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Phytochemical Screening, Acute Toxicity and Anti-Rabies Activities of Extracts of Selected Ethiopian Traditional Medicinal Plants

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University, College of Natural and Computational Sciences, Department of Microbial, Cellular and Molecular Biology in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (Applied Microbiology)

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Declaration

I hereby declare that this MSc thesis entitled “Phytochemical Screening, Acute Toxicity and Anti-Rabies Activities of Extracts of Selected Ethiopian Traditional Medicinal Plants” is my original work and has not been presented for a degree in any other University and all source of materials used for the thesis have been duly acknowledged.

Yeweynshet Tesera Tewabe _____ _____

Name of the Designate Signature Date

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List of acronyms/abbreviations

AJADD	American Journal of Advanced Drug Delivery
ARV	Anti-Rabies Vaccine
ATRM	African Traditional Medicines
CC50	Cytotoxicity Concentration 50%
CCEEVs	Cell Culture and Embryonated Egg-Based RabiesVaccines
CDC	Centers for Disease Control and Prevention
CNS	Central Nervous System
DALYs	Disability-Adjusted Life Years
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DRIT	Direct Rapid Immunohistochemical Test
ELISA	Enzyme Linked Immune Sorbent Assay
EPHI	Ethiopian Public Health Institute
FAT	Fluorescent Antibody Test
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
IACUC	Institutional Animal Care and Use Committee
IC_{50}	Inhibitory Concentration 50%
I/C	Intra cranial
ITM	Improved Traditional Medicine
LD_{50}	Medium Lethal Dose
MEM	Minimum Essential Medium
MOI	Multiplicity of Infection

MST	Mean Survival Time
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide
NTV	Nerve Tissue Vaccine
OECD	Organization of Economic Corporation and Development
OIE	World Organization for Animal Health
PBS	Phosphate Buffer Saline
PEP	Post Exposure Prophylaxis
PPE	Personal Protective Equipment
PrEP	Pre Exposure Prophylaxis
PV	Pasteur Virus
RABV or RV	Rabies Virus
RIG	Rabies Immunoglobulin
RNP	Ribo Nucleo Protein
Rpm	Revolution Per Minute
RTCT	Rapid Tissue Culture Infection Test
$TCID_{50}$	Median Tissue Culture Infectious Dose
THP	Traditional Health Practitioners
TMPs	Traditional Medicinal Plants
UV	Ultraviolet
VNA	Virus-Neutralizing Antibodies
WHO	World Health Organization

Abstract

Despite the existence of safe and effective vaccines, rabies disease still causes an estimated 59,000 human deaths a year in the endemic areas in Asia and Africa. In most developing countries people believe to cure rabies with different traditional and religious treatment rather than seeking effective post exposure prophylaxis. The objective of this study was, therefore, to investigate the phytochemical constituents, acute toxicity and antirabies activity of crude extracts of the leaves of *Justicia schimperiana* and *Ricinus communis* and the stem bark of *Croton macrostachyus*. Extraction was done by maceration technique. Standard procedures were used to test the presence of various phytochemicals. For the determination of acute toxicity and antirabies activities, Organization for Economic Corporation and Development (OECD) Guideline No.423 was used. Different concentrations of extracts (0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 mg/ml) were tested for their cytotoxic effect on vero cells through 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay. The mean cytotoxic concentration (CC_{50}) was estimated and the antirabies assay was carried out using the minimal cytotoxic concentration of extracts. The phytochemical screening result has revealed the presence of alkaloids, flavonoids, phenols, steroids, tannins and terpenoids in all plant extracts screened. The toxicity evaluation of the extracts revealed that they are slightly toxic. The antirabies assay result showed that all plant extracts had a moderate to good antirabies potential. The 80 % methanol extracts exhibited higher antirabies activity compared to the other extracts under investigation. The present study concluded that these medicinal plants have possessed different phytochemicals that helps in the anti-rabies properties of the studied plants commonly used in Ethiopia. Further study on the mechanism of those phytochemicals must be elucidated for the potential as antirabies agent. Evaluation of those medicinal plants for their long-term toxicity would also be very important.

Keywords/phrases: Anti-rabies**,** Cytotoxicity, Pathogenicity, Phytochemicals, Traditional medicinal plants

1. Introduction

Rabies is a fatal viral zoonosis, which causes encephalitis in warm-blooded animals and humans (Zulu *et al*., 2009). It is caused by rabies virus, a neurotrophic virus which kills or causes physiological disorders by infecting the neurons at the central nervous system (Vural *et al*., 2016). All mammals are susceptible to rabies, but only a limited number of species also act as reservoir hosts. They include members of the families Canidae (dogs, jackals, coyotes, wolves, foxes and raccoons), Mustelidae (e.g., skunks), Viverridae (mongooses), and the order Chiroptera (bats) (Balcha Chernet and Nejash Abdela, 2016). Having a large population of stray dogs in a community is considered to be a risk factor for spreading of zoonotic diseases such as rabies (Abraham *et al*., 2017). In developing countries, the majority of confirmed and reported cases and over 90% of human exposures are from domestic dogs (Kaare *et al*., 2009). It is prevalent throughout the world and endemic in many countries except in Islands like Australia and Antarctica. It is most common in the world where stray dogs are presented in large numbers, particularly in under developing countries (Neevel *et al*., 2018). Individuals from rural, remote areas or developing countries are at high risk of dog bite which may be due to greater unvaccinated stray dog (Chaudhary *et al*., 2018). In India, rabies affects mainly people of lower socio-economic status and children between the ages of 5 and 15 years. Indian children often play near stray dogs, which are many and roam freely, and are used to sharing their food with them, which results in frequent bites (Kole *et al*., 2014). In Africa, the highest recorded human death due to the disease for the year 1998 was reported from Ethiopia. The magnitude of the problem is higher in big cities like Addis Ababa linked with the presence of large number of uncontrolled dogs and the absence of regular vaccination of dogs (Tamiru Dabuma *et al*., 2017). According to Richard Pankhurst, the first and only recorded of rabies case in Addis Ababa occurred in August 1903.The main cause for an increase in the number of dog bites is its high breeding rates. Sterilization is one of the methods for controlling the population of dogs (Abraham *et al*., 2017).

Until 1885, when Louis Pasteur and Emile Roux developed a vaccine, all human cases of rabies were fatal as the case fatality rate almost 100% (Abebe Mengesha *et al*., 2016). Several modern cell culture and embryonated egg-based rabies vaccines (CCEEVs) containing inactivated rabies virus are available. The first licensed human rabies vaccine developed from cell culture was the primary hamster kidney cell vaccine, which was created by cultivating viruses in primary

hamster kidney cells (Kissling, 1958). The disease is 100% preventable by either pre-exposure prophylaxis (PrEP) or post-exposure prophylaxis (PEP) which together effectively prevent approximately 372,000 deaths yearly. In resource-poor settings, however, these prophylaxes are frequently not accessible, incomplete or delayed and consequently, almost 96% of all human rabies cases occur in Africa and Asia despite the fact that rabies virus circulates worldwide (Neevel *et al*., 2018). Specifically, prompt administration of vaccines in conjunction with rabies-immunoglobulins and proper wound management after exposure prevent rabies even after high-risk exposure (Nie *et al*., 2017). However, death is almost always inevitable in unimmunized patients; only supportive measures are recommended after the onset of neurological signs and symptoms. Older nerve tissue vaccines should no longer be used as they may induce severe adverse reactions and are less effective than CCEEVs (Crowcroft and Thampi, 2016). The vaccines recommended by WHO include those produced in Vero cells, available since the 1980s. Unfortunately, the cell culture rabies vaccines are expensive and not readily available to individuals living in developing countries where rabies is endemic in dogs. Sheep brain derived Fermi type rabies vaccine is still being manufactured and utilized for the majority of exposed patients in Ethiopia, even though this vaccine has been discouraged by the WHO (Birhanu Hurisa *et al*., 2013). Hence, a continuing search for antirabies agents that is selectively virucidal, accessible and cost effective remains crucial. Many traditional medicinal plants have been reported to have strong antiviral activity and some of them have already been used to treat animals and people who suffer from viral infections inhibiting the replication cycle of various types of DNA or RNA viruses. Additionally, different secondary metabolites, including lignans, tannins, saponins, flavonoids and phenolic acids exhibit promising antiviral activity. In the search for such antiviral agents, the antirabies activity of medicinal plant extracts, including South American plants, was evaluated (Müller *et al*., 2007).

According to a World Health Organization (WHO, 1993) report, at least 80 % of the populations in most developing countries rely for their primary health care on traditional forms of health care. Several countries of Africa have realized the need and importance to develop improved traditional medicines (ITM) from native and endemic plants that are traditionally used at various places for various ailments. Medicinal plants have been used in diagnosis, treatment or prevention of various ailments since past decades in the name of traditional systems of medicine such as Ayurvedic medicine, Chinese traditional medicine, Tibetan medicine, Unani medicine,

Japanese medicine and African traditional medicine (Chaudhary *et al*., 2018). Nearly 25% of modern medicines are derived from plants first used traditionally (Petros Admasu *et al.*, 2014). In plants, the naturally occurring chemical compounds are phytochemicals. They give organoleptic properties and color to the plant and they are beneficial to boost up immunolatory responses and provide immunity against many diseases (Khalid *et al*., 2018). The phytochemical analysis of the plants is very important commercially and has great interest in pharmaceutical companies for the production of the new drugs for curing of various diseases (Wadood *et al*., 2013).

Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Tiwari *et al*., 2011). *Phytolacca dodecandra, Justicia schimperiana, Ricinuscommunis, Brucea antidysenterica, Croton macrostachyus* and *Cucumis ficifolius* were the most cited medicinal plant species utilized for the management of rabies (Asfaw Meresa *et al.*, 2017). Therefore, evaluations of the antirabies activity of such medicinal plant extracts are a necessary and highly desirable task. The present study had the objectives of assessing the phytochemical constituents, acute toxicity and antirabies potential of extracts from the leaves of *Justicia schimperiana* and *Ricinus communis* as well as the stem bark of *Croton macrostachyus.*

1.1. Statement of the problem

Rabies claims the lives of more than 24,000 people in Africa annually, but efforts to control the disease are still not sufficient, particularly in sub-Saharan Africa such as Ethiopia. Prevention of rabies in humans is complicated because those most commonly exposed to canine rabies lack the resources necessary to treat or prevent exposure. As a result, individuals who are exposed to the rabies virus often see traditional healers for the diagnosis and treatments of the disease. Understanding the relationship among medicinal plants used in traditional medicine systems can help identify plant materials with potential constituents applicable to modern medicine. Knowledge of medicinal plants that has been passed on from generation to generation has been proven to help millions in Ethiopia, but most of the traditional healers have poor knowledge on the dosage, safety and antidote while prescribing remedies to their patients.

