳር ክፍል መገፋት፣ የጉበት ሕዋሳት መሰብሰብ፣ እና የዚሁ የጉበት ሕዋሳት መከፋፈልን ያሳየ ሲሆን በ400 ሚግ/ኪግ ሜታኖሊክ የቅጠል ቅሪት ላይ እና በውሃ የቅጠል ቅሪት ሁሉም መጠኖች ላይ በታከሙ አይጦች ጉበትና ኩላሊት ላይ ምንም ዓይነት ለውጦች አልታዩም። የአይጦች የኩላሊት ሕብረ ሕዋስ ክፍሎች በተመሳሳይ የቬ.ባይፖንቲኒ ሜታኖሊክ የቅጠል ቅሪት በ400 ሚግ/ኪግ ላይ ይህ ነው የሚባል ከፍተኛ ለውጥ አላሳዩም። ይሁን እንጂ በ800 ሚግ/ኪግ ላይ የኩላሊት ክፍሎች የኩላሊት የደም አጣሪ ክፍል በሕዋሶች መከበቡ እና የሽንት መውሰጃ ቱቦ ክፍተቱ መጥበቡን አሳይተዋል።

በማጠቃለያ ይህ ጥናት የዚህ ተክል የውሃ ቅጠል ቅሪት በከፍተኛ የመድኃኒት መጠን (800 ሚግ/ኪግ) ለ45 ቀናት ሲወሰድ እንኳን ደህና ሊሆን እንደሚችል ይጠቁማል። ይህ ደግሞ የቬ.ባይፖንቲኒ ቅጠሎች የውሃ ዝግጅትን አስመልክቶ ከሚሰጠው ባህላዊ አስተያየት ጋር የሚጣጣም ነው። ይሁን እንጂ የሜታኖሊክ ቅጠል ቅሪት በጉበት ላይ የመርዛማነት ባህሪ ሊያሳይ የሚችል ሲሆን ይህ ደግሞ በከፍተኛ የመድኃኒት መጠን ከ45 ቀናት በኋላ በኤኤስቲ፣ ኤኤልቲ እና ኤኤልፒ መጠኖች ላይ እድገትን አሳይቷል። ለእንዲህ አይነት መርዛማነት ምክንያት የሆኑትን ግብዓቶች ለመለየት ተጨማሪ ጥናቶች ያስፈልጋሉ።

ቁልፍ ቃላት፡ ቬ.ባይፖንቲኒ፣ የስዊዝ አልቢኖ አይጦች፣ ጉበት፣ ኩላሊት፣ የደምና የባዮ ኬሚካል ጥናት መለኪያዎች

1. INTRODUCTION

1.1. BACKGROUND

Medicinal plants are reservoirs of potentially useful chemical compounds that serve as basis for many standard drugs used in modern medicine (Debella, 2002). In recent years, the clinical importance of herbal drugs has received considerable attention (Gupta and Flora, 2005). However, poisoning of animals with plants is a common clinical occurrence (Abebe *et al*., 2003; Nwafor, 2004), and some medicinal plants may give serious adverse reactions and produce long-term effects such as hepatotoxicity (Steenkamp *et al*., 2006; WHO, 1988).

V. bipontini is a herb claimed to be useful for the treatment of malaria and malaria related symptoms, and it was found to be effective at 400mg/kg/day of mice, and its inhibitions against *Plasmodium berghei* in both aqueous and methanolic leaf extracts were 52.7% and 40%, respectively (Assefa *et al*., 2007). In Ethiopia, among medicinal plants, people use *V. bipontini* against malaria (Assefa *et al*., 2007) without the knowledge of its side effects especially, on tissues of liver and kidney, and blood components, which are commonly affected by toxic medicinal plant (Oboh, 2006; WHO, 2000).

Blood has a unique composition and many functions (Martini, 1995). Its composition exposes it to a wide range of abnormal conditions in the profile of blood parameters due to changes in metabolism. These abnormal alterations in blood parameters are results of changes in cellular integrity and exposure of toxic chemicals (Choudhari and Deshmukh, 2007).

Liver is the largest organ and the major site of intense metabolic activities. The liver injury caused by toxic chemicals and certain drugs, alcohols and multitude environmental toxins has recognized as a toxicological problem, and all of which place a burden on this vital organ (Oboh, 2006; Venukumar and Latha, 2002). Pharmacological and chemical agents may also be hepatotoxic and produce variety of liver ailments in hepatic tissues

such as necrotic and profound steatosis, centrilobular necrosis, ballooning degeneration, nodule formation, and fibrosis (Aydin, *et al*., 2003; Venukumar and Latha, 2002).

Kidneys are the second selected organs due to their prime functions as a blood-containing organ and through which a wide variety of toxic agents are filtered during the excretory process (Aughey *et al*., 1984). In addition to this, the tubular components of nephron may be primarily damaged as a result of organic and inorganic toxins (Aydin *et al*., 2003; Burkitt *et al*., 1996).

1.2. MEDICINAL PLANTS

Medicinal plants refer to plants, which are used medicinally for specific ailments (Assefa *et al*., 2006). According to WHO, "traditional medicine is the sum total of knowledge and practices used in diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing" (Berhanu, 2002). From the above definitions, one can understand that traditional medicine is practiced in almost all cultures and play an important role in all tradition. The use of traditional medicine has expanded globally and is gaining popularity. In Ethiopia, traditional remedies represent not only part of the struggle of the people to fulfill their essential drug needs but also they are integral components of their cultural beliefs and attitudes (Assefa *et al*., 2006). Many parts of a plant that are leaf, root, fruit, and stem-bark have important roles in treating many ailments including leprosy, asthma and malaria. Especially, the fruit and fresh leaves are applied for tropical ulcer and malaria (Assefa *et al*., 2006) and 87% of the remedies are originated from different parts of plants (Abebe and Ayehu, 1993).

For various reasons, including limitations of conventional or modern medicines and lack of formal health care facilities Berhanu, (2002); Shinde and Dhalwal, (2007), many developing countries still rely mainly on traditional practitioners and local medicinal plants for primary health care. This is also true in Ethiopia; where 80% of the total rural population depends on traditional medicine. Ethiopians have used traditional medicines at least as early as the 17^{th} century; however, very little is documented about this field. This is partly due to the reluctance of traditional practitioners to reveal their trade secrets (Berhanu, 2002).

In many countries, experience has been accumulated in the use of locally available medicinal plants, and some of them have been used effectively. Drugs from natural source, however; are not necessarily safe; some have given rise to serious adverse reactions and some contain chemicals that may produce long-term effects. Some countries may therefore wish to include traditional drugs in their national drug policy. Therefore, it is important to evaluate the use of traditional drugs and simultaneously strengthen explorative and developmental research in traditional medicine (WHO, 1988). Although *V. bipontini* is used in traditional medicine as antimalaria, antispasmodic, antisnake bite, antivenereal diseases, purgative and vermifuge Assefa *et al*., (2007), only a preliminary antimalaria investigation of *V. bipontini* has been carried out to evaluate its effectiveness.

WHO has been promoting traditional medicines as a source of less expensive, comprehensive medical care, especially in developing countries and 80% of the world's population relies on medicinal plants for their primary health care (Shinde and Dhalwal, 2007; WHO, 1999). The traditional use of plants in the treatment of different infections is widely practiced in developing countries. Therefore, various medicinal plants have also been studied using modern scientific approaches because many medicinal plants have a variety of properties and various biological components that can be used to treat various diseases (Pieme *et al*., 2006).

Although medicinal plants are important elements of Ethiopian traditional medicine (Gedif and Hahn, 2003), the indigenous knowledge of medicinal plants is decreasing in alarming rate and the vast knowledge on traditional uses of plants is not fully documented because most of the knowledge is conveyed from generation to generation by word of mouth (EPA, 2005). Herbal medicine become a topic of global importance and plays a central role in the health care system of large proportions of world's population. Therefore, the indigenous knowledge of medicinal plants should be fully documented and left for next generation (WHO, 1998).

1.2.1. ANTI-MALARIAL MEDICINAL PLANTS

Malaria is one of the most life-threatening infectious diseases worldwide and claims the life of millions of people each year (Kalauni *et al*., 2006), and is also one of the leading public health problems in Ethiopia, about 75% of the total area of the country is malarious (Endeshaw *et al*., 2008). It remains the greatest human killer among parasitic infections, despite the continuous worldwide efforts to combat this disease and the attempts to eradicate the causative organisms. Protozoan parasite is the causative agent in the genus *Plasmodium* (*P*) and transmitted by Anepheles mosquitoes. *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* are species of *plasmodium*. These species can produce malaria in human. Among these four species, *P. falciparum* is the most widespread and dangerous one (Endeshaw *et al*., 2008; Kalauni *et al*., 2006).

The historical discovery of quinine was from *Cinchona* tree and the recent discovery of artemisinin was from *Artemisia annua* that is from *Asteraceae* family of *Vernonia*. Therefore, there is anticipation that new leads may emerge from the tropical plant sources (Kalauni *et al*., 2006). Moreover, medicinal plants have in the past been the source of some of the most successful anti-malarial agents (JOEP, 2005).

It has been reported that more than two-third of the world's populations live in malaria endemic areas and about 200 million people have signs and symptoms of malaria every year. Of these, an estimated 1 to 2 million people succumb to the disease annually (JOEP, 2005). The majority of those who die from malaria are infants and children living in Sub-Saharan Africa. In Sub-Saharan Africa, more than 80% of the population relies on traditional medicines mainly plants and healers as the primary source of health care. This is mainly because of the accessibility and affordability of consulting with healers, and their cultural sensitivity (JOEP, 2005). The continuous infection and the spread of resistance among parasites to commonly used quinoline antimalarial drugs are causing a progressive increase in severity of the disease and mortality. Therefore, traditional method of malaria treatment could be a promising source of new anti-malarial compounds (EPA, 2005).

1.3. *VERNONIA bipontini*

1.3.1 ETHNOBOTANY OF *VERNONIA bipontini*

V. bipontini is discovered by Linnaea (Linnaea, 1875) and its name was verified in 1990 by Wattimah (Wattimah and Tadesse, 2008). *V. bipontini* species used in this study are found in tropical Africa (Linnaea, 1875). It is also indicated that it is found in Ethiopia under the family *Compositae*, genus *Vernonia*, and epithet *Caccaensis* (Wattimah and Tadesse, 2008). The genus *Vernonia*, consists of more than 1000 species, is distributed in tropical and subtropical regions of Asia, Africa, and America (Ojiako and Nwanjo, 2006). *Vernonia* is a species of forbs and shrubs in the family *Asteraceae*. Some of these species are sometimes known as ironweeds (Paulo, 1998). The genus is named after the English botanist William Vernon. They are known for having intense purple flowers. *V. bipontini* is a rarely erect and too straggly, woody herbs, of 0.6-1.5m height. Although the exact common location of *V. bipontini* is not well known, it is found in Shewa region above and to the west of 1000m up and down (Mesfin, 2004), Bale namely Delomenna (Fig.1) and it is locally known as "Reji" (Assefa *et al*., 2007).

Specimens of *V. bipontini*, are found at Addis Ababa University Herbarium collected from different areas, are also indicated that it is widely distributed in the flora of Ethiopia and Eriterea such as Tigray, Asmara, Gondar, Wollo, Shewa, Ambo, Muger, Bale, and Debre Birhan road.

Figure 1: Photograph of *Vernonia bipontini* showing the leaves and stem arrangement

1.3.2. ETHNOMEDICAL USE OF *VERNONIA bipontini* AND RELATED SPECIES

V. bipontini traditionally has the following indigenous use: significant anti-malaria, anti-spasmodic, anti-snake bite, anti-venereal diseases, purgative, and vermifuge (Assefa *et al*., 2007). People living in areas, where *V. bipontini* grows use methods of water preparation of the plant leaves for treating malaria and malaria related symptoms and take in the form of drinking. From the species of *Vernonia*, *V. amygdalina* is one of the pharmacologically useful plants. Both aqueous and alcoholic extracts of the stem-bark, the roots, and the leaves of *V. amygdalina* are also reported to be extensively used as anti-malaria, purgative, and in the treatment of ecezema or inflammatory conditions of the skin particularly with vesiculation in the acute stage (Ojiako and Nwanjo, 2006). Microscopic observation of the tissue sections of liver and kidney has showed no morphological abnormalities as compared to the controls after 42 days of oral administration of aqueous leaf extract of *V. amygdalina* (Amole *et al*., 2006). The phytochemical analysis indicated that the presence of antioxidant agents (sesquiterpene) such as saponins, flavonoids, oxalates, alkaloids and vernoniosides (glucosides) in the methanolic leaf extract (Eleyinmi *et al*., 2006; Eleyinmi *et al*., 2008 ; Nwanjo, 2005). However, in aqueous leaf extract vernodalin, vernolide, hydroxyvernolide, and glucosides (vernonioside) in related species *(V. amygdalina)* (Osinubi, 2005). Nwanjo (2005) in his study also showed the presence of tannins in addition to alkaloids, saponins, flavonoids, and glycosides chemical constituents in the fresh leaf of related plant *(V. amygdalina).* According to Diwan *et al*., (2000) the presence of saponin in the extract can cause from mild to severe diarrhea. The availability of active ingredient (Dichloromethane/DCM) in methanolic leaf extract that can induce enlargement of spleen has also indicated in *V. scorpioides (Lam.) Pers* (Pagno *et al*., 2006).