1.2. Objectives

1.2.1. General objective

To determine the phytochemical constituents, acute toxicity and anti-rabies activities of the most commonly used traditional medicinal plants used for the management of rabies in Ethiopia.

1.2.2. Specific objectives

To assess active ingredients within the ethanol, methanol and water extracts of selected Ethiopian traditional medicinal plants

To evaluate the potential acute toxicity of the ethanol, methanol and water extracts of plants collected from different parts of Ethiopia

To evaluate the in vivo and in vitro antirabies potential of ethanol, methanol and water extracts of selected Ethiopian traditional medicinal plants

1.3. Significance of the study

Traditional medicine is used throughout the world as it is culturally acceptable, economically affordable and effective against certain type of diseases as compared to modern medicines. The findings of this study provide information on the phytochemical constituents, acute toxicity and anti-rabies activities of the selected Ethiopian traditional medicinal plants.

2. Literature Review

2.1. Rabies Etiology and Epidemiology

Rabies virion, a bullet shaped enveloped infectious particle (180 nm x 75 nm in size), having 12 Kb negative sense single-stranded RNA genome, belongs to the Lyssavirus genus of the Rhabdoviridae family and Mononegavirale order (Singh *et al.,* 2017). Rabies is a central nervous system (CNS) disease that is almost invariably fatal. The causative agent is rabies virus (RV), a negative-stranded RNA virus of the rhabdovirus family, which has a relatively simple, modular genome organization and encodes five structural proteins: a RNA-dependent RNA polymerase (L), a nucleoprotein (N), a phosphorylated protein (P), a matrix protein (M) and an external surface glycoprotein (G) (Dietzschold *et al*., 2008). All the lyssaviruses share many biological and physicochemical features as well as amino acid sequence characteristics that classify them with other rhabdoviruses. These include the bullet shaped morphology helical nucleocapsid or ribonucleoprotein core. The name Rhabdo comes from the Greek and identifies the characteristic bullet or rod-shape of the viruses (Semayat Oyda and Bekele Megersa, 2017). Lyssaviruses are usually confined to one major reservoir species in a given geographic area, although spillover to other species is common. Identification of different virus variants by laboratory procedures such as monoclonal antibody analysis or genetic sequencing has greatly enhanced understanding of rabies epidemiology (Gemechu Regea, 2017). Rabies virus is very fragile outside of the animal host, and is rapidly inactivated by drying or exposure to ultraviolet (UV) light (Tariku Jibat *et al*., 2018). The ssRNA of rabies virus contains five monocistronic genes relate to five viral proteins whose order is highly conserved (Singh *et al*., 2017). The negative-sense RNA genome is tightly encapsulated by N, P, and L proteins to form a ribonucleoprotein complex that is responsible for virus replication in the cytoplasm within infected cells. The RABV G protein is the only viral protein exposed on the surface of the virus and is not only the major determinant of viral pathogenicity, but also the major protective antigen responsible for inducing protective immunity against rabies (Zhu and Guo, 2016).

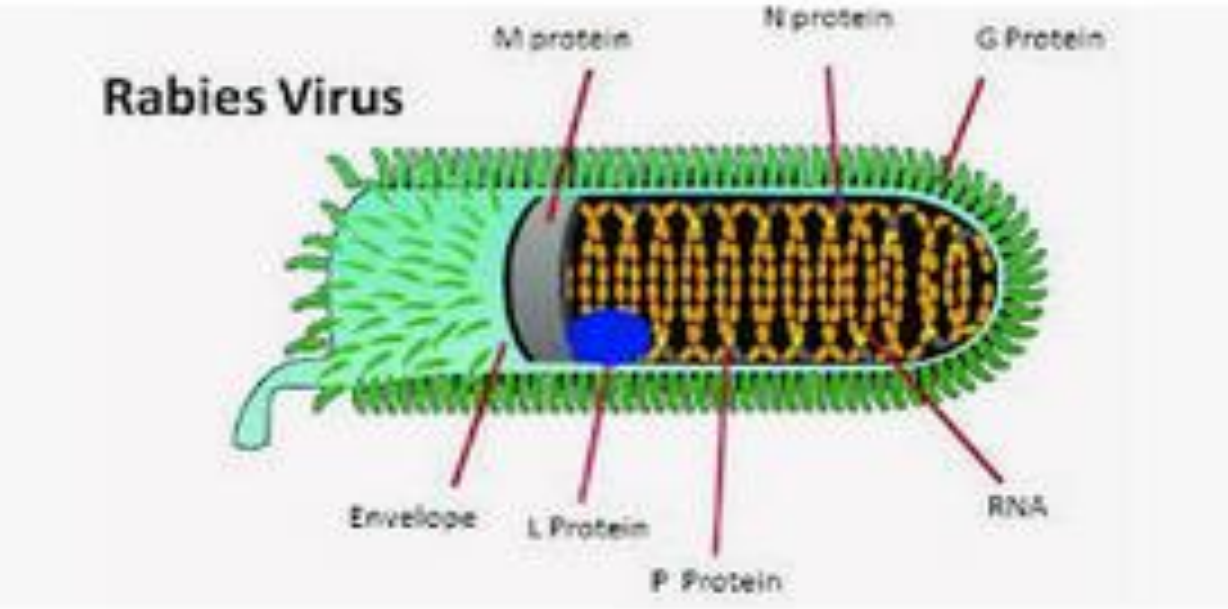

Figure 1: Rabies-virus-shape-and-its-structures

Source: https dokuwiki.png. Accessed on 22 May, 2019

The RABV is not viable outside the host and can be inactivated by sunlight, heat and desiccation (Singh *et al.,* 2017). The virus affects virtually all mammals and infected species invariably die from the disease once clinical signs are manifested and is a fatal viral zoonotic disease which causes encephalitis in all warm blooded animals and humans (Semayat Oyda and Bekele Megersa, 2017). Among the viral diseases, rabies is unique and it can affect a wide range of victims including humans as well as a wide variety of wildlife species that act as reservoirs for infection predominantly and it influences the population dynamics accordingly (Singh *et al.,* 2017). According to the Center for Disease Control (CDC), all mammal species have a risk of becoming infected by the rabies virus. All mammals are susceptible to the virus, with reservoir species most notably among carnivores of the family Canidae (Taylor and Nel, 2015). From an epidemiologic perspective, the name of the mammalian species acting as the reservoir and vector is used as an adjective to describe involvement in the infection process. For example, rabies maintained by dog-to-dog transmission is termed canine rabies, whereas rabies in a dog as a result of infection with a variant from a different reservoir mammal, e.g., skunk (or raccoon or fox), would be referred to as skunk (or raccoon or fox, etc.) rabies in a dog (Gemechu Regea,

2017). Persons having frequent contact with wildlife, such as mammalogists, are at greater risk than the general population for exposure to rabid animals (Krebs *et al.*, 1995).

2.2. Global Distribution and Burden of Rabies

In the world, it has been estimated that about 59, 000 people die from rabies each year, of which the highest death is in Africa and Asia due to the presence of endemic canine rabies and dogs remain the major animal reservoirs in such areas (Wunner & Briggs, 2010). It is prevalent throughout the world except in Islands (Singh, *et al.*, 2017). The virus is a highly opportunistic pathogen and canine rabies continues to spread to new areas, demonstrated by the last decade's epidemic across much of the People's Republic of China and its emergence on previously rabies free islands such as Flores and Bali (Taylor and Nel, 2015). The United States as with other developed countries have seen a dramatic decrease in the number of human infections and deaths due to the rabies virus. According to the Centers for Disease Control and Prevention (CDC) the stark reduction in the number of rabies cases is attributable to the elimination of canine rabies through vaccination, the vaccination of wildlife, education of the virus, and timely administration of post exposure prophylaxis. Currently, in the U.S. only one to three cases of rabies are reported annually (CDC, 2017).

Though rabies is prevalent throughout the globe, many countries and islands have got rabies-free status due to strict quarantine or by virtue of their water locked geographical location. Among the African countries Cape Verde, Congo, Libya Mauritius, Reunion and Seychelles are free from rabies (Singh *et al.*, 2017). Most of the human death cases occur in Asian and African countries. Of these countries, Ethiopia is one of the worst affected, with domestic dogs being the major sources of the infection to humans (Tariku Jibat *et al.*, 2018). The first major outbreaks of rabies in dog were reported in many parts of Ethiopia in 1884, especially in the former province of Tigre, Begemder, Gojjam and Wollo. The reviewed rabies situation in Ethiopia revealed that 2172 cases of animal rabies had been confirmed in and around Addis Ababa during 1990-2000, where dogs constituted 89.83 % with the incidence rate of 73.2 % (Semayat Oyda and Bekele Megersa, 2017).

The global burden of canine rabies is not distributed equally, and it disproportionately affects regions with limited resources, that are least capable of responding to the disease. It causes economic loses directly or indirectly on local and national economy. There are two pathways

following human exposure: the individual either seeks medical treatment or is given PEP, incurring direct and indirect costs, or he does not receive PEP, and either remains well or dies from rabies, leading to further costs (Gemechu Regea, 2017). Rabies causes at least 24000 deaths per year in Africa. The high death rates reported in poor rural communities and children. The major cause of spread of rabies in this region is urbanization (Niloufer, 2003).

In Ethiopia, the national annual estimates from official reports indicate 12 exposure cases and 1.6 rabies deaths per 100,000 populations. Quantification of the health burden enhances the understanding of its long term effects and of the comparative advantages of different levels of treatment and prevention (Tariku Jibat *et al.,* 2018). The annual reports of the EPHI (Ethiopian Public Health Institute) indicated that a total of 488 human deaths had occurred from 1964 to 1975. During the period between 1996 and 2000, a total of 9593 post exposure, and a total of 153 fatal human rabies cases were recorded. The cases were originated from Addis Ababa and its surroundings and other regions in the country (Semayat Oyda and Bekele Megersa, 2017).

2.3. Rabies Transmission

The lyssavirus infection is transmitted by all animals who are considered as warm-blooded, while the lyssavirus can also grow up in cells of cold-blooded animals (Bano *et al.,* 2017). The disease is communicable during the period of salivary shedding of rabies virus. It is transmitted when the virus is introduced into bite wounds, into open cuts in skin, or onto mucous membranes from saliva or other potentially infectious material such as neural tissue (CDC,2011). On rare occasions human rabies has been acquired by inhalation of airborne virus in laboratories working with live rabies virus and in caves with millions of bats. The common mode of transmission of rabies in man is by bite of a rabid animal or the contamination of scratch wounds by virus infected saliva (Balcha Chernet and Nejash Abdela, 2016). Spread of the disease is often seasonal, with high incidence in late summer and autumn because of large scale movement of wild animals at the mating time and in pursuit of food. Respiratory and oral transmission can also occur. The main determinant of transmission is the population density of non-immunized susceptible key host species that are free roaming within an ecosystem (Semayat Oyda and Bekele Megersa, 2017). It affects all warm blooded mammals and the virus shades in the saliva of clinically ill animals and is transmitted through a bite. The main reservoir for humans is known to be carnivores (Tariku Jibat *et al.,* 2018). In some experimental models, virus was

found to immediately enter nerves at the site of inoculation and to appear in the CNS within a very short time. In other systems, virus entered peripheral nerves after local replication in non-nervous tissue. Transport to the CNS occurs by retrograde axoplasmic flow at an estimated rate of 15 to 100 mm per day (Smith, 1996).