Antimicrobial activity of the leaf extract of *V. amygdalina* has been reported for treatment of malaria, gastrointestinal ailments (Abosi and Raseroka, 2003), and various infectious diseases (Erasto *et al*., 2006). Aqueous leaf extract has been shown to reduce blood sugar levels in rabbits. It is also reported that the leaf and root-bark extracts of this species produced large decreases in parasitaemia, resulting in chemosuppression ranging between 41.5% and 67.0% for the leaf extract and 38.5% and 53.5% for the root-bark

extract (Abosi and Raseroka, 2003). The effects of various concentrations of aqueous extract of *V. amygdalina* leaves on some biochemical indices of liver function in albino Wistar rats showed that *V. amygdalina* has nutritional, clinical and veterinary relevance considering the diverse applications of the plant in almost all African populations (Ojiako and Nwanjo, 2006). The crude extracts of *V. amygdalina* produce significant changes in Pcv, RBC, and Hgb concentration. This extract also significantly reduces the fecal egg count of the helminthes. Therefore, it could be a potential source of a new lead on anthelmintic agent (Adedapo *et al*., 2007). On the other hand, it is also indicated that the aqueous leaf extract of *V. amygdalina* produced no significant changes in Pcv, WBC and platelet counts (Amole *et al*., 2006).

1.4. BLOOD AND ITS COMPONENTS

Blood is a unique type of connective tissue, for it is a fluid tissue. Numbers of cells are suspended in the fluid (plasma), and varieties of solutes are dissolved in it (Landau, 1980). The total volume of blood in the normal adult is about 6 liters, which accounts for 7 to 8% of the total body weight (Ross and Pawlina, 2006). Blood consists of plasma about 55% by volume, and a solid part or the formed elements about 45% by volume. The formed elements are formed from bone marrow (Young *et al*., 2006). These blood cells are red blood cells (erythrocytes), white blood cells (leukocytes), and fragmented cells called platelets or thrombocytes (Carola *et al*., 1992). The major non-fluid elements of the blood are the red and white blood cells, with the red cells making up, by far, the greatest portion (Burkitt *et al*., 1996).

Erythrocytes or red blood cells (RBC) are anucleate cells devoid of typical organelles. They contain large amounts of oxygen carrying haemoglobin, which are involved in the transport of O_2 and CO_2, and function exclusively within the vascular system (Young *et al*., 2006). There are about 25 trillion erythrocytes in the body (Carola *et al*., 1992). The normal range of erythrocytes in a typical blood sample is from 4.5-5.5 million/cm^2 in male and 3.8-4.8 million/cm^2 in female (Kawthalkar, 2006).

Leukocytes or white blood cells (WBC) are sub-classified into two general groups that are granulocytes and agranulocytes based on the presence or absence of specific granules in the cytoplasm. Neutrophils are the most numerous WBC as well as the most common granulocytes (Ross and Pawlina, 2006) and constitute 40-75% of circulating leukocytes. They generally only leave the circulation in large number in response to disease and tissue injury in the acute inflammation (Young *et al*., 2006). Eosinophils are named for the large, eosinophilic, fragile granules in their cytoplasm (Ross and Pawlina, 2006) and they constitute 1-6% of a typical blood sample (Kawthalkar, 2006). Basophils are the least numerous of the WBCs accounts for less than 1% of the total leukocytes (Kawthalkar, 2006). Lymphocytes and monocytes are the two types of agranulocytes. Lymphocytes are the main functional cells of the lymphatic or immune system and they are the most common agranulocytes accounts for about 20-40% of the total blood leukocytes (Kawthalkar, 2006). They are found in both blood and lymph (Ross and Pawlina, 2006). Monocytes are the precursors of the cells of the mononuclear phagocytotic system. They constitute 2-10% in a total leukocytes and differentiate into the various phagocytes that are called perisinusoidal macrophages (kupffer cells) when they are found in liver (Ross and Pawlina, 2006). The normal adult leukocytes count is 4,000 to 11,000 per cubic millimeters (Carola *et al*., 1992) which constitute an important part of the defense and immune system of the body, and act mainly outside blood vessels in the tissues (Burkitt *et al*., 1996).

The term “formed elements” is used because it includes tiny fragments, known as platelets (thrombocytes) which are not actually whole cells (Landau, 1980). There are about 350,000 per cubic millimeters or a trillion platelets in adult (Carola *et al*., 1992).

These platelets also play an important role in the control of bleeding (homeostasis) by plugging defects in blood clotting successive reaction (Burkitt *et al*., 1996).

A typical sample of plasma is about 90% water, 7% protein constituent, and 3% electrolytes, amino acids, glucose and other nutrients, various enzymes, hormones, metabolic wastes, and trace of many other organic and inorganic compounds (Carola *et al*., 1992). Serum is a clear yellow liquid of plasma without clotting factors (fibrinogens) (Junqueira and Carneiro, 2005). In the serum biochemical profiles, liver associated enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), as well as others gamma-glutamyl transferase (GGT), sorbital dehydrogenase (SDH) are commonly used as makers of hepatic injuries (Gaskill *et al*., 2005; Haldar *et al*., 2007; James *et al*., 2007; Osman and Al-Busadah, 2003). When these enzymes increased in the serum, it is reflection of liver dysfunction due to liver injuries (Adebayo *et al*., 2005; Ege *et al*., 2008; Gaskill *et al*., 2005; Seifert *et al*., 2008; Shahraki *et al*., 2007). The increase serum ALT (cytoplasmic enzyme) activity is typically associated with hepatocellular membrane damage and leakage of the enzyme from hepatocytes (Ege *et al*., 2008; Gaskill *et al*., 2005; James *et al*., 2007). The increased serum ALP (membrane-bounded enzyme) activity is also associated with a pathological occurrence in the liver (Haldar *et al*., 2007). Normally kidneys filter serum urea and other substances present in the blood. The presence of high serum urea and creatinine concentration in the blood indicates more deteriorious effect on the kidneys, which can adversely affect the functioning of kidneys (Adebayo *et al*., 2003).

1.5. LIVER

1.5.1. HISTOPHYSIOLOGY OF LIVER

The liver is the largest organ in the body (Moore and Dalley, 2006; Sherlock and Summerfield, 1988) and accounts for about 2% of the body weight in adults and 5% in

infants (Moore, 1992). The liver lies mainly in the right upper quadrant, where it occupies almost all of the right hypochondriac region and part of the epigastric region and extends into the left hypochondriac region (Moore, 1992). The outer surface of the liver is covered by a capsule composed of collagenous tissue called Gilsson's capsule over which is a layer of mesothelial cells from the peritoneum (Young *et al*., 2006).

The basic structural components of the liver are the liver cells, or hepatocytes. These epithelial cells form groups in interconnected plates and constitute two-third of the mass of the liver (Junqueira and Carneiro, 2005). Structural units of the liver are liver lobules, which are visible in light microscope. Each liver lobule is a polygonal mass of tissue about 0.7 x 2mm in size, with portal spaces located at the corners of the lobules. These spaces contain connective tissue, bile ducts, lymphatics, nerves, and blood vessels. Hepatocytes in the liver lobule are radially disposed, and are arranged like the bricks of a wall (Junqueira and Carneiro, 2005).

Microscopic structures of the liver shows that sinusoids are pale-stained spaces between the plates of the hepatocytes. Sinusoid lining cells include endothelial cells, Kupffer cells and hepatic lipocytes. Endothelial cells are the majority of the cells lining the hepatic sinusoids with flat darkly stained nuclei and thin fenestrated cytoplasm. Kupffer cells are large and scattered phagocytic cells with ovoid nuclei. Hepatic lipocytes are fat storing cells (Young *et al*., 2006). Most of the collagenous connective tissues in the liver are in the form of portal triads. A typical portal triad contains hepatic portal vein, hepatic artery and bile duct. Hepatic portal vein is the largest and thin wall, lined by endothelial cells. Hepatic artery is thick walled vessel with smaller diameter. Bile duct is a network of bile canaliculi located within plate of hepatocytes (Young *et al*., 2006).

Functionally, liver has four major tasks: secretory, storage, metabolic, and excretory. It serves to secrete normally 600 to 1000 ml of bile per day for emulsification of the large fat particles of food into smaller particles (Carola *et al*., 1992). Liver cells can store large amounts of glycogen, amino acids, (Guyton and Hall, 2006), fat-soluble vitamins (Carola *et al*., 1992) and serve as storage of fat until it is needed elsewhere in the body. It also breaks down glycogen into glucose to supply the body with energy whenever it is needed (Guyton and Hall, 2006). Liver also converts glucose into fat in the fat cells. Liver works

as a lipid metabolic organ that degrades fatty acids into small compounds, which can be used for energy. It also detoxifies and eliminates drugs such as pesticides, herbicides, environmental pollutants and toxic substances (Effendy *et al*., 2006; Carola *et al*., 1992). Liver also works as urea forming organ to regulate ammonia present in the blood, and essentially all urea formed in the human body is synthesized in the liver (Guyton and Hall, 2006). Liver also manufactures most of the plasma proteins, blood clotting-factors (Effendy *et al*., 2006) and fetal erythrocytes (Carola *et al*., 1992).

1.5.2. HISTOPATHOLOGY OF LIVER

Hepatocytes, with their high degree of metabolic activities, are readily disturbed by toxins, especially drugs or alcohol, and may demonstrate the histological cell responses known as cloudy swelling, fatty changes, and necrosis. With more severe metabolic disruption, the hepatocytes undergo hydropic degeneration and become swollen and vacuolated (Burkitt *et al*., 1996). Fatty change is another manifestation of sub-lethal metabolic disarrangement seen in certain cell types with high-energy demand. It is most common in the liver. The fatty changes due to metabolic injury to hepatocytes are also manifested by large cytoplasmic vacuoles within some hepatocytes, which usually displace the nucleus to one side. The dead cells stain a bright pink (eosinophilia) and standout from the other cells. This is due to degeneration of structural proteins which form a compact homogenous mass, whereas living cell's nucleus is smaller, condensed, and intensely stained with hematoxylin (basophilia) (Burkitt *etal*., 1996). Apoptosis is a normal highly organized physiological mechanism to destroy injured or abnormal cells (Pagliar *et al*., 2003; Taraphdar *et al*., 2001).

Viral hepatitis, toxins, drugs, and systemic infections are the most important groups of conditions causing acute liver inflammation. Acute inflammation of the liver parenchyma is usually marked by focal accumulations of inflammatory cells in the site of necrotic hepatocytes (Burkitt *et al*., 1996). Liver sections may appear portal tracts enlargement with inflammatory cellular infiltration mainly plasma cells (Sherlock and Summerfield, 1988), vacuolated hepatocytes, dilated sinusoids, and increased number of Kupffer cells in histopathological study with H & E stains (Ebaid *et al*., 2007). Certain diseases of the liver cause obliteration of the normal sinusoidal arrangement and this then cause

impairment of the liver function (Young *et al*., 2006). Cirrhosis is the end stage of many types of liver damage. It is characterized by destruction of normal liver architecture, which is replaced by regenerative nodules of hepatocytes separated by bands of fibrous tissue (Burkitt *et al*., 1996). Progressive liver damage is also characterized by lobular hepatitis where infiltrations of the hepatic lobules occur by inflammatory cells in the surrounding apoptotic hepatocytes (spotty necrosis) (Burkitt *et al*., 1996).

In chronic hepatitis, the portal triads are infiltrated and usually expanded by lymphocytes. In some cases, the metabolic disruption may be irrecoverable and some hepatocytes undergo necrosis (Burkitt *et al*., 1996).

1.6. KIDNEYS

1.6.1. HISTOPHYSIOLOGY OF KIDNEY

Kidneys are paired reddish-brown, retroperitoneal, bean-shaped organs lying in the posterior wall of the abdominal cavity between the level of the twelfth thoracic and the third lumbar vertebrae, with an adrenal gland sitting on the top (superior pole) of each like a small cap (Landau, 1980). The left kidney lies slightly higher than the right kidney. A typical single adult kidney measures approximately 10cm in length, 5.5cm in width, 3cm in thickness, and 150g in weight (Martini, 1995). Kidney is medially concave and laterally convex (Carola *et al*., 1992). The kidneys are closely invested by a strong fibrous capsule, which gives the fresh kidney a glistening appearance. Each kidney has anterior and posterior surfaces, medial and lateral borders, and superior and inferior poles (Moore, 1992; Waugh and Grant, 1988). Coronal or frontal section of the kidney reveals three distinct regions called renal pelvis, medulla and cortex (Carola *et al*., 1992).

Uriniferous tubules are the functional units of the kidney. They are densely packed so that the connective tissue stroma of the kidney is scant. The entire uriniferous tubule is epithelial in nature and is, therefore, separated from the connective tissue stroma by an intervening basal lamina (Gartner and Hiatt, 2001). Uriniferous tubule is a highly convoluted structure that modifies the fluid passing through it to form urine as its final output. This tubule consists of two parts, each with a different embryological origin, the nephron and the collecting tubule. There are approximately 1.3 million nephrons in each

kidney. They are shorter cortical and longer juxtamedullary (Gartner and Hiatt, 2001). Each nephron consists of a dilated portion the renal corpuscle and the renal tubule i.e. proximal convoluted tubule, the thin and thick limbs of Henle's loop and the distal convoluted tubule (Junqueira and Carneiro, 2005). Several nephrons are drained by a single collecting tubule, and multiple collecting tubules join in the deeper aspect of the medulla to form larger and larger ducts (Gartner and Hiatt, 2001).

The renal corpuscle is composed of glomerulus and Bowman's capsule. The glomerulus is the dilated, pouch-like proximal end of the nephron, which invaginates into Bowman's capsule. The space inside Bowman's capsule is known as Bowman's space (urinary space). Bowman's capsule has parietal (external) and visceral (internal) layers. The parietal layer of Bowman's capsule consists of a simple squamous epithelium supported by a basal lamina and a thin layer of reticular fibers. At the urinary pole, the epithelium changes to simple cuboidal, that is, epithelium characteristic of convoluted tubule (Junqueira and Carneiro, 2005). The glomerulus initiates contact with the visceral layer of Bowman's capsule that can be modified to epithelial cells called podocytes. Podocytes are highly modified to perform a filtrating function (Gartner and Hiatt, 2001; Waugh and Grant, 1988).

The glomerulus is supplied by the short, straight afferent glomerular arteriole and drained by the efferent glomerular arteriole. The connective tissue component of the afferent arteriole does not enter Bowman's capsule, and special cell types known as mesangial cells, that are located at the vascular pole, replace the normal connective tissue cells. Pericyte-like intragranural mesangial cells are also situated within the renal corpuscle. Basal lamina, which invests the glomerulus, consists of three layers: the lamina rarae externa, lamina densa and lamina rarae interna (Gartner and Hiatt, 2001; Young *et al*., 2006).

Proximal tubule consists of much of the renal cortex and has two regions. Proximal convoluted tubule, which is located near renal corpuscles, and the pars recta (descending thick limb of Henle's loop), which descends in medullary rays within the cortex and then in the medulla to become continuous with the loop of Henle at the junction of the outer and inner stripes. The convoluted portion of proximal tubule is composed of a simple

cuboidal type of epithelium with an eosinophilic granular cytoplasm (Gartner and Hiatt, 2001).

Limbs of Henle's loop have three regions. The pars recta of the proximal tubule continue as the thin limbs of Henle's loop. It is composed of squamous epithelial cells. The nuclei of the cells composing the thin limbs bulge into the lumen of the tubule, hence, in paraffin section, these limbs resemble capillaries in cross-section. They may be distinguished from capillaries, in that their epithelial lining cells are slightly thicker, with less densely stained nuclei and their lamina contain no blood cells (Gartner and Hiatt, 2001).

The distal tubule has three regions the ascending thick limb of Henle's loop, the macula densa, and the pars convoluta. The ascending thick limb of Henle's loop is formed from cuboidal epithelial cells and Juxataglomerular apparatus. This apparatus has three components the macula densa of the distal tubule, juxstamacular cells of the afferent glomerular arteriole, and extraglomerular mesangial cells (Gartner and Hiatt, 2001; Young *et al*., 2006).

Collecting tubules are composed of a simple cuboidal epithelium that convey and modify the ultra filtrate from the nephron to the minor calyces of the kidney. The distal convoluted tubules of several nephrons join to form a short connecting tubule that leads into the collecting tubule. Collecting tubules have three recognized regions. These are cortical collecting tubules, medullary collecting tubules, and papillary collecting tubules (Gartner and Hiatt, 2001).

Renal interstitium is the space between uriniferous tubules, and blood and lymph vessels. It occupies a very small volume in the cortex but increases in medulla (Junqueira and Carneiro, 2005). The renal interstitium is also a very flimsy, scant amount of loose connective tissue housing three types of cells. These cells are fibroblasts, macrophages, and interstitial cells (Gartner and Hiatt, 2001).

Kidneys, to accomplish their functions, rely on three distinct processes such as filtration, reabsorption, and secretion (Martini, 1995). Everyday the kidneys filter about 1700 liters of blood. The kidneys consist of compound tubular glands that clear the blood plasma of

metabolic wastes. They regulate osmolality and volume, fluid and electrolyte balance, eliminate foreign chemicals, and help maintain the acid base balance of the body (Krause, 1996). Excretion of the metabolic products includes creatinine (from muscle creatine phosphate), uric acid (from nucleic acid), urea (from amino acids) and metabolites of hemoglobin are performed by the kidneys. The kidneys are instrumental in eliminating the by-products of the metabolism such as drugs, pesticides, and other environmental factors from the blood plasma (Effendy *et al*., 2006). In addition to the excretory functions, the kidneys have properties of endocrine organs and release two substances such as rennin and erythropoietin directly into the blood stream (Krause, 1996; Waugh and Grant, 1988). Nephrons filter water and soluble components from the blood and they selectively reabsorb some of the components back into the blood to maintain the normal blood concentration. In this way, useful substances are conserved and recycled. They also selectively secrete inorganic ions (Na^+, K^+, and H^+) in order to maintain stable concentrations of these ions in the extracellular fluid. The water and other substances not reabsorbed are the true wastes, which constitute the urine. Therefore, kidneys are the remarkable organs that maintain constant water balance (Carola *et al*., 1992).

1.6.2. HISTOPATHOLOGY OF KIDNEY

Renal disorders can be divided into total and partial disorders. Total disorder is chronic disorder or renal failure in which all functions of the nephron are impaired, whereas partial disorder is minor or precursor stages in which certain functions are disturbed (Burkitt *et al*., 1996). The structural units responsible for the excretion of nitrogenous by products of body metabolism and the maintenance of water and electrolyte homeostasis are the nephrons, which have two components the renal corpuscle and the renal tubules. The glomerulus, which is part of the renal corpuscle, consists of a highly specialized capillary network from which water, electrolyte ions and nitrogenous waste products are filtered into the lumen of the renal tubule (Burkitt *et al*., 1996). Glomerulus in response to damaging stimuli appears to react in one or more of the following ways. Swelling or proliferation of the normally fat endothelial cells lining the glomerular capillaries, proliferation of the epithelial cells investing the outer surface of the glomerular capillary tuft (the podocytes) and the cells lining Bowman’s capsule, thickening of glomerular

basement membrane, proliferation of the mesangial cells, and excessive production of acellular mesangial material (Burkitt *et al*., 1996). In chronic glomerulonephritis acellularity and complete hyalinised glomerular tufts, thickened blood vessels, fine interstitial fibrosis, inflammatory cells and a tubular atrophy can be seen (Mohan, 2007).

The tubular components of the nephron may be primarily damaged because of hypovolaemic shock, inorganic and organic toxins, or as a result of infection. In hypovolemic and intoxication states, tubular epithelial cells may exhibit marked cytoplasmic degenerative changes leading to acute tubular necrosis and producing the clinical syndrome of acute renal failure. Tubular epithelial cells have considerable power of recovery and regeneration (Burkitt *et al*., 1996). It is also indicated that the kidney sections may show some cellular inflammations, shrinkage of glomeruli, congestion of glomerular capillaries, and oedema and vacuolations of the tubular cells due to toxic agents (Aydin *et al*., 2003; Ebaid *et al*., 2007).

1.7. SIGNIFICANCE OF THE STUDY

Some herbs contain hazardous compound that might be harmful to the host system (Effendy *et al*., 2006). It is therefore, necessary to investigate the toxicity of local medicinal plants usually employed by herbalists in the treatment of diseases (Oyewole and Massaquoi, 2008).

To date, a study on the toxic effects of *V. bipontini* on some blood profiles and liver and kidney tissues due to oral administration of aqueous and methanolic leaf extracts of *V. bipontini* is not available. It could also provide important information regarding chronic effects of *V. bipontini* on some blood parameters and at cellular level on liver and kidney tissues of mice. To this effect, this study is imperative to evaluate the toxic effects of *V. bipontini* on some blood parameters and on liver and kidney tissues. The outcome of this study would also provide some additional evidence for further study on the medicinal plant.

2. OBJECTIVES

2.1. GENERAL OBJECTIVE

To investigate the toxic effects of *V. bipontini* on blood profiles, liver and kidney tissues in

mice.

2.2. SPECIFIC OBJECTIVES

- To observe signs of toxicity in experimental animals
- To determine the LD_{50}s of both aqueous and methanolic leaf extracts of *V. bipontini*
- To observe the effects of aqueous and methanolic leaf extracts of the plant on body weight
- To assess any hematological and biochemical changes on blood profiles at different doses of the leaf extracts

- To investigate the effects of the extracts on the weight of the liver and kidney
- To evaluate the effects of aqueous and methanolic leaf extracts of *V. bipontini* on histopathology of liver and kidney tissues

3. MATERIALS AND METHODS

3.1. PLANT MATERIAL COLLECTION AND EXTRACTION

The fresh leaves of *V. bipontini* plant (Fig.1) were collected based on Ethno-botanical description and with the help of local traditional healers around Shisha River in Harenna forest, Delomenna awraga, Bale region 524 kms southeast of Addis Ababa in December 2007. This plant grows with *celtis*, *croton*, and *syzygium* plants. The plant specimen of collected plant was identified and deposited at Herbarium of Department of Drug Research (DDR), Ethiopian Health and Nutrition Research Institute (EHNRI).

The method of Debella (2002) was used for extraction. Fresh leaves of *V. bipontini* were cleaned from extraneous materials, dried under shied at room temperature, and ground by

manual stick grinder to obtain fine particles. The powdered plant material was weighed (4.5kgs) using SCIENTECH Mode No SL 3100D Rev-c accuracy class (II).

3.1.1. AQUEOUS EXTRACT PREPARATION

Powdered materials (2.2 kgs) obtained from the *V. bipontini* were macerated with water for 24 hours with intermittent agitation by Orbital shaker DS-500. Then, the supernatant part of agitated materials was separated from the un-dissolved portion of the plant (marc). The supernatant portion filtered with $0.1mm^2$ mesh guaze and 15 cm Whatman grade1 filter paper. The filtrate of the *V. bipontini* was lyophilized at lower temperature (-46 - 51^oC) and lower pressure (133×10^{-3} mbr) to form crude extract. A yield of 25g of crude extract (8.93%) was obtained from 2.2kg dry powder. It was then kept in a desiccator at room temperature until used (Debella, 2002).

3.1.2. METHANOLIC EXTRACT PREPARATION

Powdered *V. bipontini* (1.8 kg) was macerated with 80 % methanol for 48 hours with intermittent agitation by Orbital shaker DS-500. The supernatant part of agitated material filtered with 15 cm Whatman grade1 filter paper two times. The filtrate of *V. bipontini* was then concentrated using Rotary evaporator (BÜCHI R-250) at 41^oc to remove 80% methanol and further dried using Water Bath (BÜCHI B-490) to remove 20% of water. A yield of 15.5g of crude extract (6.59%) was obtained from 1.8kgs of dry power and kept in refrigerator till used (Debella, 2002).

3.2. EXPERIMENTAL ANIMALS PREPARATION

The animals employed for this study were adult male and female Albino Swiss mice (25-35g). The mice were obtained from DDR, EHNRI and bred in the Research Animal Breeding Laboratory of DDR, EHNRI at Addis Ababa, Ethiopia. The mice were acclimatized to a laboratory condition for a week before the commencement of the experiment. Mice of the same sex were grouped into 8 experimental and two control groups for pilot experiment, 17 experimental and 2 control groups for LD_{50s} determination, and 40 experimental and 20 control groups for long term administration of

both aqueous and methanolic leaf extracts. Finally, all mice were housed in common metallic cage under 23 ± 2^0C. They had unrestricted access to a standard pellet diet and tap water. The animals were maintained under 12 hrs light-dark cycle throughout the duration of the study.