2.4. Pathogenesis and Incubation

Rabies virus entry occurs through wounds or direct contact with mucosal surfaces. The virus cannot cross intact skin. The virus then either replicates in non-nervous tissues or directly enters peripheral nerves and travels by retrograde axoplasmic flow to the central nervous system (CNS) (WHO, 2004). The virus replicates in the bitten muscle (local viral proliferation in non-neural tissue) and gains access (viral attachment) to motor endplates and motor axons to reach the central nervous system (Semayat Oyda and Bekele Megersa, 2017). The virus is highly neurotropic, and once it enters the body through a break in the skin or mucous membrane, it migrates along the nerves from the site of infection to the brain where it causes fatal encephalitis. From the brain, the virus travels back out to the organs of the body, eventually causing them to shut down. The development of the virus is influenced by concentration of the virus, number of bites and distance from head (Taylor and Nel, 2015).

The rabies virus (RABV) causes relatively slow but progressive disease without initial clinical signs which turns fatal after onset of clinical signs. The virus at the injected site remains hidden (eclipse) for variable time (a threshold must exceed to cause disease) (Singh*et al.,* 2017). The RAV G protein is the only viral protein exposed on the surface of the virus and the major determinant of viral pathogenicity, and also the major protective antigen responsible for inducing protective against it applied things (Tariku Jibat *et al.,*2018). The speed of virus uptake, the ability of the virus to spread efficiently from cell-to-cell and the rate of virus replication are the major factors that determine the pathogenicity of a RV. Neuroinvasiveness and neurotropism are the main features that define the pathogenesis of rabies. Although RV pathogenicity is a multigenic trait involving several elements of the RV genome, the RV glycoprotein plays a major role in RV pathogenesis by controlling the rate of virus uptake and trans-synaptic virus spread, and by regulating the rate of virus replication (Dietzschold*et al.,*2008). The incubation period is highly variable. In domestic animals, it is generally 3 to12 weeks, but can range from several days to months, rarely exceeding 6 months (CDC,2011). In 98% of the cases, the

incubation period is under one year. The incubation period is only 12-21days when the bite site is on the head and neck, while it is 25 days to two years when on the extremities. Generally, it takes as short as 12 days to as long as two years for the development of clinical rabies (Susilawathi *et al.,* 2012).

2.5. Clinical Signs

Rabies infection in humans is triphasic, i.e., prodromal, acute neurologic, and coma proceeding to death. The prodromal signs are mostly non-specific, including fever, headache, myalgia, nausea, vomiting, and abnormal sensation around bite sites. During acute neurologic stage patients show anxiety, agitation, dysphagia, hyper salivation, paralysis, and episodes of delirium (Susilawathi *et al.,* 2012). It is investigated that as the disease becomes advanced, the animal shows strange behavior. The primary clinical signs are frequently non-specific and can comprise anxiety, restiveness, anorexia or an improved appetite, nausea, diarrhea, a minor fever, dilation of the pupils, hyperactivity to any stimuli in addition to extreme salivation. Symptoms are produced after completion of the incubation period (Bano *et al.,* 2017). Once clinical signs of rabies appear, the disease is nearly always fatal, and treatment is typically supportive. Symptoms of rabies are variable, but some distinctive signs including severe anxiety, fear of water (hydrophobia), and fear of air (aerophobia) are recognizable in writings from millennia ago (Taylor and Nel, 2015). It causes inflammation of the brain in humans and other mammals. In Paralytic (dumb) phase, the gullet and masseter muscles turn into paralyzed; the animal might be incapable of swallowing, and salivating abundantly. In addition to that, there might be facial paralysis along with dropping of the lower jaw. Furthermore, this stage is also characterized by dropping of foamy salivary secretion and paralysis of hind limbs eventually leading complete paralysis followed by death (Bano *et al.,* 2017). During the final stages of disease, the virus is excreted in the saliva, and it is transmitted between hosts primarily through bites or scratches from infected animals, but more rarely transmission can occur through viral contact with mucous membranes (Taylor and Nel, 2015).

2.6. Diagnosis of Rabies

Rabies diagnostic tests were born with routine inoculation of rabies virus (RABV)-infected brain or saliva samples to rabbits in 1880 followed by the identification of Negri bodies reported in 1903 (Mani & Madhusudana, 2013). Initial capture or confinement of a rabies-suspect animal

can be quite problematic. Rabid animals can be unpredictable, sometimes alternating from an apparently weakened neurologic state to one of dangerous aggression (Hanlon and Nadin-Davis, 2013). The standard methods for rabies diagnosis in humans are virus isolation, antigen detection, and viral genome detection (Susilawathi *et al.,* 2012). The disease can only be identified following the onset of the symptoms and it is not easy to diagnose via ante-mortem. The virus is usually undetectable during the incubation period, and infections can also be difficult to diagnose when the clinical signs first appear. The diagnosis of rabies virus is made by taking some part of tissue from the brain of suspected animal. But mostly for confirmatory diagnosis samples from the brain stem and cerebellum are taken (Bano *et al.,* 2017). The clinical signs of rabies are confused with other neurological sings caused by other neurotropic etiological agents. The animals showing abnormal behavior should be kept in isolation where they cannot bite others for 10 days (Singh *et al.*,2017).

One million to two million animal bites per year are treated by physicians in the United States. Fortunately, only a small proportion of these bites involves a risk of rabies infection, and it is the function of the rabies laboratory to rapidly and accurately identify rabies virus-infected animals (Smith, 1996). Historically and in the research setting, RABV infection is identified by infecting cells and detecting virus. This can be done either through the Mouse Inoculation Test (MIT) or by inoculation of samples onto cultures of murine neuroblastoma or other cells (Rapid Tissue Culture Infection Test - RTCT). Following intracerebral inoculation of mice aged 3-4 weeks; MIT test results are available after an incubation period of up to 28 days. Some strains are associated with a longer incubation period. In laboratories with cell culture facilities and appropriate level of bio-containment, RTCT provides results within 24-48 hours, which is far quicker than intracerebral inoculation (Mani and Madhusudana, 2013).

Diagnosis of rabies should not be assumed without laboratory confirmation as similar clinical signs may be caused by a number of other viral or bacterial pathogens. Most commonly the confirmation is made by detection of lyssavirus antigens in the mouse brain via direct fluorescent antibody (dFA) test, although other methods for antigen or RNA detection can be implemented as well (Kuzmin, 2015). Rabies diagnosis can be performed on fresh specimens from several different tissue sources or on appropriate specimens stored at proper temperatures, preferably refrigerated. Impressions (or smears) of tissue samples from brainstem, thalamus, cerebellum,

and the hippocampus (Ammon's horns) are recommended for increased sensitivity of the test (WHO, 2005). The direct fluorescent antibody (dFA) testis rapid, sensitive, specific, easy to perform, and relatively inexpensive (Smith, 1996) and it has served as the cornerstone of rabies diagnosis for the past half century. The test is based upon microscopic examination under ultraviolet light of impressions, smears or frozen sections of tissue after they have been treated with anti-rabies serum or globulin conjugated with fluorescein isothiocyanate. The diagnostic conjugate should be of high quality and the appropriate working dilution must be determined in order to detect the different genotypes of lyssavirus (WHO, 2005).

The sensitivity of techniques for rabies diagnosis varies greatly according to the stage of the disease, antibody status, intermittent nature of viral shedding and the training of the technical staff. Molecular methods, although useful and extremely sensitive, may not always give positive results for patients with rabies. This may be due to the intermittency of virus shedding, the timing of sample collection, and the type of specimens collected (Hemachudha and Wacharapluesadee, 2004). Laboratory diagnosis is critical to confirm the status of a suspect case, in part, to justify prophylaxis in exposed persons or animals, to measure objectively the impact of disease prevention programs, and to support certification of a country as free of disease (Rupprecht *et al.,* 2017). Current techniques for the definitive laboratory diagnosis of rabies usually are nondiagnostic in the early days of infection, become useful only a week or more after the onset of illness, and are of little benefit for the patient because no effective therapy is available. However, the diagnosis of rabies should be confirmed as quickly as possible so the number of persons exposed to the infection can be limited, and therapy for those exposed can be initiated promptly (Wilkerson, 1995).

2.7. Prevention and Control of Rabies

Rabies is 100% fatal but it is also completely preventable with application of existing vaccination technology (Garg *et al.,* 2017). The majority of animal and human exposures to rabies can be prevented by raising public awareness on rabies transmission routes, and avoiding contact with wildlife (Balcha Chernet and Nejash Abdela, 2016). The earliest effective control measures against rabies were based on movement restrictions and muzzling of dogs, and the elimination of stray dogs. These methods were the backbone of the program that resulted in canine rabies elimination from the United Kingdom in 1902 (Taylor and Nel, 2015). All rabies

vaccines registered for human and animal use must conform to established potency standards. A minimum antigenic potency of 2.5 IU per dose is mandatory (Balcha Chernet and Nejash Abdela, 2016).

2.7.1. Prevention of Human Rabies

Prevention of rabies in humans is complicated because those most commonly exposed to canine rabies (e.g., children, the poor) also lack the resources necessary to treat or prevent exposure (Gemechu Regea, 2017). Human rabies can be prevented by a) eliminating exposure to rabies virus, b) providing appropriate rabies pre-exposure prophylaxis, and c) prompt local treatment of bite wounds combined with appropriate rabies post exposure prophylaxis (Balcha Chernet and Nejash Abdela, 2016). Currently, the disease is vaccine- preventable with pre- and post-exposure prophylaxis (PrEP and PEP) (Shwiff *et al.,* 2019). Pre-exposure vaccination may be offered to high risk groups like laboratory staff handling the virus and infected material, clinicians and persons attending human rabies cases, veterinarians, animal handlers and catchers, wildlife wardens, quarantine officers and travelers from rabies free areas to rabies endemic areas (Gemechu Regea, 2017). The first effective treatment for the rabies virus was a vaccine in 1885 by Louis Pasteur and Emile Roux. The vaccine was made from the nerve tissue of rabbits infected with rabies (Petros Admasu *et al.,* 2014). Rabies could potentially be eliminated from the human population due to availability of efficacious vaccination tools which have been validated across the world (Garg *et al.,* 2017).

Modern rabies vaccines produced on cell cultures or embryonated eggs are both safe and efficacious. Human rabies treatment is expensive and comprises a significant portion of the overall economic impact of rabies. Treatment usually consists of a series of four to five vaccinations over a span of several weeks, accompanied by a dose of rabies immunoglobulin (RIG) (Shwiff *et al.,* 2019). The essential components of rabies post exposure prophylaxis are immediate thorough cleansing of all wounds with soap and water and the administration of anti-rabies immune globulin and vaccine (Smith, 1996). Although there is an efficient vaccine, which can also be administered after the exposure, once the clinical manifestation sets in death is inevitable. The Milwaukee protocol for management of clinical rabies also has not produced encouraging results. A major lacuna has been the non-availability of a successful specific anti-viral. Ribavirin and interferon-α have proved to be disappointing agents for the therapy of rabies.

There is insufficient evidence to support the continued use of ketamine or amantadine for the therapy of rabies (Sandeepan *et al.,* 2017).

2.7.2. Animal Rabies Control

The majority of human rabies deaths globally occur as a result of being bitten by dogs. Adherence to a regular rabies vaccination schedule is critical to protect animals against recognized and unrecognized rabies exposures (Gemechu Regea, 2017). Vaccination of pet animals provides a barrier to transmission of rabies to humans. This has provided a major mechanism for prevention by breaking the link between rabies cycles in wildlife and transmission to domestic animals; the latter providing a ready means to pass the infection on to humans (Krebs *et al.,* 1995). According to the World Organization for Animal Health (OIE) and the WHO recommendations, the critical percentage of dogs to be vaccinated to prevent rabies cases should be at least 70% (WHO, 2005). Although rabies can be well controlled among domesticated animals by different types of useful and widely available vaccines, canine rabies continued to be a serious problem in Africa, including Ethiopia (Petros Admasu *et al.,* 2014).

In Ethiopia, 94.01 percent of rabies cases are caused due to the bite of rabid dogs and the rest cases incriminate domestic and wild animals (Tariku Jibat *et al.,* 2018). Public education on the risks of rabies transmission from wild animals is paramount to effective disease prevention. A number of recently developed, highly-effective, thermostable, inactivated vaccines are available for veterinary use. The vaccines may be used in young pups, but they must be boosted at three months of age and again within the following year. Revaccination must be carried out every three years thereafter. Cattle and sheep may be vaccinated annually or every two to three years, depending on the vaccine manufacturer's instructions. Following an outbreak in domestic livestock, vaccination of animals without visible bite wounds is strongly recommended (Balcha Chernet and Abdela Nejash, 2016). Rabies vaccines may be administered under the supervision of a licensed veterinarian to animals held in animal shelters before release. Within 28 days after initial vaccination, a peak rabies virus antibody titer is expected, and the animal can be considered immunized (Gemechu Regea, 2017).

2.8. Rabies Treatment

Rabies infection is always fatal unless prompt post exposure treatment is administered before symptoms begin. Fortunately, rabies can be a vaccine-preventable disease, provided that post-exposure prophylaxis (PEP) is given promptly and correctly. Protection against rabies correlates

with the presence of rabies-specific virus-neutralizing antibodies (VNAs). The recent advance of modern cell cultivation techniques has made the production of high-quality rabies vaccines from cell culture feasible (Zhu and Guo, 2016). Effective post exposure therapy includes wound cleansing and active and passive immunization in a previously unimmunized individual. Active immunization is achieved with five doses of a modern cell culture vaccine, including purified chick embryo cell culture vaccine or human diploid cell vaccine (administered intramuscularly in the deltoid muscle on days 0, 3, 7, 14, and 28). Passive immunization is performed with human rabies immune globulin at a dosage of 20 IU/kg with local infiltration into and around the wound(s); any remaining dose should be given intramuscularly into the gluteal area (Jackson, 2009). Just once rabies warning signs have appeared, the treatment is generally supportive. The patients are sedated to manage their fear and pain (Bano *etal.,* 2017).

Ribavirin and interferon-alpha are broad-spectrum antivirals that have significant *in vitro* activity against rabies but *in vivo* experiments and treatment of several human patients with rabies infection did not show any beneficial activity. Two other FDA approved drugs with some *in vitro* anti-rabies activity are the N methyl-D-aspartate (NMDA) receptor antagonist ketamine and amantadine (Jochmans and Neyts, 2017). Despite the wide availability of different types of useful vaccines used for rabies treatment, a continuing search for new compounds having antirabies agent remains crucial (Petros Admasu *et al.,* 2014). Although live-attenuated virus-based vaccines represent the most promising approach for rabies control and treatment, some other novel modalities have also been investigated for their potential role in rabies treatment, such as protein and peptide vaccine, nucleic acid-based vaccine, RNA interference (RNAi), and RIG, coupled with BBB permeability enhancing agents such as monocyte chemotacticprotein-1 (MCP-1) (Zhu and Guo, 2016).

2.9. Costs and Cost-Effectiveness of Control Measures

The highest financial expenditure in any country is the cost of rabies post exposure prophylaxis. The type of vaccine, vaccine regimen and route of administration as well as the type of immunoglobulin used all significantly influence the cost of treatment (WHO, 2005). The total cost of canine rabies to a country comprises a number of different aspects, including the direct costs of vaccinating animals and people, the indirect costs (often borne by the patients and dog owners) of lost salary and travel to seek vaccination, losses due to livestock that die of rabies, surveillance costs, dog population management costs, and productivity losses. There is a strong

logical argument that mass dog vaccination is a more cost-effective long-term strategy than relying on PEP provision alone (Taylor and Nel, 2015). Deaths due to rabies are responsible for 1.74million disability associated life years (DALYs) lost each year with 0.04 million DALYs are lost through morbidity and mortality following side-effects of unsafe nerve-tissue vaccines (Abebe Mengesha *et al.,* 2016). About 563 million United States dollars are spent annually in the world on measures to prevent rabies, yet in countries of south-eastern Asia the disease is still an important public health problem (Kole *et al.,* 2014). The need to pay for transport and expensive post-exposure prophylaxis for rabies exposed family or community members can lead to the unplanned sale of production animals and livelihoods assets, further impacting food and economic security (Gemechu Regea, 2017). Veterinary services in Africa usually report very limited budgets and often have to divert resources during outbreaks of other diseases. This is clearly the most significant constraint to effective rabies control (Lembo *et al.,* 2010). The cost of upgrading nerve tissue based vaccine production facilities in order to produce cell culture rabies vaccines is beyond the financial budget of most developing countries. Therefore, banning the use of nerve tissue based vaccines alone will not solve the problem as this would remove access to the post-exposure treatment (PET) available to many patients' unable to afford modern cell culture vaccines (Abebe Mengesha *et al.,* 2016). Effective vaccination campaigns need to reach a sufficient percentage of the population to eliminate disease and prevent future outbreaks, which for rabies is predicted to be 70%, at a cost that is economically and logistically sustainable (Kaare *et al.,* 2012).

2.10. Risks of Rabies vaccine

Evidence suggests that rabies vaccine may have non-specific protective effects in animals and children. Non-specific effects of vaccines are defined as those effects on the immune system of the recipient that alter the risk of illness or death from conditions other than the specific infectious disease the vaccine is designed to prevent (Knobel *et al.,* 2017). The use of nerve tissue vaccine from rabies virus–infected goat or sheep brain tissue has led to severe adverse reactions and is currently being phased out in developing countries. Most reported adverse events following immunization with rabies vaccine are local reactions such as pain at the injection site, swelling, redness, and induration (Kang *et al.,* 2018).

2.11. Traditional Medicinal Plants

Traditional medicines have a very long history: it is the sum total of the practices based on the theories, beliefs and experiences of different cultures and times, often inexplicable, used in the maintenance of health, as like in the prevention, diagnosis, improvement and treatment of illnesses (Firenzuoli and Gori, 2007). Traditional medicine is largely transmitted as oral knowledge and around 4,000 species are used in ATRM which is predominantly (90%) plant based (Payyappallimana, 2016). Medicinal plants have an unbelievable history in terms of serving humanity in almost all continents of the world. Traditional healers have transferred that incredible knowledge from generation to generation. Even modernity or cultural revolutions have not altered the in-depth wisdom of this natural medical paradigm (Khan, 2014).

The World Health Organization (WHO) defines traditional medicinal plants as natural plant materials which are used at least or in the absence industrial processing for the treatment of diseases at a local or regional scale (Jamshidi-kia *et al.*,2018). The use of medicinally active plants predates modern history. A large number of plants used in the traditional medicine have now become a part of the modern world health care system because of their unique ability to synthesize a wide array of compounds with diverse health-related benefits (Naithani *et al.*,2010). Since the ancient period plants have been utilized by human beings as medicinal agents on the basis of ethnomedical background (Divya *et al.,* 2011). Plants that possess therapeutic properties or exert beneficial pharmacological effects on the human body are generally designated as medicinal plants. The therapeutic use of plants goes back to the Sumerian and the Akkadian civilization in about the 3rd millennium B. C (Kutama *et al.,* 2018). Medicinal plants are used for discovering and screening of the phytochemical constituents which are very helpful for the manufacturing of new drugs (Wadood *et al.,* 2013). Most developing countries, especially those in Asia, Africa, Latin America and the Middle East, 70%–95% of their population rely on traditional medicines for treatment of different diseases. The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant which used as sources of medicines throughout history and continued to serve as the basis for many pharmaceuticals used today (Petros Admasu *et al.,* 2014).

More than a tenth of the plant species (over 50000 species) are used in pharmaceutical and cosmetic products. However, the distribution of medicinal plants across the world is not uniform and medicinal herbs are mainly collected from the wildlife population. In traditional methods,

plant materials are tested for pharmaceutical purposes. If any evidence of activity is observed, the extract is fractioned, and the active compound is isolated and identified. Each step of decomposition and isolation is usually guided by biological test, which is referred to as bioassay-guided fractionation (Jamshidi-kia *et al.,* 2018). Modern drug discovery which has its roots in traditional medicine provides avenues to newer phytomolecules based therapies. Nowadays major pharmaceutical industry are reducing their research focus and are indulging towards profit-making venture by extracting and selling phyto-constituents (Kapoor *et al.,* 2017). In Ethiopia, medicinal plants contribute, to about 80% of the traditional medicines used in the country (the others being animal and mineral origins). Most of these plants are obtained from local sources in the wild by knowledgeable traditional practitioners (Worku Abebe, 2016).