3.3. ADMINISTRATION OF THE EXTRACTS

Each group of mice was given different doses of aqueous and methanolic leaf extracts orally using intragastric catheter. These extracts were given once after they fasted for 18 hrs for LD_{50s} determination. However, in long-term toxicity study, mice were administered with different doses of both aqueous and methanolic leaf extracts for 45 days (WHO, 2000) after 7 days acclimatization. The aqueous and methanolic leaf extracts were dissolved in distilled water and 4% tween, respectively. A volume of 2.5ml for 5 mice (0.5ml per mouse) of the extracts were administered orally to experimental groups using a blunt intragastric catheter fitted to a 3ml syringe in 24 hrs intervals for 45 days. Some volume of distilled water and 4% tween were also given for control groups in 24 hrs intervals for 45 days. The blunt intragastric catheter was cleaned and placed in an oven after each administration to avoid any contamination.

3.3.1. PILOT EXPERIMENT

The pilot experiment was performed on 40 Swiss albino mice of both sexes using the method of Maxwell *et al*., (2007) to evaluate the dose ranges that would be lethal. The animals were divided into five groups (one control and four treated) for each extract. Each group consists of four animals. Each treated group received designated doses of

either aqueous or methanolic leaf extracts of *V. bipontini* as indicated in table 1 and 2 below.

Table 1: Doses and number of mice used for aqueous leaf extract of *V. bipontini* for pilot experiment

Group	Dose (mg/kg)	Number of mice used
Control	-	4
1	10	4
2	100	4
3	1000	4
4	10000	4

Table 2: Doses and number of mice used for methanolic leaf extract of *V. bipontini* for pilot experiment

Group	Dose (mg/kg)	Number of mice used
Control	-	4
1	10	4
2	100	4
3	1000	4
4	10000	4

3.3.2. LD_{50s} DETERMINATION

Lethal doses for fifty percent of the mice (LD_{50s}) for both aqueous and methanolic leaf extracts were determined using a total number of 190 Swiss albino mice that were divided into 19 groups of 10 mice (see table 3 and 4). After the pilot experiment, two months old mice ranging from 25 to 35g in weight were selected. Nine groups of mice were formed to administer the aqueous leaf extracts of *V. bipontini* at a dose interval of 250mg/kg. These doses were 1250mg/kg, 1500mg/kg, 1750mg/kg, 2000mg/kg, 2250mg/kg, 2500mg/kg, 2750mg/kg, 3000mg/kg, and 3250mg/kg/bw of mice. Eight groups of mice were also administered with methanolic leaf extracts of the same plant at doses of 1250mg/kg, 1500mg/kg, 1750mk/kg, 1875mg/kg, 2000mg/kg, 2250mg/kg, 2500mg/kg, and 2750mg/kg/bw of mice after 18 hours of fasting (Maxwell *et al*., 2007).

Two control groups were administered with distilled water and 4% tween for aqueous and methanolic leaf extracts, respectively. Eight experimental groups were also administered with particular doses of methanolic leaf extract dissolved in 4% tween. The methanolic leaf extract was dissolved in 4% tween for it was not fully miscible with distilled water. The number of death in each group within 24 hrs was recorded. Besides, delayed mortality up to 3 days was considered as lethal dose. This was done by observing the mice for toxicity signs (Nwinyi *et al*., 2004). The dose increment was limited to 3250mg/kg for aqueous leaf extract and 2750mg/kg for methanolic leaf extract because all mice died at these doses. The LD_{50s} of both aqueous and methanolic leaf extracts were estimated by using SPSS version 11 software and the probit method was applied to calculate the lethal doses of fifty percent of the population for each extract.

Table 3: Doses of aqueous leaf extract of *V. bipontini* and number of mice used for LD_{50} Determination

Group	Doses mg/kg	No. of mice used
Control	-	10
1	1250	10
2	1500	10
3	1750	10
4	2000	10
5	2250	10
6	2500	10
7	2750	10
8	3000	10
9	3250	10

Table 4: Doses of methanolic leaf extract of *V. bipontini* and number of mice used for

LD_{50} determination

Group	Doses mg/kg	No. of mice used
Control	-	10
1	1250	10
2	1500	10
3	1750	10
4	1875	10
5	2000	10
6	2250	10
7	2500	10
8	2750	10

3.3.3. LONG-TERM TOXICITY

The long-term toxicity study was carried out using 60 female and male albino mice (25-35g). Animals were kept in a temperature-controlled environment 23 ± 2^0C with 12 hrs light-dark cycle. Food and water were freely available for a week before the beginning of administration of both aqueous and methanolic leaf extracts of *V. bipontini*. Out of the 60 mice, 40 were randomly assigned to 4 experimental groups of 10 mice each and the male and female mice were placed in separate cages. The remaining 20 mice were also randomly assigned to 2 control groups of 10 mice each. Then, the animals were randomly assigned into two (1^{st} and 2^{nd}) control groups and four (3^{rd}, 4^{th}, 5^{th} and 6^{th}) experimental groups for aqueous and methanolic leaf extracts administration.

The 1^{st} control group for aqueous leaf extract received 0.5ml of distilled water and the 2^{nd} control group assigned for methanolic leaf extract received 0.5 ml of 4% tween. From the four experimental groups, two (3^{rd} and 4^{th}) groups were administered with aqueous leaf extract at doses of 400mg/kg and 800mg/kg, respectively. The remaining two (5^{th} and 6^{th}) groups were administered with methanolic leaf extract at doses of 400mg/kg and 800mg/kg, respectively. One male mouse died from the 6^{th} group one week before 45 days. Both aqueous and methanolic leaf extracts were administered in 24 hrs intervals for

45 days (Amole *et al*., 2006; WHO, 2000). Standard pellet (132g) was consumed within 24 hrs intervals by a cage of 5 mice. All groups were closely observed for any physical, food intake, behavioral alterations and signs of abnormalities throughout the study. Blood samples were collected through heart puncture of each mouse into different sample bottles for blood parameter analysis. Finally, tissues were taken from all groups of mice for histopathological evaluation.

3.4. BODY WEIGHT MEASUREMENT

Body weights of all groups of mice were taken before the commencement of the first oral administration using SCIENTECH Mode No SL 3100D Rev-c accuracy class (II). These were considered to be the initial body weight. The body weights of all groups were also taken on the last day of oral administration and these were considered to be the final body weight.

3.5. BLOOD COLLECTION

3.5.1. BLOOD COLLECTION FOR HEMATOLOGICAL INVESTIGATION

Blood collection was performed by placing each animal in airtight dissector jar with cotton soaked in diethyl ether. Blood was collected from each animal by cardiac puncture using sterile needle and 5ml syringe. The sample was put in an ethylene-diamine-tetra-acetic acid (EDTA) bottles to prevent adhesion proteins (coagulation factors) in cell-cell and cell-matrix interactions for hematological determinations (Gabriel *et al*., 2008) using automated hematological analyzer, SYSMEX KX-ZIN (Adebayo *et al*., 2005) at EHNRI, Addis Ababa, Ethiopia. Hematological parameters including total counts of RBC and WBC, hemoglobin (Hgb), hematocrit (Hct), mean cell volume (Mcv), mean corpouscular hemoglobin (Mch), mean corpuscular hemoglobin concentration (Mchc), lymphocytes (L), and platelet were measured (Selmanoglu *et al*., 2001; Tuffery, 1987).

3.5.2. BLOOD COLLECTION FOR BIOCHEMICAL ANALYSIS

Biochemical investigation was performed after blood sample was collected by using cardiac puncture and jugular veins with sterile needle and 5ml syringe. The sample was

kept at 4 °C for 4 hrs to let it clot. The clotted blood was centrifuged (using Humax 4k bench top Centrifuge with a capacity of 12x15ml; Germany, Max-Planck-ring 21D-65205 Wiesbaden) at 5000 RPM maximum speed for 10 minutes to obtain the serum. The serum samples were kept in -22^{o}C refrigerator until used for biochemical analysis (Mohan, 2007). Biochemical parameters including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and urea were measured (Ege *et al*., 2008; Selmanoglu *et al*., 2001).

3.6. ANIMAL DISSECTION, ORGANS WEIGHT MEASUREMENT AND TISSUE SAMPLING

Animals of each group were sacrificed at the end of 45 treatment days after body weight of mice were taken one by one on a digital electronic balance while under diethyl ether anesthesia. Animals lay up on a clean paper towel and had all four extremities pinned to thin corkboard. A vertical midline incision with scissors cut from the neck to pubis and opens the peritoneum. Then, 3-4mm wide strips of tissue samples were randomly taken from right lobe of liver and coronal section of right kidneys were cut lengthwise with a scalpel through the renal pelvis after each of these organs was weighed with 0.001 precision balance. These tissue samples were taken from each organ and transferred by a blunt forceps to a test tube containing 10% buffered formalin that completely immerses the tissues for the purpose of fixation (Lamberg and Rothstein, 1978).

3.7. TISSUE PROCESSING AND ROUTINE STAINING

A. FIXATION

Fixation is the primary step of tissue processing. This process prevents degeneration of tissue. Sample tissues were taken immediately after sacrifice from the right lobe of liver and coronal section of right kidney and immersed in 10% buffered neutral formalin over night at 22±2^{o}c after blood collection performed (Lamberg and Rothstein, 1978).

B. WASHING / HYDRATION

The formalin fixed tissues were washed in running tap water for 8 hrs to allow paraffin wax to infiltrate into the tissue easily (Singh, 2006).

C. DEHYDRATION

Following washing, tissues were dehydrated in a series of an increase graded ethanol i.e. in 70%, 80%, 95%, 100% I and 100% II (see appendix II) for 1 hour each (Mohan, 2007).

D. CLEARING / DEALCOHOLIZATION

In this step, two changes of xylene were used for one hour each to remove ethanol from the tissue and replace it with fluid that is miscible with paraffin (Singh, 2006).

E. INFILTRATION / IMPREGNATION

Tissues were infiltrated by two changes of paraffin wax (see appendix II) which had a melting point of 56^{o}C (52-64^{o}C) for 1½ hrs in each changes (Mohan, 2007).

F. EMBEDDING

The tissues were embedded in paraffin wax with the help of Electro-thermal Wax Dispenser to form tissue blocks in squared metallic plates block moulds. The blocks were then labeled, sealed in plastic bags with examining surface downward prior to sectioning, and placed in refrigerator until sectioned (Mohan, 2007). This process enables the specimens too small and/or delicate to be surrounded with some suitable materials that impart firmness without producing any injuries on the tissue (Singh, 2006).

G. SECTIONING

Rotary microtome was used for sectioning of tissue blocks manually at a thickness of 5 µm. The paraffin block having tissue was put in the rotary microtome. The ribbon of sections was carefully picked from the knife by a blunt forceps to float in a water-bath of 40^{o}C (slightly below the melting point of wax) to remove folds in the sections. Unfolded sections were picked by clean microscopic glass slides and were placed in an oven maintained at a temperature of 56^{o}C for 20-30 minutes for proper drying and better adhesion. At this stage, the sections are ready for staining (Mohan, 2007).

H. STAINING

Staining solutions were prepared using the formula given by Clopton (2006). The paraffin wax was removed from the tissue sections using xylene. The sections were then immersed in a series of descending alcohol concentration to remove xylene after which

distilled water was used to hydrate the tissue. The hydrated sections were immersed in hematoxylin for 3-5 min with an eosin counterstained and agitated with acid alcohol to prevent over staining. Sections were immersed in a mixture of sodium bicarbonate, ethanol, and distilled water (see appendix I) and tap water to give blue color to the nucleus. Finally, it was immersed in 95% alcohol and eosin to give pink color to the cytoplasm (Clopton, 2006; Mohan, 2007).

Finally, tissue sections were dehydrated in 95% alcohol, cleared in xylene, and mounted by adding a drop of DPX (Dibutyl phthalate in xylene) mounting medium on the section to cover the microscopic glass with cover glass and to increase the refractive index of the tissue under light microscope. This was done with care to prevent bubble formation between the tissue and the glass cover (Singh, 2006).

3.8. PHOTOMICROGRAPHY

Using light microscope, sections of the liver and kidney were examined at different magnification (x25, x40, and x100) objectives using MC 80 DX Microscope Camera (Carl Zeiss). The selected sections of liver and kidney tissues of the control and experimental groups were evaluated. Photomicrographs were taken and examined using Axiostar Binocular light microscope at magnification of x4216 (objective (x40) eye piece (x10) Axiostar Binocular light (x2.53) picture (15cm)/ Fuji film (3.6cm)) and x10541(objective (x100) eye piece (x10) Axiostar Binocular light (x2.53) picture (15cm)/ Fuji film (3.6cm))

3.9. STATISTICAL ANALYSIS

Data were digitally analyzed using the statistical software package SPSS version 11.0. All values were expressed in mean ± SEM. Treatment effects over time were compared between control and treated groups by analysis of covariance. The results were analyzed statistically using probit analysis of regression to determine LD_{50s} and analysis of variance one-way ANOVA to identify possible difference of body, liver, and kidney weights, and hematological and biochemical values. P values less than 0.05 were considered statistically significant. The correlation between body weight and organs

weight of liver and kidney was statistically analyzed by using scatter plots of Pearson correlation (Ege *et al*; 2008; Maxwell, 2007).