2.11.1. Phytochemicals and Pharmacological Properties

Phytochemicals are chemical compounds formed during the plants normal metabolic processes. These chemicals are referred to as secondary metabolites which comprises of several classes and these includes; alkaloids, flavonoids, phenols, tannins, coumarins, glycosides, gums, polysaccharides, terpenes, terpenoids (Kutama *et al.,* 2018). There are chemical compounds with complex structures and with more restricted distribution than primary metabolites (Zohra *et al.,* 2012). Plants and many of their secondary metabolites because of the healing properties have been in traditional use throughout the world since ancient times. They provide us diverse bioactive phytochemicals which play synergetic role in maintaining human health. Plants produce a diverse array of more than 100,000 secondary metabolites and can be classified, on the basis of composition and the pathway through which they are synthesized (Kapoor *et al.,* 2017). The medicinal plants are useful for healing as well as for curing of human diseases because of the presence of phytochemicals. Phytochemicals are naturally occurring in the plants, leaves, stem and roots that have defense mechanism and protect from various diseases (Wadood *et al.,* 2013). There has been increasing interest in the research on flavonoids from plant sources because of their versatile health benefits reported in various epidemiological studies. Many flavonoids are shown to have antioxidative activity, free radical scavenging capacity, coronary heart disease prevention, hepatoprotective, anti-inflammatory, and anticancer activities, while some flavonoids exhibit potential antiviral activities (Kumar and Pandey, 2013). The alkaloids, substances extracted natural sources, show promising pharmacological activities, including pharmacological activities for the treatment of neurodegenerative diseases such as Alzheimer's

disease, whose treatment is based on the use of various drugs (Chaves *et al.,* 2016). Active substances that have phenolic groups in their structure have great pharmacological potential (Siqueira *et al.,* 2012).

2.12. The Use of Traditional Medicinal Plants in Ethiopia

Traditional medicine plays an important role in the healthcare of the majority of the people in developing countries, including Ethiopia, and medicinal plants serve as valuable sources of natural therapeutic agents (Worku Abebe, 2016). In Ethiopia, about 800 species of plants are used in the traditional health care system to treat nearly 300 mental and physical disorders (Asmare Amuamuta *et al.,* 2014). Traditional Ethiopian medicine is commonly used to treat a variety of diseases including gastrointestinal disturbances, respiratory disorders, sexually transmitted infections, tuberculosis, impotency, hemorrhoids, rabies, intestinal parasites, skin problems, liver diseases, mental disorders, hypertension, diabetes, gynecological conditions rheumatism, malaria and others (Kebede Deribe *et al.,* 2006).

It is widely believed in Ethiopia that the skill of traditional health practitioners is 'given by God' and knowledge on traditional medicines is passed orally from father to a favorite child, usually a son or is acquired by some spiritual procedures. There is lack of accurate quantitative information on rabies both in humans and animals and little is known about the awareness of the people about the disease to apply effective control measures in Ethiopia (Semayat Oyda and Bekele Megersa, 2017). The number of dog to human ratio is approximately assumed to be 1:6 and 1:8 in urban and rural areas, respectively. This indicates that rabies, which is maintained and disseminated mostly by dogs, is a threat to public health in Ethiopia. People and traditional rabies healers in Ethiopia claim that traditional plants can cure both animals and humans that are exposed to rabies (Petros Admasu and Yalemtsehay Mekonnen, 2014). In rural Ethiopia, individuals who are exposed to rabies often prefer to see traditional healers for the diagnosis and treatment of the disease because of cultural background, lack of knowledge or limited accessibility to medical treatment. These widespread traditional practices of handling rabies cases might interfere with medical treatment seeking practice, resulting in an underreporting of the actual number of rabies cases and its related health burden (Tariku Jibat *et al.,* 2018).

According to Dabuma Tamiru *et al.,* (2017), some of the interviewees believed that rabies is caused by starvation; thirst and prolonged exposure to coldness and 30.6% of the respondents didn’t knew the cause of rabies. A little more than the half (52.6%) believe that it is possible to

manage rabies by Holy water. Around 36.5% respondents accepted that there was specific medical drug therapy in the Health Centre. Only 9.8% of the respondents were aware of the presence of post exposure prophylaxis (PEP). Several traditional herbs have been formulated by traditional healers to treat human and animal rabies and individuals who are exposed to rabies virus often see traditional healers for the diagnosis and treatment of the disease (Petros Admasu and Yalemtsehay Mekonnen, 2014). These widespread traditional practices of handling rabies cases are believed to interfere with timely seeking of PEP. Rabies victims especially from rural areas seek PEP treatment after exhausting the traditional medicinal intervention and usually after a loss of life from family members (Tadesse Guadu *et al.,* 2014).

Various traditional antirabies plants were reported in different indigenous people and parts of Ethiopia which were used for the treatment of rabies in humans and animals. Among the reported plants, *Phytolacca dodecandra* is widely used for the traditional treatment of rabies in both humans and animals in Ethiopia (Petros Admasu *et al.,* 2014). *Phytolacca dodecandra, Justicia schimperiana, Ricinus communis, Brucea antidysenterica, Croton macrostachyus, Cucumisficifolius, Salix subserrata, Calpurnia aurea* and *Euphorbia abyssinica* were the most cited medicinal plant species utilized for the management of rabies by the Ethiopian traditional health care system (Asfaw Meresa *et al.,* 2017).

The folk drugs of rabies such as *Cucumisficifolius, Datura stramonium, Dorsteniabarnimiana, Dracaenasteudneri, Euphorbia abyssinica*, *Gnidiaglauca, Justicia schimperiana*, *Phytolacca dodecandra, Salix subserrata*, *Silene macroselen*, *Vigna membrancea* and *Zehneriascabra* were one of the most widely used remedies in different parts of Ethiopia. Of these folk drugs, only three (*Salix subserrata, Silene macroselen* and *Phytolacca dodecandra*) were evaluated *in vivo* to see their efficacy against rabies virus (Petros Admasu and Yalemtsehay Mekonnen, 2014). About twelve traditional antirabies plants in different indigenous people from different parts of Ethiopia were reported by different investigators for the treatment of rabies in humans and animals (Petros Admasu *et al.,* 2014). However, efficacy of these plants against rabies were not evaluated with modern pharmaceutical practices except findings of Asefa Deressa and his colleagues who evaluated the efficacy of antirabies activities of crude extract of *Salix subserrata* and *Silene macroselen* plants in mice which improved the survival period (Days) of experimental mice compared to control group of mice.

2.13. Problems associated with traditional medicine

Some plants used in traditional medicines, such as taenicides, are widely known to be toxic. For example, blindness and changes in central nervous system function have repeatedly been found in people who took over dosage of *Hagenia abyssinica.* Traditional healers may cause delays in the treatment of communicable diseases such as TB if they fail to refer patients to modern health services (Kebede Deribe *et al.,* 2006). Though there is lack of published evidences, adverse fatal side effects and cases of rabies deaths after traditionally treated with medicinal plants were the most common problems reported by some health centers/institutes in Ethiopia including EPHI, owning to the non-standardization of constituents, quality and efficacy of these traditionally used antirabies herbal remedies (Petros Admasu and Yalemtsehay Mekonnen, 2014).

2.14. Description of selected medicinal plants used for treatment of rabies

Of the estimated 250,000 species of higher plants existing throughout the world, only a fraction have been examined for pharmacological activities (Ali *et al.,* 1996). According to Chaudhary *et al.,* (2018), the leaves of *Justicia schimperiana*, root and stem bark of *Croton macrostachyus* and leaves and seed of *Ricinus communis* are mostly used to cure dog bite, used as ethno medicine by different tribal society of the globe.

Justicia schimperiana (syn. *Adhatoda schimperiana*; *Gendarussa schimperiana*), belongs to the genus Justicia of the family Acanthaceae. It is a shrub with much branched stems 2-3 m high, with slightly unpleasant smell. Its ethnobotanical information indicated that the plant is used for the treatment of various health problems such as scabies, fever, asthma, excessive pellagra and constipation, gonorrhea, rabies and headache in Ethiopia (Jemal Abdela *et al.,* 2014). It is known by the common names in Amharic: Sensel and Simiza. Its stem is brittle i.e. it breaks easily. Its leaves are simple and opposite, long oval to 13 x 4 cm, tip pointed, narrowed to a short stalk. Its flowers are in conspicuous terminal heads on long stalks seen clearly above the leaves, each small flower lies inside a green-yellow leafy bract 1.5 cm long, its edge clear and membranous, flowers white or yellow white, tubular to 3 cm long, two-lipped with dark purple throat or lines on the lip (Shemsu Umer *et al.,* 2010). The plant has slightly unpleasant smell. The result of the study by Tadesse Birhanu and Dereje Abera (2015) also indicated that the leaf and root of *Justicia schimperiana* is used in the treatment of rabies and coccidiosis at Horro Guduru district, western Ethiopia (Habtamu Abebe *et al.,* 2014). In Ethiopia, *Justicia Schimperiana* is locally

utilized to heal ailments such as stomachache, burning, constipation, skin lesion, tooth ache and Scabies (Asfaw Meresa *et al.,* 2017).

Figure 2: Photo of *Justicia schimperiana* plant

Source: Yeweynshet Tesera. "*Justicia schimperiana* plant." Menagesha, Ethiopia. 2019. JPEG file.

Croton macrostachyus Hochst. ex Del. (Euphorbiaceae) another plant selected for this study is one of the eight *Croton* species found in Ethiopia. The wide range medicinal uses of *Croton macrostachyus* led scientists to isolate compounds from its different parts (Hadush Gebrehiwot *et al.,* 2018). It is widely distributed in Ethiopia and has been utilized as a remedy for malaria, abdominal pain, gonorrhea, wounds, ringworm infestation, hemorrhoids, ascariasis, epilepsy, rabies venereal diseases, cough, rheumatism, liver problem and other ailments in Ethiopian traditional medicines (Asfaw Meresa *et al.,* 2019). The leaves are large and green, turning to orange before falling. It is also characterized by creamy to yellow-white colored flowers with green (when young) to grey (at maturity) fruits (Abraham Fikru *et al.,* 2016). It has a rounded crown with slender trunk and massive spreading branches. It has a long taproot and numerous side-roots, which makes it adapted to dry climates (Getu Alemayehu, 2018). It is widely used as herbal medicine by the indigenous people of tropical Africa (Maroyi, 2017). It is regarded as a multipurpose tree by subsistence farmers in Ethiopia, Kenya, and Tanzania and the species has

potential in playing an important role in the primary healthcare. The bark, fruits, leaves, roots, and seeds of *Croton macrostachyus* are reported to possess diverse medicinal properties and it is used as herbal medicine for at least 61 human and 20 animal diseases and ailments (Obey *et al.*, 2018). The plant *Croton macrostachyus* has been traditionally used to cure dog bite in Ethiopia (Chaudhary *et al.*, 2018).