4. RESULTS

4.1. PILOT EXPERIMENT

A pilot experiment was conducted to determine the ranges of the median lethal doses of aqueous and methanolic leaf extracts of *V. bipontini*. Intragastric administration of both extracts of the plant at doses from 10 to 1000 mg/kg did not produce any death in male and female mice during 24 hrs experiment (Table 5 and 6). These results showed that at doses from 10 to 1000mg/kg, there are no signs of toxicity of *V. bipontini*. This indicated that the medium lethal doses (LD_{50s}) are higher than 1000mg/kg for both extracts. However, at 10,000mg/kg dose of both extracts, all animals died (Table 5 and 6). Therefore, the ranges of the LD_{50s} of both extracts were between 1,000 and 10,000mg/kg.

Table 5: Effects of aqueous leaf extract of *V. bipontini* at different doses on number of mice death for pilot experiment

Doses mg/kg/bw	Number of mice used	Number of mice died
0	4	-
10	4	-
100	4	-
1000	4	-
10000	4	4

Table 6: Effects of methanolic leaf extract of *V. bipontini* at different doses on number of mice death for pilot experiment

Doses mg/kg/bw	Number of mice used	Number of mice died
0	4	-
10	4	-
100	4	-
1000	4	-
10000	4	4

4.2. PHYSICAL SIGNS OF TOXICITY

Mice were observed for signs of abnormalities before and after sacrificed. Mice showed low locomotion, weakness, erection of hairs, and white color of the eyes in the course of acute study to determine LD_{50s}. During long-term administration of the extracts both treated and untreated groups showed no physical changes in their appearances and signs of toxicity at 400 and 800mg/kg bw of aqueous leaf extract of *V. bipontini* and at 400mg/kg methanolic leaf extract of the plant. However, almost all mice treated with 800mg/kg methanolic leaf extract of *V. bipontini* showed swellings on the left lateral part of abdominal region related to spleen, weakness, frequent defecation, mild diarrhea, and enlargement of spleen as compared to the control group.

4.3. LD_{50s} DETERMINATION

The acute toxicity study in mice showed LD_{50s} values of 2500.62±5.24 mg/kg and 2130.6±5.1 mg/kg body weight of mice for aqueous and methanolic leaf extracts, respectively (Fig 2 and 3). Moreover, this result showed that methanolic leaf extract of *V. bipontini* is more toxic than the aqueous leaf extract of the plant. The probit responses are indicated in vertical line and doses are indicated in horizontal line (Fig 2 and 3).

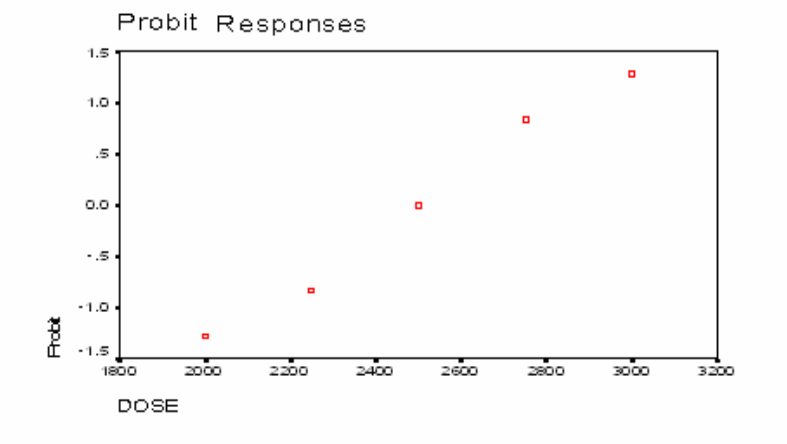


Figure 2: LD $_{50}$ curve for aqueous leaf extract of *V. bipontini* results (taken from SPSS)

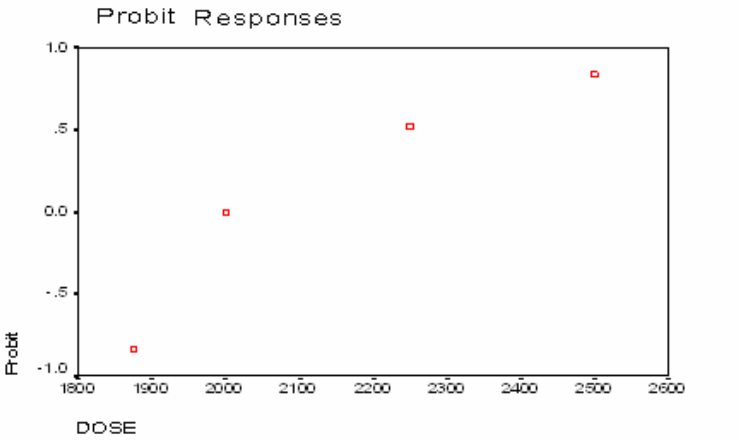


Figure 3: LD $_{50}$ curve for methanolic leaf extract of *V. bipontini* results (taken from SPSS)

4.4. EFFECTS OF AQUEOUS LEAF EXTRACT OF *V. bipontini* ON THE BODY WEIGHT OF MICE

The chronic effect of aqueous leaf extract of *V. bipontini* on the general body weight of the mice is illustrated in Table 7. The aqueous leaf extract of the plant has no significant effect on the mean values of the body weights of the mice at all doses. Even though it was not statistically significant, increased body weights in the mean values of the mice treated with 400 and 800mg/kg bw of the extract were observed (Table 7).

Table 7: Effect of aqueous leaf extract of *V. bipontini* on the body weight of mice treated at doses of 400 and 800mg/kg

Group	Treatment (mg/kg/bw)	Initial weight (in g)	Final weight (in g)	Weight change(in g)

Control	-	27.49±2.01	32.29±2.09	4.71±3.54
1	400	30.71±3.48	32.7±4.12	1.99±3.47 (0.069)
2	800	31.5±3.05	33.6±2.46	1.91±2.49 (0.062)

Values are mean ± SEM. P<0.05. The figures under brackets indicate P values.

4.5. EFFECTS OF METHANOLIC LEAF EXTRACT OF *V. bipontini* ON THE BODY WEIGHT OF MICE

The changes in the mean values of the initial and final body weights of the mice treated with 400 and 800mg/kg of the methanolic leaf extract of the plant is shown in Table 8. The result showed statistically insignificant in the body weights of mice treated at 400mg/kg bw (Table 8). However, mice treated at a dose of 800mg/kg of the plant extract showed significant decrease (P<0.05) in the final body weight (Table 8).

Table 8: Effects of methanolic leaf extract of *V. bipontini* on the body weight of mice treated at doses of 400 and 800mg/kg

Group	Treatment (mg/kg/bw)	Initial weight in g	Final weight in g	Weight change in g
Control	-	30.6±2.15	32.75±2.19	1.54±2.07
1	400	32.61±3.13	32.5±2.9	0.28±1.2(0.06)
2	800	30.77±2.6	28.33±2.36*	-1.98±0.46* (0.001)

Values are mean ± SEM. * The mean difference is significant at the P < 0.05 level. The figures under brackets indicate P values.

4.6. EFFECTS OF AQUEOUS LEAF EXTRACT OF *V. bipontini* ON HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS

The chronic effect of the aqueous leaf extract of *V. bipontini* on hematological and biochemical parameters of blood is illustrated in Table 9. There was no significant difference in hematological composition of blood parameters between control and mice treated at doses of 400 and 800mg/kg bw of the plant extract. However, a decrease in RBC (M/UL) from 8.03±0.62 to 7.66±0.49 and 7.63±0.43 for mice treated at doses of 400 and 800mg/kg bw respectively was observed. Total WBC (K/UL) also decreased insignificantly from 5.3±0.8 to 4.89±0.59 and 4.8±0.55 at doses of 400 and 800mg/kg bw of the extract, respectively. Moreover, the platelet count (K/UL) has also shown insignificant decrease from 1030.6±23.83 to 987.95±160 and 935.9±112.64 at doses of 400 and 800mg/kg bw of the extract, respectively. Similarly, Hgb concentration insignificantly decreased from 12.24±0.79 to 11.52±0.51 and 11.57±1.08 at doses of 400 and 800mg/kg bw of the extract, respectively. Hct percentage also showed non-significant decrease from 40.25±1.78 to 38.42±1.5 and 37.73±5.05, respectively at 400 and 800mg/kg bw of the extract. Slight non-significant decrease in MCV from 51.99±1.87 to 50.3±1.82 and 50.59±2.46 was also observed for mice treated at a dose of 400 and 800mg/kg bw of the extract, respectively. Lymphocyte count has shown insignificant decrease from 83.7±3.27 to 81.84±8.86 in mice treated at a dose of 400mg/kg bw of the extract. But lymphocyte percentage of mice treated at 800mg/kg dose increased from 83.7±3.27 to 88.1±5.37 ($P>0.05$). However, the mean values of MCH and MCHC remained relatively the same as compared to the control group (Table 9).

Similarly, there was no significant difference between the various biochemical parameters of blood in the three groups of mice at all doses (Table 9). As shown in the same Table, serum AST level of mice treated at a dose of 400mg/kg bw of the extract increased insignificantly (from 123.6±26.54 to 139±41.65) but AST level of mice treated at a dose of 800mg/kg bw of the extract showed non-significant decrease (from 123.6±26.54 to 127±38.3). In the same way, serum ALT level decreased from 49.6±5.62 to 48.9±6.48 and 45.8±4.54 in a dose dependent manner when treated with 400 and

800mg/kg bw of the extract, respectively (Table 9). Even though it was not statistically significant, an increase in serum ALP level from 46.7±47.67 to 63.0±85.99 and 72.2±138 was observed at doses of 400 and 800mg/kg bw of the extract. The increase in ALP level was relatively high (from 46.7±47.67 to 72.2±138) for those treated at a dose of 800mg/kg bw of the extract. However, ALT serum level insignificantly decreased from 49.6±5.62 to 48.9±6.48 and 45.8±4.54 at 400 and 800mg/kg of the plant extract, respectively as compared to the control. Similarly, at 800mg/kg AST serum level insignificantly declined from 132.6±26.54 to 127±38.3. Blood urea also insignificantly decreased from 55.9±7.01 to 54.9±9.89 and increased from 55.9±7.01 to 60±6.6 in mice treated with 400 and 800mg/kg bw of the extract, respectively (Table 9).

Table 9: Hematological and biochemical parameters between aqueous leaf extract of *V. bipontini* treated groups at doses of 400mg/kg, 800mg/kg, and control group

Hematological & Biochemical Parameters	Control with distilled water	Aqueous treated groups	
		400mg/kg	800mg/kg
RBC(M/UL)	8.03±0.62	7.66±0.49 (0.13)	7.63±0.43 (0.09)
WBC(K/UL)	5.3±0.8	4.89±0.59 (0.13)	4.8±0.55 (0.06)
Platelet(K/UL)	1030.6±23.83	987.95±160 (0.41)	935.9±112.64 (0.07)
Hgb (g/dl)	12.24±0.79	11.52±0.51 (0.06)	11.57±1.08 (0.08)
Hct (%)	40.25±1.78	38.42±1.5(0.21)	37.73±5.05(0.09)
Mcv(fl)	51.99±1.87	50.3±1.82(0.08)	50.59±2.46(0.14)
Mch (pg)	12.48±0.47	12.05±0.54(0.01)	12.05±0.69(0.1)
Mchc (g/dl)	25.35±1.03	24.7±0.54 (0.09)	24.85±0.85(0.19)
L (%)	83.7±3.27	81.84±8.86 (0.51)	88.1±5.37(0.13)
AST (IU/L)	132.6±26.54	139±41.65(0.8)	127±38.3 (0.7)
ALT(IU/L)	49.6±5.62	48.9±6.48(0.7)	45.8±4.54(0.1)
ALP(IU/L)	46.7±47.67	63.0±85.99(0.71)	72.2±138(0.56)
Urea(mg/dl)	55.9±7.01	54.9±9.89(0.8)	60±6.6(0.13)

Values are mean ± SEM. P<0.05. The figures under brackets indicate P values.

4.7. EFFECTS OF METHANOLIC LEAF EXTRACT OF *V. bipontini* ON

HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS

The chronic effects of methanolic leaf extract of *V. bipontini* on hematological and biochemical parameters of blood are illustrated in Table 10. As can be seen from the Table, there was no significant difference in hematological and biochemical composition of blood between control and mice treated at a dose of 400 mg/kg bw of the extract. However, significant changes were observed in mice treated with 800mg/kg bw of the plant extract as compared to the control group. RBC count (M/UL) significantly decreased ($P<0.05$) from 7.85 ± 0.73 to 2.91 ± 2.6 in mice treated with 800mg/kg bw of the extract. Platelet count (K/UL) also decreased considerably ($P<0.05$) from 1042.8 ± 57.3 to 516.24 ± 300 with 800mg/kg bw of the extract, while a dose of 400mg/kg bw showed no significant change in the platelet count as compared to the control group (Table 10). Moreover, both 400 and 800mg/kg bw of the extract considerably decreased ($P<0.05$) MCH and MCHC as compared to the control. Besides, 800mg/kg bw of the methanolic leaf extract caused a significant increase ($P<0.05$) in serum AST, ALT, and ALP levels, while 400mg/kg bw of the extract showed no change in AST, ALT and ALP levels when compared to the control group. Even though there was no statistically significant serum ALP, an increase in serum ALP level was observed at 400mg/kg methanolic leaf extract of *V. bipontini*. Moreover, blood urea concentration non-significantly increased in mice treated with 400 and 800mg/kg bw of methanolic leaf extract of *V. bipontini,* respectively. The results also revealed no change in the total WBC (K/UL) and lymphocyte percent at all doses ($P>0.05$) (Table 10). Changes in Hgb, Hct and Mcv were not significant in mice treated with 400mg/kg of methanolic leaf extract of *V. bipontini*. However, these hematological parameters significantly decreased ($P<0.05$) in mice treated with the extract at a dose of 800mg/kg bw as compared to the control (Table 10).

Table 10: Hematological and biochemical parameters between methanolic leaf extract of *V. bipontini* treated groups at doses of 400mg/kg, 800mg/kg, and control group

Hematological & Biochemical Parameters	Control with 4% tween in distilled water	Methanolic treated groups	
		400mg/kg	800mg/kg
RBC(M/UL)	7.85±0.73	6.44±1.16(0.07)	2.91±2.6* (0.001)
WBC(K/UL)	4.28±0.69	4.1±1.84(0.8)	3.36±1.82(0.21)
Platelet(K/UL)	1042.8±57.3	912.97±93.2(0.11)	516.24±300* (0.001)
Hgb (g/dl)	11.24±0.62	9.64±2.49(0.15)	7.58±3.4* (0.003)
Hct (%)	37.42±1.25	32.68±3.03(0.161)	24.56±12.78* (0.001)
Mcv(fl)	49.96±1.76	47.53±3.97(0.08)	46.8±2.83(0.031)
Mch (pg)	12.54±0.72	10.52±1.11* (0.001)	8.74±0.93* (0.001)
Mchc (g/dl)	23.78±1.16	22.22±1.72(0.027)	19.2±1.53* (0.001)
L (%)	82.04±3.77	78.24±11.1(0.50)	75.5±18.9(0.27)
AST (IU/L)	115±23.68	88.5±15.99 (0.42)	211.5±128* (0.008)
ALT(IU/L)	40±4.71	45.5±8.95 (0.47)	69.4±28.33* (0.001)
ALP(IU/L)	65.5±28.4	108±159.56(0.35)	161±55.4* (0.04)
Urea(mg/dl)	53.5±6.25	58.5±8.51(0.14)	59.1±7.13(0.10)

Values are mean ± SEM. *The mean difference is significant at the $P < 0.05$ level. The figures under brackets indicate P values.

4.8. EFFECTS OF AQUEOUS LEAF EXTRACT OF *V. bipontini* ON THE WEIGHTS OF LIVER AND KIDNEY OF MICE

The chronic effect of aqueous leaf extract of *V. bipontini* on the weights of liver and kidney of mice is shown in Table 11. There were no significant changes in the liver and kidney weights of the mice treated with 400 and 800mg/kg doses of the extract as compared to the control group. However, a slight non-significant decrease in the mean values of the weights of liver from 1.66±0.07 to 1.59±0.07 and kidney from 0.25±0.13 to 0.24±0.01 of the mice treated with 800mg/kg of the plant extract was observed when compared with the control group (Table 11).

Table 11: Effects of aqueous leaf extract of *V. bipontini* on the weights of liver and kidney of mice

Group	Treatment (mg/kg/bw)	Liver weight	Kidney weight
Control	-	1.66±0.07	0.25±0.13
1	400	1.62±0.07 (0.172)	0.24±0.08(0.23)
2	800	1.59±0.07(0.054)	0.24±0.01(0.08)

Values are given as mean ± SEM statistically significant at value P<0.05 level different from control by Post Hoc test.
The figures under brackets indicate P values.

4.9. EFFECTS OF METHANOLIC LEAF EXTRACT OF *V. bipontini* ON THE WEIGHTS OF LIVER AND KIDNEY OF MICE

The mean values of the weights of the liver and kidney of both control and experimental groups treated with methanolic leaf extract of the plant is indicated in Table 12. The result showed no significant change in the liver and kidney weights of those treated with 400mg/kg of the plant extract. However, mice treated at 800mg/kg dose showed significant decrease (P<0.05) in the liver and kidney weights as compared to the control group (Table 12).

Table 12: Effects of methanolic leaf extract of *V. bipontini* on the weights of liver and kidney of mice

Group	Treatment (mg/kg/bw)	Liver weight	Kidney weight
Control	-	1.71±0.06	0.24±0.01
1	400	1.67±0.07(0.21)	0.23±0.02(0.06)
2	800	1.33±0.1*(0.001)	0.13±0.03*(0.001)

Values are given as *mean ± SEM statistically significant at value P<0.05 level different from control by Post Hoc test.
The figures under brackets indicate P values.

4.10. CORRELATION BETWEEN BODY WEIGHT AND ORGANS WEIGHT OF LIVER AND KIDNEY AT 800mg/kg METHANOLIC LEAF EXTRACT OF *V. bipontini*

Final body weight and organ weight of liver and kidney of mice treated with methanolic leaf extract of *V. bipontini* at a dose of 800mg/kg bw showed significant ($P<0.05$) decrease in mean values when compared with the control groups (Table 8 and 12). Even though the decrease is significant, the decrease in body weight did not correlate ($P>0.05$) with the decrease in liver and kidney weights at 800mg/kg body weight methanolic leaf extract of the plant. Final body weight is indicated in vertical line, and liver and kidney weights are indicated in horizontal line in Figure 4 and 5, respectively.

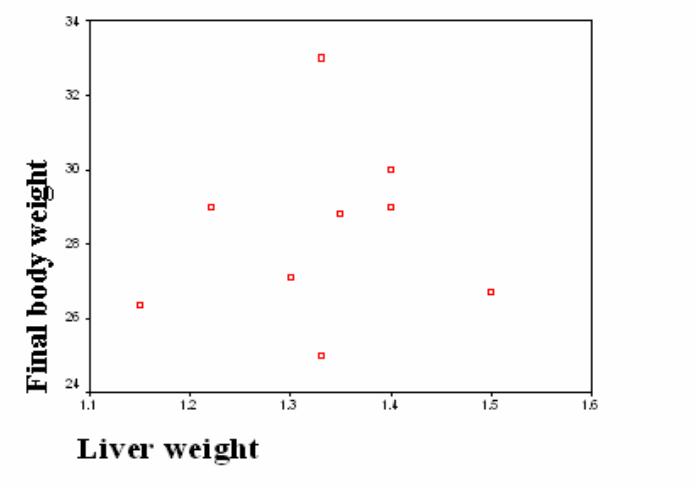


Figure 4: Pearson correlation between body weight and liver weight of mice treated with 800mg/kg bw of methanolic leaf extract of *V. bipontini* by Scatter plot

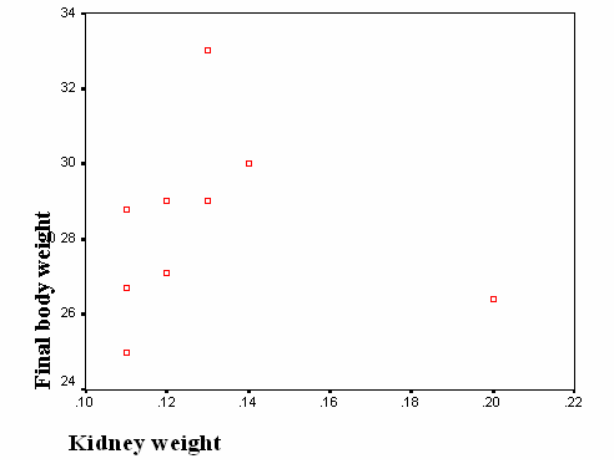


Figure 5: Pearson correlation between body weight and kidney weight of mice treated with 800mg/kg bw of methanolic leaf extract of *V. bipontini* by Scatter plot

4.11. MICROSCOPIC OBSERVATION

4.11.1. EFFECTS OF AQUEOUS LEAF EXTRACT OF *V. bipontini* ON HISTOPATHOLOGY OF LIVER

Microscopic examination of the liver sections of different groups of mice showed a normal architecture. Mice treated orally with the aqueous leaf extract of *V. bipontini* for 45 days (Fig. 6. B and C) showed no significant changes in their liver architecture at all doses as compared with the control (Fig. 6. A). The liver histology of both the control and mice treated with 400 and 800mg/kg of aqueous leaf extract showed normal features:

there is no significant difference in the shape of the central vein, size of hepatic sinusoids and hepatocytes (Fig. 6 A, B, and C) (H and E, x 4216).

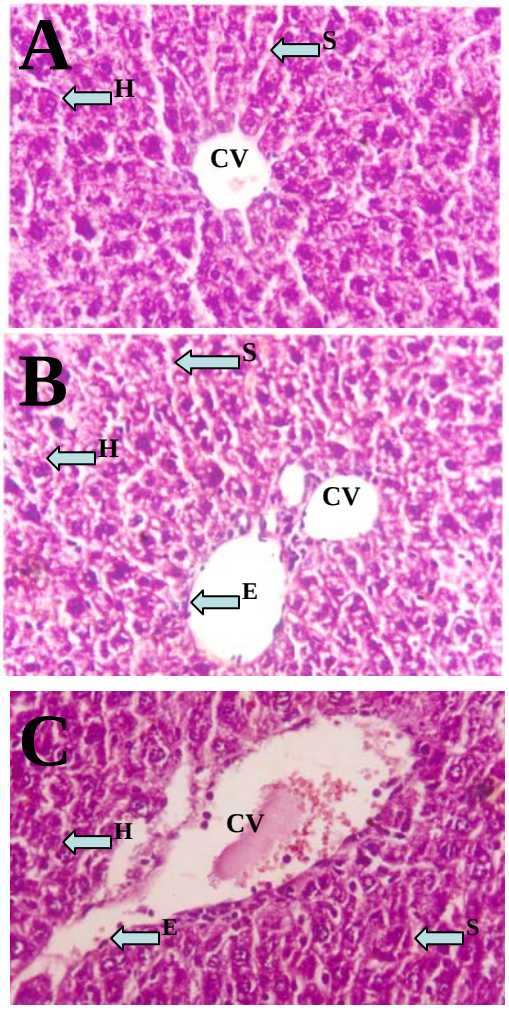


Figure 6: Photomicrographs of liver sections of the mice treated with 400mg/kg/bw (B) and 800mg/kg/bw (C) aqueous leaf

extract of *V. bipontini* showing no histopathological changes as compared to the control groups (A) (H&E, x 4216).

H = Hepatocyte S = Sinusoid CV = Central vein E = Endothelial cell

4.11.2. EFFECTS OF AQUEOUS LEAF EXTRACT OF *V. bipontini* ON HISTOPATHOLOGY OF KIDNEY

The histopathological effect of the aqueous leaf extract of *V. bipontini* was done on the kidney section that was stained with hematoxylin and eosin. Microscopic observation indicated that there was no difference observed between the kidney sections of the

control (Fig. 7. A) and mice treated with doses of 400mg/kg and 800mg/kg aqueous leaf extract of the plant (Fig.7. B and C). Renal histology of both treated and untreated groups exhibited normal features: no tubular necrosis was seen, size of Bowman's space and convoluted tubules and glomeruli were normal and clearly visible as compared to the control (Fig. 7. A, x4216).