Due to its drought hardiness and fast growth, *Croton macrostachyus* is considered useful for afforestation of shifting sand dunes, degraded waste land, hill slopes, ravines and lateritic soils. Throughout its distribution area a decoction, infusion or maceration of leaves, stem bark or root bark is taken as a purgative and vermifuge (Pagadala *et al.*, 2015). *Croton macrostachyus* has compounds isolated from its root; fruit and bark and it has a medicinal value for treatment of malaria, headache, skin rash, internal worms, abdominal pain, rabies, ringworm infestation, hemorrhoids, ascarasis, and sexually transmitted diseases (Getu Alemayehu, 2018). It is traditionally used for the treatment of wounds malaria, rabies, and gonorrhea, Tineaversi color, diarrhea, hepatitis, jaundice, and scabies (Tigist Minyamer & Getnet Belay, 2018). It is used to treat rabies, epilepsy, cough, skin disease, dysentery, lung complaints, plain full eyes, toothache and others (Pagadala *et al.*, 2015).

Figure 3: Photo of *Croton macrostachyus* plant

Source: Yeweynshet Tesera. "*Croton macrostachyus* plant." Menagesha, Ethiopia. 2019. JPEG file.

The other plant species used to treat many diseases and disorders traditionally is *Ricinus communis*; Family: *Euphorbiaceae* popularly known as 'castor plant' and commonly known as ‘palm of Christ’, Gulo (Amharic). The plant is widespread throughout tropical regions as ornamental plants (Jena and Gupta, 2012). The plant *Ricinus communis* is probably native to Africa, and is cultivated in many tropical and subtropical areas of the world, commonly appearing spontaneously (Kumar *et al.*, 2015). The castor bean plant is a tropical perennial shrub that originated in Africa, but is now cultivated in many tropical and subtropical regions around the world. It can be self- and cross-pollinated and worldwide studies reveal low genetic diversity among castor bean germplasm (Chan *et al.*, 2010). The preliminary phytochemical study of *Ricinus communis* showed the presence of steroids, saponins, alkaloids, flavonoids, and glycosides. Its stem and leave extracts produce antioxidant activity due to the presence of flavonoids in their extracts (Jena and Gupta, 2012).

Figure 4: Photo of *Ricinus communis* plant

Source: Yeweynshet Tesera. *“Ricinus communis* plant.” Menagesha, Ethiopia. 2019. JPEG file.

3. Materials and Methods

3.1. Plant material collection and identification

The leaves of *Justicia schimperiana* and *Ricinus communis* and stem bark of *Croton macrostachyus* were collected in January 2019 from Menagesha District, which is located 20km from west of Addis Ababa, Ethiopia. The botanical identification and authentication was done by Melaku Wondafrash, of the National Herbarium, Department of Plant Biology and Biodiversity management, Addis Ababa University, Ethiopia, where voucher specimens were deposited (*Justicia schimperiana* 001; *Croton macrostachyus* 002 and *Ricinus communis* 003). Plant species used in the present investigation were given in Table 1.

Table 1: The botanical identification of the plant specimens used in this study

Scientific name	**Family**	**Amharic name**	**Location**	**Part/s used**	**Voucher number**
Justicia schimperiana (Hochst. ex Nees)	Acanthaceae	Sensel	Menagesha	Leaves	001
Croton macrostachyus Del.	Euphorbiaceae	Bisana	Menagesha	Stem bark	002
Ricinus communis L.	Euphorbiaceae	Gulo	Menagesha	Leaves	003

3.2. Processing of the plants

The collected leaves of *Justicia schimperiana*, *Ricinus communis* and the stem bark of *Croton macrostachyus* were properly washed with tap water and separated from foreign material. For evaporating the water content the washed plant samples were kept in a shaded area for two weeks. The stem bark of *Croton macrostachyus* was cut into small pieces using a penknife and was air-dried for additional one week. All the plant samples were then chopped, crushed and powdered with electrical grinder and then the dried powdered samples were stored in polyethylene bags at room temperature for further processes (Hussain *et al.*, 2011).

3.3. Extract preparation

The Plant samples were subjected to sequential solvent extraction using ethanol, 80% methanol and water accordingly at room temperature and with agitation at 130 rpm on a rotary shaker and the solvents were removed under reduced pressure to obtain the extracts. All solvents were of analytical grade. The extracts of the tested medicinal plants were prepared according to the procedures previously described by (Jemal Abdela, 2014). Briefly, the powder of each plant (200 g in 1600 ml) was separately extracted with ethanol by maceration for 72 h with frequent agitation on a rotary shaker at 130 rpm and the resulting liquid was filtered (Whatman No. 1 filter paper). Accordingly the marc was re-macerated twice using the same volume of 80% methanol for 72 h and finally with water for 72 h to exhaustively extract the plant material. After exhaustive extraction, the filtered organic (ethanol and 80 % methanol) extracts were concentrated under reduced pressure (Rota vapor at 72 rpm) at a temperature not exceeding 40°C and the filtered water extracts were concentrated by lyophilization or freeze-drying. The concentrated extracts were then dried in an oven at 40°C for about 48 h. Finally, the yields of the extracts were stored at +4°C in airtight container throughout the study period until use.

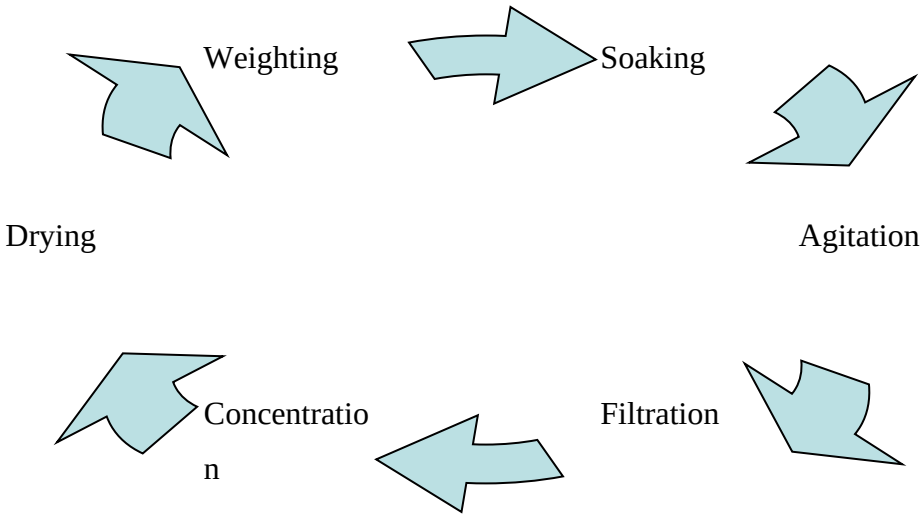

Figure 5: A brief summary of extraction procedure

3.4. Preliminary phytochemical screening

Preliminary qualitative screening for phytochemicals of all the three plant species was carried out according to (Sasidharan *et al.,* 2011; Zohra *et al.,* 2012; Wadood *et al.,* 2013; Banu and Cathrine, 2015; Khalid *et al.,* 2018). The determination of metabolites was done by differential coloring reaction and/or precipitation of the major families of chemical compounds contained in plants.

3.4.1. Test for Alkaloids (Wagner's test)

Alkaloids were identified by Wagner's test; a few drops of Wagner's Reagents (iodine solution in potassium iodide) were added to a few ml of plant extract (Banu and Cathrine, 2015).

3.4.2. Test for Flavonoids

Flavonoids identification was carried out by alkaline reagent test; the plant extract was treated with 2-3 drops of sodium hydroxide solution and observed the formation of acute yellow color. To this mixture some drops of sulphuric acid were added (Khalid *et al.,* 2018).

3.4.3. Test for Phenol (Ferric chloride test)

The extract (50 mg) was dissolved in 5 ml of distilled water. To this few drops of neutral 5% ferric chloride solution were added (Banu and Cathrine, 2015).

3.4.4. Test for Steroids (Libermann Burchard Test)

To 1ml of extract, 1ml of chloroform, 2-3 ml of acetic anhydride and 1 to 2 drops of concentrated sulphuric acid were added (Kumar *et al.,* 2007 as cited in Sasidharan *et al.,* 2011).

3.4.5. Test for Saponins

The saponins study was based on froth test; 50 mg extract was diluted with distilled water and made up to 20 ml. The suspension was shaken in a graduated cylinder for 15 minutes (Banu and Cathrine, 2015).

3.4.6. Test for Tannins (Ferric chloride test)

Two ml of plant extract was added to 2 ml of water, a 1 to 2 drops of diluted ferric chloride solution was added (Zohra *et al.,* 2012).

3.4.7. Test for Terpenoids

For the confirmation of terpenoids in the selected plants, 0.4 g of selected plant sample was diluted with 5 ml of methanol, Then 2 ml chloroform and 3 ml sulphuric acid were added in selected sample extract (Wadood *et al.,* 2013).

3.5. Cell culture and virus

Vero cells obtained from Vaccine and Diagnostics production directorate, EPHI were cultured using the T-75 culture flasks following the procedure described in (Ammerman *et al.,* 2008). Briefly, vero cells kept in liquid nitrogen were recovered from frozen stock in a 37°C water bath. The cell suspension was transferred from the cryovial into a 15ml conical tube containing 10ml of DMEM supplemented with FBS. The cells were pelleted by centrifugation at 200× g for 5 minutes at room temperature and the supernatant was removed and discarded. The cells were resuspended in 5-10 ml DMEM supplemented with 10% FBS and transferred to tissue culture flask with vented cap. Flasks were incubated in 37°C incubator with 5% CO_2 for 2-3 days until a monolayer was obtained. The growth medium from confluent monolayer of vero cells was removed and cells were washed with 10 ml 1X DPBS. Five ml of 1X trypsin-EDTA was added and the cells were incubated at 37°C for 2-3 minutes, until cells start to streak as they detached from the flask. To inactivate the trypsin-EDTA, 5 ml DMEM with 10% FBS were added and cells were washed down in media. The cell suspension was removed from flask and transferred to a sterile 15 ml conical tube and centrifuged at 200 × g for 5 minutes at room temperature. The cells were resuspended in 10 ml DMEM with 10% FBS after supernatant was removed and discarded. The desired dilution of cells in a total of 15 ml DMEM with 10% FBS was prepared and added to 75 cm^2 cell culture flasks with vented caps. Flasks were incubated in 37°C incubator with 5% CO_2 until a monolayer was obtained. Finally, cells were collected within a volume of 20-30 ml in cell culture medium with 10% heat-inactivated FBS. Cells were maintained at 37 °C under a humidified 5 % CO_2 atmosphere for bioassay.