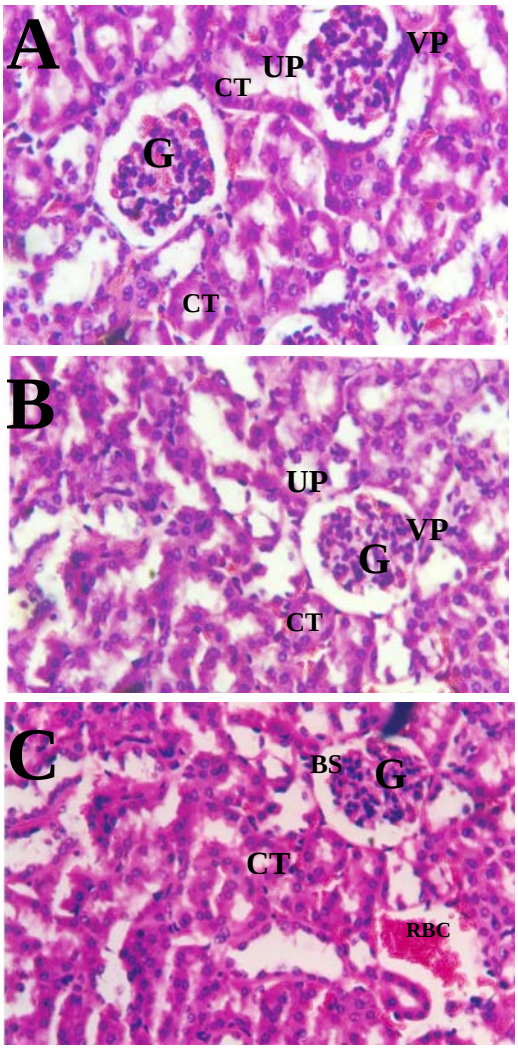
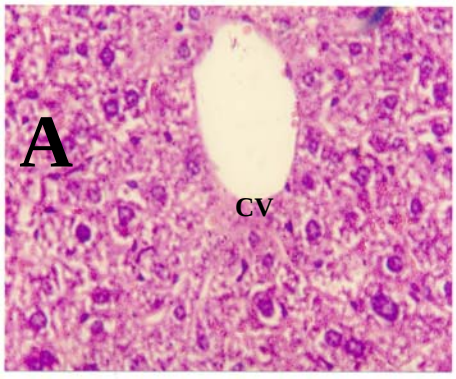


Figure 7: Photomicrographs of kidney sections of control mouse (Fig.7. A) showing no histopathological change as compared to the mice treated with 400 (B) and 800mg/kg (C) aqueous leaf extract of *V. bipontini* (H&E, x4216). RBC = Red blood cells G = Glomerulus UP =Urinary pole CT = Convoluted tubule VP =Vascular pole BS = Bowman's space

4.11.3. EFFECTS OF METHANOLIC LEAF EXTRACT OF *V. bipontini* ON HISTOPATHOLOGY OF LIVER

The histopathological effect of methanolic leaf extract of *V. bipontini* on liver tissue (Fig. 8 C and D) of mice treated at 800mg/kg of the extract as compared to the control (Fig. 8 A) showed some histopathological abnormalities such as dilated sinusoid, nuclear enlargement, bi-nucleation of hepatocytes, peripheral cramped chromatin, shrinkage of hepatocytes (single cell death), fragmentation of hepatocytes (apoptosis) (Fig. 8. D, H&E, x10541). These findings are; however, not seen in the control (Fig. 8 A) and in mice treated with 400mg/kg/bw of methanolic leaf extract of the plant (Fig. 8. B, H&E, x4216).



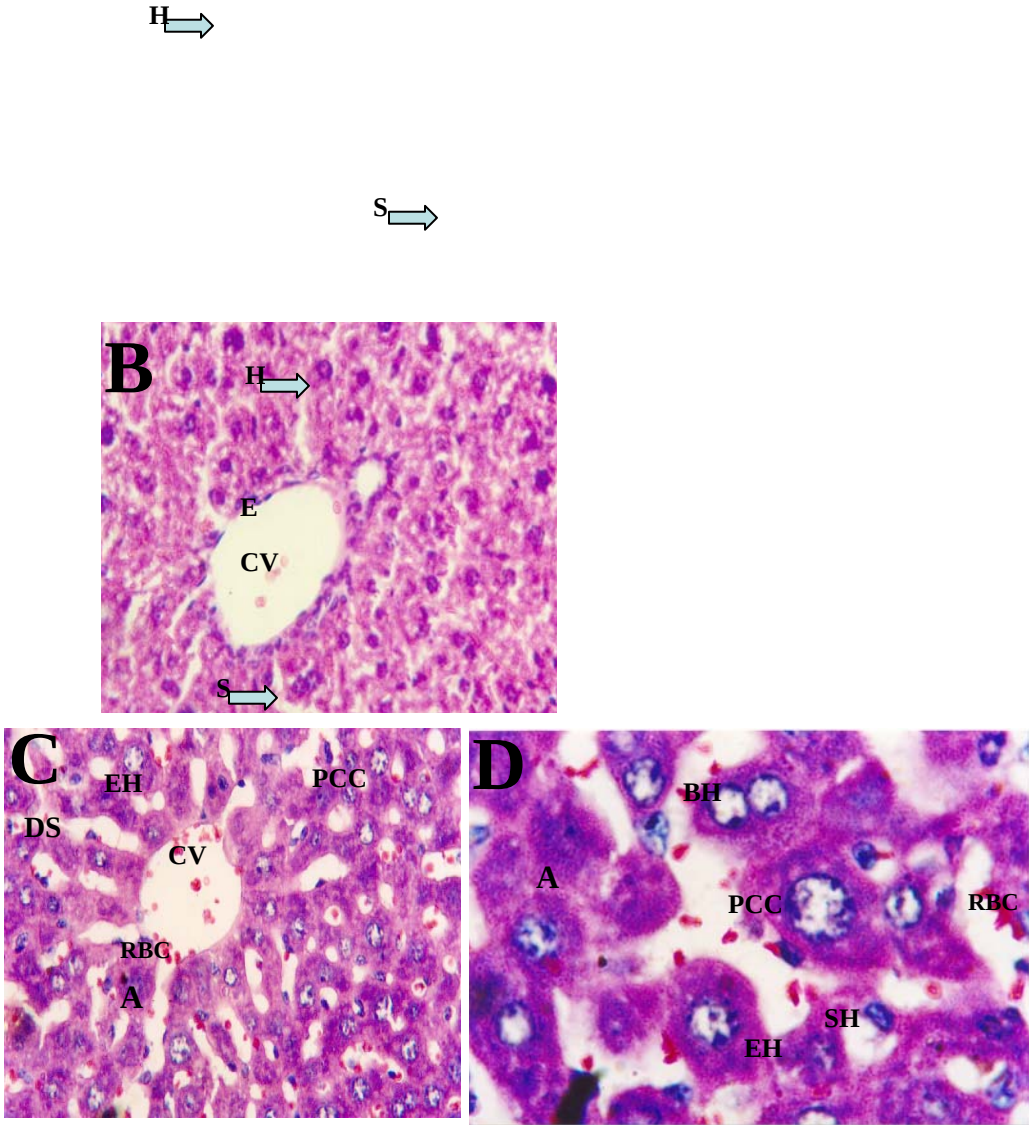


Figure 8: Photomicrographs of liver sections A = control mice showing no histopathological change, (H & E, x4216).

B = the mice treated with 400mg/kg/bw methanolic leaf extract of *V. bipontini*, showing no histopathological changes. C and D = the mice treated with 800mg/kg/bw of methanolic leaf extract of the plant at (x4216, C) and (x10541, D) magnification showing dilated sinusoid, nuclear enlargement, bi-nucleation of hepatocytes, peripheral cramped chromatin, shrinkage of hepatocytes (single cell death), fragmentation of hepatocytes (apoptosis).

H = Hepatocyte S = Sinusoid DS = Dilated sinusoid E = Endothelial cells SH = Shrinkage of hepatocyte

EH = Enlargement of hepatocyte CV = Central vein FH = Fragmented hepatocyte (A = Apoptosis)

BH = Bi-nucleated hepatocyte PCC = Peripheral cramped chromatin RBC = Red blood cells

4.11.4. EFFECTS OF METHANOLIC LEAF EXTRACT OF *V. bipontini* ON HISTOPATHOLOGY OF KIDNEY

Light microscopic observation showed that there was no significant difference between the kidney sections of the control (Fig. 9. A) and mice treated with methanolic leaf extract of *V. bipontini* at doses of 400mg/kg (Fig. 9 B). In the control and mice treated with 400mg/kg urinary pole, vascular pole, glomerulus, convoluted tubules were normal and clearly visible (Fig. 9. A and B, H&E, x4216). The common toxicity structural changes such as tubular necrosis, inflammation, fatty changes, and WBC infiltration were not observed. Tissue section of mice treated with the methanolic leaf extract of *V. bipontini* at a dose of 800mg/kg/bw showed narrowing of Bowman's space and increase in cellularity of glomerulus (Fig. 9. C). However, convoluted tubules and Bowman's space were normal (Fig. 9 C) as compared to the control (Fig. 9 A) and to mice treated with 400mg/kg methanolic leaf extract of the plant (Fig. 9. B, H&E, x4216).

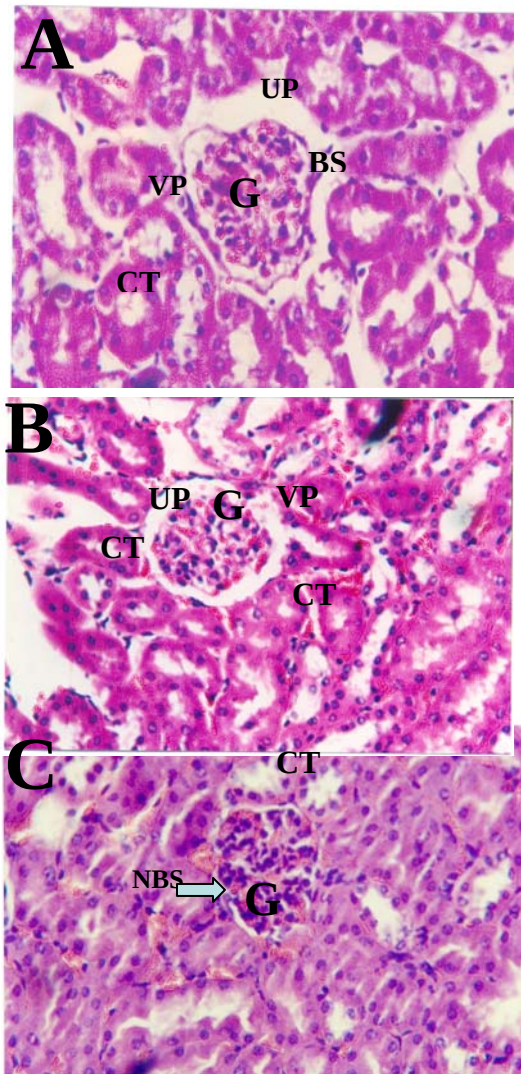


Figure 9: Photomicrographs of kidney of control mouse (Fig. 9. A) and of mice treated with 400mg/kg bw of methanolic leaf

extract of *V. bipontini* showing no histopathological change (B) (H&E, x4216). Mice treated with 800mg/kg showed some morphological changes, cellularity of glomerulus and narrowing of Bowman's space (Fig. 9. C, H&E, x4216).

G = Glomerulus UP = Urinary pole VP = Vascular pole

NBS = Narrow Bowman's space BS = Bowman's space CT = Convoluted tubule

5. DISCUSSION

The widely used medicinal plants have formed the basis of health care throughout the world since the earlier days of humanity and have considerable importance (Ebong *et al*., 2008). The majority of Ethiopians depend on medicinal plants as their source of health care especially in rural areas. Thus, knowledge of uses and side effects of medicinal plants provide a vital contribution to human health care. *Vernonia* species are the sources of many local medicines (Amole *et al*., 2006). People living in areas, where *V. bipontini* grows use the plant for treating malaria and malaria related symptoms. According to Assefa *et al* ., (2007) leaf extract of *V. bipontini* in vivo anti-malarial activity, in 4-day, suppressive assays against *Plasmodium berghei* in mice reduced parasitemia by more than 50% when tested at an oral dose of 400mg/kg/day indicating that the median effective dose (ED_{50}) is 400mg/kg/day. The inhibitions of this plant in both aqueous and methanolic leaf extracts were 52.7% and 40%, respectively (Assefa *et al*., 2007).

It is for this reason that this study is primarily designed to determine $LD_{50s,}$ and long-term effects of both aqueous and methanolic leaf extracts of *V. bipontini* at doses of 400mg/kg and 800mg/kg that might probably have effects on hematological and biochemical parameters and on liver and kidney tissues.

Physical signs of toxicity showed that frequent defecation, mild diarrhea, weakness, and enlargement of spleen were observed at a dose of 800mg/kg methanolic leaf extract of the plant during long-term experiment. The mild diarrhea might be due to the presence of bioactive chemical (saponins) in methanolic leaf extract (Diwan *et al*., 2000). The

presence of saponins may create some health hazard (Igile *et al*., 1995). The observed splenomegaly may also be induced by the active ingredients (Dichloromethane/DCM) found in the plant extract (Pagno *et al*., 2006).