The Vaccine and Diagnostics Production Directorate, Ethiopian public health Institute, provided rabies virus Pasteur virus strain (RV PV) used in the present study. The virus (PV) was propagated in vero cells as previously described by (Webster and Clow, 1937) and titer of infectious virus was obtained by the limit-dilution method and expressed as 50% tissue culture infections dose per ml ($TCID_{50}$/ml). Briefly, serial 10-fold dilutions (10^{-1} to 10^{-7}) of virus in

serum-free MEM were added into the confluent monolayer in 96-well tissue culture plate and were incubated for 72 h at 37°C. After incubation, the medium were decanted and the cells were fixed by adding cold acetone (50 µl per well) and kept at 4°C for 30 minutes. After discarding the acetone, cells were stained by direct polyclonal florescent-labeled antibody for 30 minutes and washed three times with 0.01 M phosphate buffer saline (PBS), air dried, and visualized under an inverted fluorescence microscope. The titer was calculated by using Spearman-Karber method and expressed as $TCID_{50}$. The virus titration was also performed in mice through intracerebral inoculation using serial ten-fold dilutions at a volume of 0.03 ml. Six mice were inoculated per virus dilution, and the end point was death. The statistical method of Spearman-Karber was used and the mortality at each dilution was calculated to determine the 50% end point titer (Ramakrishnan, 2016).

3.6. *In vivo* acute toxicity and anti-rabies screening of crude extracts

3.6.1. Experimental animals and their management

Female Swiss albino mice aged 3-4 weeks and with weight of 15-25g obtained from the laboratory animal unit of Ethiopian Public Health Institute (EPHI) were used for the studies. The mice were maintained at a temperature of 22^0 C ± 2°C and with a 12 h light/12 h dark cycle and were given clean pellet diet and water ad libitum. As described by (OECD, 2001), all experimental mice were treated under similar feeding management and left under controlled condition for three days to acclimatize before conducting the experimental procedure and each mouse were used only for one experiment. The handling of mice was done following internationally accepted ethical principles.

3.6.2. *In vivo* acute toxicity test

The extracts from the leaves of *Justicia schimperiana* and *Ricinus communis* and stem bark of *Croton macrostachyus* plants, proposed for their antirabies activity, were assessed for their toxicity in non-infected female Swiss albino mice according to the standard guideline of Organization for Economic Cooperation and Development (OECD). In the acute toxicity study, a single administration of the extracts at doses of 1000, 2000, 3000, 4000 and 5000 mg/kg respectively, was given orally. A total of 276 Swiss albino mice were used and the extract was administered to mice by using oral gavage in a volume of 20 ml/kg body weight. All mice were randomly divided into control and treatment groups; six mice per cage. They were fasted for 4 h

prior to dosing and 2 h after the administration of the extract. Following the period of fasting, the mice were weighed and the extracts were administered. The mice in the treatment groups (A, B and C) received 0.5 ml of the ethanol, methanol and water extracts of each plant respectively, at doses of 1000, 2000, 3000, 4000 and 5000 mg/kg. And the mice in the control group received 0.5 ml of respective vehicle of each extract (4% Polysorbate-80) (OECD, 2001).

The mice were observed individually after dosing at least once during the first 30 minutes, and daily thereafter, for a total of 14 days (Assefa Deressa *et al.,* 2010). For determination of LD_{50} of extracts, an exploratory assay with each tested mouse strain was performed in order to exactly determine the dose range to be used with accuracy. The LD_{50} value was expressed as micrograms of extract per mouse body weight. All observations were systematically recorded, with individual records being maintained for each mouse. Weight changes were calculated and recorded and at the end of the test surviving animals were weighed by a sensitive digital weighing balance and then humanely killed by cervical dislocation (OECD, 2001).

3.6.3. *In vivo* anti-rabies screening of crude extracts

Mice were randomly assigned into three treatment groups and two controls, six mice per cage, and each mouse was used only for one experiment. The extracts were diluted before administration to the mice using a 4% Polysorbate-80 solvent and sterile distilled water. Except group I, all groups of mice were inoculated with PV virus strain. As described by Assefa Deressa *et al.,* (2010) after virus inoculation, the mice were allowed to stay in their respective cages for about 1 hour so as to make them calm. Then, the diluted extracts were orally administered to the mice in the treatment groups by using an intra-gastric needle based on the animal's body weight in 1ml vehicle via gauge. Briefly, Group I was negative control and received water as a placebo. Group II was inoculated intracerebrally (30 µl) with 10 LD_{50} RV PV. Group III, IV and V were inoculated intracerebrally (30 µl) with the challenge dose of 10 LD_{50} RV PV followed by the oral administration (after 1 h) of the ethanol, methanol and water extracts (20 mg/ml) respectively, at dose of 3000 mg/kg. Dosage was setup based on the acute toxicity assay result i.e. the highest dose which showed the lowest toxicity would be selected. Volume administered was calculated based on individual mouse body weight and 0.5 ml was the maximum volume administered. Extracts were administered via the oral route using gavage and all the groups of mice were observed for mortality up to 28 days.

3.6.4. Determination of mean survival time

Anti-rabies activities of the selected medicinal plants were evaluated on mice survival period compared to negative control group. Mortality rates as a result of rabies virus challenge were determined by clinical signs and direct fluorescent antibody test (Petros Admasu *et al.*, 2014). The mice were monitored daily for the sign of paralysis and mortality for about 28 days after inoculation of the virus. Death was recorded for each mouse in the treatment and control groups throughout the follow up period on the mouse history cards. Confirmatory diagnosis of rabies through direct FAT was conducted by opening of the skulls and collection of the brain of mice was done according to the procedure specified by Dean and Albelseth, 1973. Briefly, a midline incision was made on the dorsal surface of the head using scalpel and blade. The brain sample consists of cerebellum, hippocampus and brain stem and any available brain tissues were taken and an impression smears were made for direct FAT. Standardized protocol for the direct FAT was carried out in accordance with the procedures described by Kissling, 1975. The number of days each mouse survived was recorded for the mice in each group and mean survival time were calculated using the formula (Nafiu *et al.*,2013).

$$MST = \frac{sum\ of\ days\ of\ survival\ of\ mice/group}{total\ number\ of\ mice\ in\ the\ group}$$

3.6.5. Fluorescent antibody test

Fluorescent antibody test was done to confirm the presence of the virus. Brain impressions were made upon microscope slides, which were fixed by acetone and incubated with fluorescein isothiocyanate (FITC)-labeled antibodies to rabies virus. The stained impressions were viewed using fluorescence microscopy (Mayes and Rupprecht, 2015). Briefly, the mice infected with PV-RV were observed closely for 2 hours after treatment with different extracts. After that, the mice were checked daily for 28 days to register clinical signs. The mice with rabies related signs were killed humanely by placing them into the airtight plastic container. The skull was grasped ‘by forceps in the orbits, and the cranium was cut away by curved scissors or scalpel to expose the brain. The brain was removed from the skull; a section of the brain just anterior to the cerebellum was homogenized gently and very thin impression smears were made on slides. The smears were allowed to air dry for 30 minutes and then fixed in acetone at -20^0c for about 1h and air dried for about 5-10 minutes. A sufficient quantity of clarified conjugate (FITC) were added

on each smear and incubated at 37°c for 30 minutes. The smears were soaked in PBS, rinsed with distilled water and air dried. Finally, the slides were examined under a fluorescence microscope. When labeled antibody was incubated with rabies suspect brain tissue, it would bind to rabies antigen (Hosseini and Asgary, 2015).

3.7. Screening for *in vitro* cytotoxicity and anti-rabies activities of crude extracts

3.7.1. Cytotoxicity assay

Evaluation of the cytotoxicity of ethanol, methanol and aqueous extracts towards the vero cells was performed using MTT [3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide] assay. Briefly, vero cells were seeded in a 96-well flat-bottom microtiter plate at a density of 2×10^5 cells/ml and allowed to adhere for 24 h at 37°C in a CO_2 incubator. After incubation, culture medium was replaced with a fresh medium and different concentrations (0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 mg/ml) of extracts were serially diluted in DMEM and added to each culture wells in triplicate. To the control cells only 100 µl of the culture medium was added. The plates were incubated for 3 days at 37°C with 5% CO_2. Subsequently, the medium was removed and the cells washed by phosphate buffer saline (PBS) followed by the addition of 100 µl of DMEM and 50µl of MTT working solution (5mg/ml in phosphate buffer solution) to each well. The plates were then incubated for 3h. After incubation, MTT was aspirated, and the formed formazan crystals were solubilized by adding 50µl of DMSO per well, followed by gentle shaking for 15 min (Fayyad *et al.,* 2014). In this regard, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay uses a water-soluble yellow tetrazolium salt (MTT), which is reduced to an insoluble purple formazan by viable cells. The absorbance of dissolved formazan, quantified spectrophotometrically, correlates with the number of intact viable cells. Finally, the intensity of the dissolved formazan crystals (purple color) was quantified using the ELISA plate reader at 490 nm (Sandeepan *et al.,* 2017). Percentage of viable cells was obtained by dividing the mean absorbance at 490 nm of treated cells (for each concentration of extract) to the mean absorbance of its control cells (Soltanian *et al.,*2017).

Cell viability (%) = Treated cells Mean OD/Control OD x 100%

The percentage of cytotoxicity is calculated as [(A-B) / A] x 100 where, A and B are the OD490 of untreated and of treated cells, respectively.

3.7.2. *In vitro* anti-rabies assay

The *in vitro* antirabies activity of medicinal plant extracts (Sensel, Bisana and Gulo) were evaluated by FAT assay. Vero cell lines were trypsinized and 2×10^5 cells/ml were seeded in a 96-well tissue culture micro plate and incubated at 37°C for 72 h. After the incubation period, 50µl of rabies virus suspension were added to confluent cell monolayers in a 96-well plate and allowed to stand for 1h to enable virus adsorption. Thereafter, different concentrations (2mg/ml, 4mg/ml and 8mg/ml) of each extract based on cytotoxicity test result were added in triplicate into all the wells with the exception of the negative control wells that contained only vero cells and the virus control that contained an equal virus concentration but lacked the plant extract. The plates were incubated at 37 °C in 5% CO_2 humidified incubator for 72 h. The medium were decanted and the cells were fixed by adding cold acetone (50µl per well) and kept at 4°C for 30 minutes. After discarding the acetone, cells were stained by direct polyclonal florescent-labeled antibody for 30 minutes and then washed (3 times) with 0.01 M PBS, air dried, and visualized under an inverted fluorescence microscope. Reading was qualitative, every well that shows specific fluorescence was considered to be positive.

3.8. Ethical Clearance

Ethical approval for the study was sought from the college of Natural and Computational Science Institutional Review Board (CNS IRB), Addis Ababa University. The animals were handled according to the international guidelines for the care and use of laboratory animals (Clark *et al.*, 1997).