The acute toxicity study in LD_{50s} determination showed that methanolic leaf extract of *V. bipontini* is more toxic than the aqueous leaf extract of the plant. This might be due to active ingredients responsible for toxic effects, which were more abundant in methanolic extract of the plant leaves than in aqueous leaf extract.

In the present study, aqueous leaf extract of *V. bipontini* did not produce any significant increase in the mean values of body weights of mice treated at all doses. Similar results were previously reported in a study of a related plant extracts (*V. amygdalina*) by Amole *et al*., (2006). The methanolic leaf extract of *V. bipontini*, however; decreased body weight in mice treated at 800mg/kg. The decreased in body weight of the treated animals with methanolic leaf extract of *V. bipontini* at 800mg/kg might be due to the fact that *V. bipontini* leaf extract contains some anti-nutritional factors, which reduce body weight. This is in agreement with the previous studies done on related species (*V. amygdalina*) by Igile *et al*., (1995); Siddhuraju *et al*., (2001), and Eleyinmi *et al*., (2006) who reported the presence of anti-nutritional factors especially, phytic acid, tannin, and oxalate, which inhibit the activities of digestive enzymes. Phytic acid, tannin, and oxalate which form complexes with metals (Ca^{++}, Zn, Mg and Fe) and proteins, reduce mineral and protein bioavailability (Eleyinmi *et al*., 2006; Eleyinmi *et al*., 2008). In turn, this may lead to low growth, notably reduced mean body weight. Since most phenolics and tannins are water soluble, they can be significantly reduced during soaking the plant material in water for aqueous extraction (Salawu, 2007). This may be the reason why only methanolic leaf extract induced decrease in the body weight of mice at 800mg/kg bw while the equivalent dose of the aqueous leaf extract did not. Kumar *et al*., (2005) also reported that weight of mice treated with methanolic leaf extract of *Caesalpinia bonducella* and *Bauhinia racemosa* decreased significantly as a reflection of low growth.

In this study, treatment of mice at 400 and 800mg/kg bw of aqueous leaf extract of *V. bipontini* did not alter hematological and biochemical composition of blood. Our findings revealed that hematological and biochemical parameters remained within normal limits

after chronic treatment with all doses of aqueous leaf extract of *V. bipontini*. These findings are supported by previous reports that described the absence of any significant effect on the hematological (Amole *et al*., 2006, Eleyinmi *et al*., 2006) and biochemical (Ojiako and Nwanjo, 2006) parameters of blood after chronic administration of related species (*V. amygdalina*) in mice.

In the present study, mice treated with methanolic leaf extract of *V. bipontini* at a dose of 400mg/kg bw showed no changes in their hematological and biochemical parameters. However, significant changes in the blood parameters were observed in mice treated at a higher dose of the methanolic leaf extract of the plant. Higher dose of the extract decreased RBC, Hgb, platelets count, Mch and Mchc and increased serum AST, ALT and ALP levels in treated animals. Other investigators following chronic treatment with different agents in plant extracts also noted reductions in hematological profiles of blood. Thus, the findings of the present study is consistent with the previous reports (Chodhani and Deshmukh, 2007; Kola, 2007) which suggested that methanolic leaf extract of related species of *V. bipontini (V. amygdalina)* possesses the potential of adversely affecting hematological indices. According to Choudhari and Deshmukh, (2007) the decreased number of RBC count and Hgb content may be due to defective haematopoiesis, inhibited erythropoiesis or an increase in destruction of red blood cells (Choudhari and Deshmukh, 2007; Selmanoglu *et al*., 2001). Methanolic leaf extract of *V. bipontini* may induce inhibition of RBC formation, which reduced hemoglobin content. The fall in hemoglobin content and RBC count can be correlated with induction of anemia in mice treated with methanolic leaf extract of the plant at the higher dose (Choudhari and Deshmukh, 2007). The reduced number of platelets (thrombocytopenia) and RBC in the circulating blood might be due to the observed enlarged spleen which could probably trap and store them excessively (Faeh *et al*., 2001; Shebl *et al*., 1999) and the platelets deficiency might also induce hemorrhage (Faeh *et al*., 2001). Low hematological values obtained in mice treated with 800mg/kg of methanolic leaf extract of *V. bipontini* might also be due to the characteristics of bioactive components present in *V. bipontini* as if sesquiterpene lactones and vernonisides may have been responsible (Eleyinmi *et al*., 2006). Moreover, the results of the current study showed chronic treatment of methanolic leaf extract of *V. bipontini* induced increase in biochemical parameters (AST, ALT and ALP) at a dose of

800mg/kg. These results are in agreement with the previous report by James *et al*., (2007). Saponin, flavonoids and tannin might elicit adverse biochemical actions when ingested by animals (Ojiako and Igwe, 2008). According to James *et al*., (2007), increase in chemistry of serum ALP, a membrane-bound enzyme, is due to release of the enzyme following a pathological phenomenon. Similarly, ALT, a cytoplasmic enzyme, found in hepatocytes normally at very low concentration, is also released into the plasma following hepatotoxic damage (Haldar *et al*., 2007; Gaskill *et al*., 2005) and apoptosis (Wu *et al*., 2007).

There was no change in the liver and kidney weights of mice treated with aqueous leaf extract of *V. bipontini* at all doses. These results are in line with the findings of Amole *et al*., (2006); Igile *et al*., 1995), and Eleyinmi *et al*., (2006) who reported no significant differences in weights of the organs.

Similarly, no changes in the actual organ weights of liver and kidney were seen in all animals treated with methanolic leaf extract of *V. bipontini* at a dose of 400mg/kg bw. However, the organ weights of the animals treated with the plant extract at a dose of 800mg/kg bw significantly decreased. The decrease in the weight of these organs might be due to the anti-nutritional bioactive components (phytic acid and tannin) probably present in the plant extract. The reduced organ weights of liver and kidney did not correlate with loss of body weight at 800mg/kg bw of methanolic leaf extract of the plant. The decreased in body weight might be due to the reduced weight of other organs.

In the present study, treatment with aqueous leaf extract of the plant did not cause any pathologic lesions in the liver and kidneys even at 800mg/kg. It might be due to the absence of cyanogenic glycoside in the plant extract that is responsible for histopathological changes (Nwanjo, 2005). This observation is in agreement with those of Amole *et al*., (2006) who described that microscopic observation of the tissue sections of liver and kidney showed no morphological abnormalities as compared to the controls after chronic oral administration of aqueous leaf extract of the related species (*V. amygdalina*). However, histological examination of the liver and kidney of mice treated with methanolic leaf extract at a higher dose revealed some histopathological changes. The changes in the liver were characterized by dilated sinusoids, nuclear enlargement, bi-

nucleation of hepatocytes, and peripheral cramped chromatin. The methanolic leaf extract also induced apoptosis in hepatocytes as demonstrated by fragmentation of hepatocytes, cell shrinkage and destruction of the cytoskeleton. Other investigators following treatment with different agents (Ebaid *et al*., 2007; Patel, 2000; Steenkamp *et al*., 2001; Taraphdar *et al*., 2001; Wu *et al*., 2007; Zuckerman *et al*., 2002) noted similar histopathological changes in liver. The histopathological lesions of organs might be due to the presence of bioactive compounds (alkaloids, tannins, saponins, flavonoids, oxalate, and glycosides) (Eleyinmi *et al*., 2006; Nerurkar *et al*., 2004; Nwanjo, 2005) which are not dissolved in methanol during extraction of the plant. The presence of oxalate in the food is also associated with acidity and toxicity (Eleyinmi *et al*., 2006). Retention of water inside hepatocyte resulting in cell enlargement (swelling) may be due to reduction of energy necessary for ion regulation in the cells (Ebaid *et al*., 2007; Effendy *et al*., 2006). The observed apoptosis is may be an important pathophysiologic mechanism for the maintenance of liver tissue, allowing hepatocytes to die without provoking a potential harmful inflammatory response through a tightly controlled and regulated process (Pagliara *et al*., 2003; Patel, 2000). Previous studies have also reported that phenolic compounds (e.g. flavonoids), nitrogen compounds (e.g. alkaloids), saponins, and tannin present in the plant extracts have antiradical activities (Rodríguez *et al*., 2008). Free radicals setup a chain reaction that can cause biological damage by stimulating glycation of protein, inactivation of enzymes and alteration in the structure and function of collagen basement and other membranes and alcoholic leaf extracts were more effective stable free radical scavengers than aqueous leaf extracts (Rodríguez *et al*., 2008). This may lead to the observed apoptotic hepatocytes.

The histopathological changes observed in the kidney sections showed increase in cellularity of glomerulus and urinary space obliteration. This finding may agree with the reports of Ebaid *et al*., (2007) that showed similar alterations in the structure of glomerulus as a result of the treatment different toxic substances (piroxicam). However, necrosis, tubular degradation, fatty changes as well as inflammatory cellular infiltration, which are signs of renal toxicity, were not observed. This shows the plant extract has no marked effect on kidney of mice at higher dose.

6. CONCLUSION

From the above investigation, it can be concluded that aqueous leaf extract of *V. bipontini* is safe at any of the doses used in the study. However, the methanolic leaf extract of the plant at higher dose (800mg/kg) might induce anemia and some histopathological alterations in liver and kidney.

7. RECOMMENDATIONS

- Investigation on the histopathology of other organs especially, on spleen, is recommended because enlargement of spleen was seen during physical observation of dissected mice treated with methanolic leaf extract of *V. bipontini* at higher dose (800mg/kg).
- Further studies are recommended on the mechanism of action of the extract for the toxic effects.
- Further work is needed to find out the active ingredients responsible for such toxicities.
- Further study is also recommended to examine the toxic effects of the plant on blood parameters and histopathology of different organs in prenatal mice.

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APPENDICES

APPENDIX I

CHEMICAL PREPARATION

10% Neutral Buffered Formalin

10% Formalin (90% distilled H2O and 37% Formalin)	1000ml
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$)	4g
Sodium monohydrogen phosphate anhydrous (Na_2HPO_4)	6.5g

Harris' Hematoxylin

Hematoxylin Crystal	2.5g

Ethanol,100%	25ml
Ammonium or Potassium Alum	50g
Distilled water	500ml
Mercuric oxide (red)	1.25g

Eosin

Eosin Y (Yellow) CI 45380	0.5g
Ethanol, 95%	100ml
Glaceial acetic acid	0.5ml

1% Acid Alcohol

Ethanol, 70%	500ml
HCl, concentrated	5ml

Bluing Solution

Sodium bicarbonate	2.5g
Ethanol	1000ml
Distilled water	500ml

APPENDIX II

TISSUE PROCESSING SCHEDULES TO FORM PARAFFIN BLOCK FOR MANUAL TECHNIQUE

Fixation

Buffered formalin, 10%	24 hrs

Washing

Running tape water	24 hrs

Dehydration

Alcohol, 70%	1 hr
Alcohol, 80%	1 hr

Alcohol, 95%	1 hr
Absolute alcohol I	1 hr
Absolute alcohol II	1 hr
Clearing	
Xylene I	1 hr
Xylene II	1 hr
Infiltration (in paraffin oven)	
Paraffin wax I 56 ^{o}C (52-64^{o}c)	1½ hrs
Paraffin wax II 56 ^{o}C (52-64^{o}c)	1½ hrs

APPENDIX III

HEMATOXYLIN AND EOSIN (H & E) STAINING SCHEDULES FOR PARAFFIN SECTIONS

Chemicals	**Duration of Staining**
Xylene I	5min
Xylene II	2min
Absolute alcohol I	2min
Absolute alcohol II	2min
Alcohol, 95%	2min

Alcohol, 70%	3min
Alcohol, 50%	2min
Distilled water	2min
Hematoxylin	3-5min
Acid alcohol	agitate
Bluing solution	3min
Tap water	2min
Alcohol, 95%	2min
Eosin	14 seconds
Alcohol, 95%	agitate
Xylene I	agitate
Xylene II	agitate

DECLARATION

I, the undersigned, declare that this thesis is my original work, has not been presented for a degree in this and any other universities and that all sources of materials used for this thesis have been duly acknowledged.

M. Sc candidate **Mebratu Alebachew**

Signature _____

Date of submission _____

Addis Ababa, Ethiopia

Advisors: **Dr. Yamrot Kinfu**

Signature _____

Date and place of submission _____
Addis Ababa, Ethiopia

Prof. Eyasu Makonnen

Signature _____

Date and place of submission _____
Addis Ababa, Ethiopia

Dr. Yonas Bekuretsion

Signature _____

Date and place of submission _____
Addis Ababa, Ethiopia

Ato Kelbesa Urga

Signature _____

Date and place of submission _____
Addis Ababa, Ethiopia