3.9. Data Analysis

Data were entered into an excel spreadsheet and then transferred to a statistical package for social sciences (SPSS version 20). Student's t test was used to compare means of treatment and control groups and to evaluate the significance of observed differences between groups of mice in the mean survival time (Days). Paired t test statistic was applied for body weight change of mice in acute toxicity determination. The 50% cytotoxic (CC_{50}), 50% inhibitory (IC_{50}) concentrations and selectivity index (SI) were calculated from concentration-effect curves after linear regression analysis.

4. Results and Discussion

4.1. Phytochemical study result of extracts

The property of extracting solvents significantly affects the phytochemical content, the higher the polarity, the better the solubility of compounds. In the present study the result of qualitative phytochemical analysis of all extracts from the three plants using different standard methods showed positive results for alkaloids, but negative for saponins. The study also revealed the presence of terpenoids in all the extracts except the ethanol extract from leaf of *Justicia schimperiana*. One of the possible causes of negative results for saponins in all plant extracts could be ecological factor. The type, content, and proportion of phytochemicals may vary depending on ecological factors in areas where the plants are grown (Liu *et al.,* 2015). In this study, the ethanol, 80% methanol and water showed differential extraction of some of the compounds within the same organ of the plant. For example, the methanol extracted most of the bioactive compounds such as, alkaloids, flavonoids, phenols, steroids, terpenoids and tannins while the ethanol extracted only alkaloids, steroids and tannins and the water extracted alkaloids, flavonoids and terpenoids in the leaf of *Justicia schimperiana*. The variations in each solvent extract may be due to degrading enzymes that may be active or denatured in any of the three extractants. For example, the enzyme polyphenol oxidase, which degrade polyphenols in water extracts, whereas in methanol and ethanol they are inactive (Tiwari *et al.,* 2011).

The 80% methanol extract of the leaf of *Justicia schimperiana* showed the presence of alkaloids, flavonoids, phenols, steroids; tannins and terpenoids, however, saponins were absent. From previous studies it was confirmed that flavonoids and alkaloids (Mukherjee *et al*., 2017); polyphenols, tannins, terpenoids and steroids (Limenew Abate and Tadesse Mengistu, 2018); phenols, tannins, flavonoids, saponins, terpenoids, glycosides, and anthraquinones (Belay Mekonnen *et al.,* 2018); saponins, alkaloids and flavonoids (Jemal Abdela *et al*., 2014) were present in the methanolic extract of *Justicia schimperiana.*

From the three plants studied, only *Croton macrostachyus* showed flavonoids in its three extracts. Flavonoids have been reported to possess a wide variety of biological activities among which are antimicrobial, anti-inflammatory, anti-allergic, antiviral, anticancer as well as antidiarrheal properties (Middleton *et al.,* 2000). The test of alkaloids, flavonoids, and terpenoids gave positive results in the three extracts of the stem bark of *Croton macrostachyus*. The

Libermann Burchard test failed to show the presence of steroids in the ethanolic extract, but gave a positive result with the methanolic and water extracts. Steroids are involved in diverse physiological functions ranging from regulation of metabolism, behavior, and fertility to inflammation and immune control (Libert and Dejager, 2014). Previously, it has been documented that the leaves fruits and stems from *Croton macrostachyus* contains broad spectrum of phytochemicals including alkaloids, aminoacids, saponins, flavonoids, steroids, triterpenoids and tannins (Letha *et al.*, 2016).

Presence of alkaloids, phenols, steroid, tannins and terpenoids were recorded in the three extracts of *Ricinus communis* while flavonoids were recorded only in the methanolic extract. Phytochemicals such as, tannins, saponins, terpenoids, flavonoids and alkaloids were detected in methanol extract of the leaves of *Ricinus communis* (Suurbaar *et al.*, 2017). Pandhure (2015) identified alkaloids, flavonoids, steroid, terpenoids, cardiac glycosides and saponins in the leaves and stem of *Ricinus communis.* Phytoconstituents detected in plant samples are shown in (Table 2).

Table 2: Different phytochemical components in the ethanol, 80% methanol and water extracts of the leaves of *Justicia schimperiana* and *Ricinus communis* and the stem bark of *Croton macrostachyus* plants

Plant name	Solvent	Secondary metabolites						
		Alkaloids	Flavonoids	Phenols	Steroids	Saponins	Tannins	Terpenoids
Justicia schimperiana	Ethanol	+	_	_	+	_	+	_
	Methanol	+	+	+	+	_	+	+
	Water	+	+	_	_	_	_	+
Croton macrostachyus	Ethanol	+	+	+	_	_	+	+
	Methanol	+	+	+	+	_	+	+
	Water	+	+	_	+	_	_	+
Ricinus communis	Ethanol	+	_	+	+	_	+	+
	Methanol	+	+	+	+	_	+	+
	Water	+	_	+	+	_	+	+

+, the secondary metabolite was present; _, the secondary metabolite was absent.

4.2. The result of acute oral toxicity test

The acute toxicity results showed no evidence of toxicity of the ethanol, 80 % methanol and water extracts of *Justicia schimperiana (leaf), Croton macrostachyus (stem bark)* and *Ricinus communis* (leaf) in mice administered at 1000 mg/kg, 2000 mg/kg and 3000 mg/kg. No abnormalities were recorded at three doses with regard to food consumption, water intake and body weight of the mice. This shows that the plant extracts could be well tolerated up to the dose of 3000 mg/kg body weight of Swiss albino mice. It was also noticed that the oral LD_{50} of *Justicia schimperiana* leaves aqueous extract (Andualem Tesfaye *et al.,* 2016) and 80% methanolic extract (Belay Mekonnen *et al.,* 2018) was greater than 2000 mg/kg as there was no mortality or remarkable signs of toxicity noted in the 14-day observation of the mice which were used for the test. From previous studies it was confirmed that the aqueous extract of *Croton macrostachyus* stem bark did not provoke death until the dose 16 g/kg during 7 days of observation; whereas the organic extract caused general behavior, adverse effects and mortality (Mbiantcha *et al.,* 2013). However, it the present study, at higher doses, i.e. 4000 mg/kg and 5000 mg/kg, mice showed common signs of toxicity like low locomotion, weakness and erection of hairs including death in the course of acute study. As a result, the LD_{50} of the extracts could be greater than 3000mg/kg body weight. It suggested that the extracts may not be completely safe at a dose higher than 3000 mg/kg. According to the Hodge and Sterner, (1943) toxicity scale, the ethanol, methanol and aqueous extracts of *Justicia schimperiana* (leaf), *Croton macrostachyus* (stem bark) and *Ricinus communis* (leaf) were placed in category IV (500 mg/kg-5000 mg/kg, p.o.), and hence classified as slightly toxic.

The percentage of mice that died at each dose was transformed to probits using Finney's method (Appendix 7). According to Finney's method the log dose at probit 5.0 (Log LD_{50}) for the ethanol, 80 % methanol and water extracts of *Justicia schimperiana* were found to be 3.60, 3.54 and 3.65 and hence, LD_{50} was calculated by taking antilog of the Log LD_{50} values of each extract and found to be 4000mg/kg, 3500mg/kg and 4500mg/kg body weight respectively (Table 3). The acute toxicity study in LD_{50} determination showed that 80 % methanol leaf extract of *Justicia schimperiana* is more toxic than the ethanol and water leaf extracts of the plant. This might be due to active ingredients responsible for toxic effects, which were more abundant in methanolic extract of the plant leaf than in ethanolic and water extracts.

Table 3: Mice treated with *Justicia schimperiana* extracts, and responses in acute toxicity testing

Group	Dose (mg/kg)	Log Dose	Death n (%)	Probits
A1	1000, 2000, 3000	3, 3.30, 3.48	0 (0%)	2.76
	4000	3.60	3 (50%)	5.00
	5000	3.70	5 (83%)	5.95
B1	1000, 2000, 3000	3, 3.30, 3.48	0 (0%)	2.76
	4000	3.60	4 (67%)	5.44
	5000	3.70	6 (100%)	7.24
C1	1000, 2000, 3000	3, 3.30, 3.48	0 (0%)	2.76
	4000	3.60	0 (0%)	2.76
	5000	3.70	4 (67%)	5.44

Where, A1= group of mice treated with ethanol extracts of *Justicia schimperiana*, B1= mice treated with 80 % methanol extracts of *Justicia schimperiana* and C1= mice treated with water extracts of *Justicia schimperiana.*

Similarly, the lethal dose (LD_{50}) of the ethanol, 80% methanol and water extracts of the leaves of *Ricinus communis* was also greater than 3000 mg/kg body weight of mice. The Log LD_{50} for the ethanol and water extracts of *Ricinus communis* was found to be 3.54 but, its methanolic extract was found to be 3.60. Thus, the LD_{50} values were found to be 3500 mg/kg for the ethanol and water extracts and 4000 mg/kg for the methanol extract of the plant which were calculated by taking antilog of 3.54 and 3.60 (Table 4). Therefore, the ethanol and water leaf extract of *Ricinus communis*is more toxic than the methanol extract of the plant. At doses of 4000 mg/kg and 5000 mg/kg, there were significant differences ($p=0.008$) in changes in calculated body weights of test animals compared to the control after acute administration of *Ricinus communis* leaf extracts. The body weight gain after treated with 4000 mg/kg and 5000 mg/kg extract in treatment group was significantly ($p<0.05$) increased compared with control group. The increase in body weight of the treated animals with leaf extracts of *Ricinus communis* at 4000 mg/kg and 5000 mg/kg might be due to the fact that *Ricinus communis* leaf extracts contain some phytochemical compounds with nutritional factors, which increase the appetite, especially in high doses.

Table 4: Mice treated with *Ricinus communis* extracts, and responses in acute toxicity testing

Group	Dose (mg/kg)	Log Dose	Death n (%)	Probits
A3	1000, 2000, 3000	3, 3.30, 3.48	0 (0%)	2.76
	4000	3.60	4 (67%)	5.44
	5000	3.70	5(83%)	5.95
B3	1000, 2000, 3000	3, 3.30, 3.48	0 (0%)	2.76
	4000	3.60	3 (50%)	5.00
	5000	3.70	5 (83%)	5.95
C3	1000, 2000, 3000	3, 3.30, 3.48	0 (0%)	2.76
	4000	3.60	4 (67%)	5.44
	5000	3.70	6 (100%)	7.24

Where, A3=group of mice treated with ethanol extracts of *Ricinus communis*, B3= mice treated with 80 % methanol extracts of *Ricinus communis* and C3= mice treated with water extracts of *Ricinus communis.*

The lethal dose (LD_{50}) of the ethanol, 80 % methanol and water extracts of the stem bark of *Croton macrostachyus* were also greater than 3000 mg/kg body weight of mice. The Log LD_{50} for the ethanol and 80% methanol extracts of *Croton macrostachyus* was found to be 3.54. Thus, LD_{50} was calculated by taking antilog of 3.54 and found to be 3500 mg/kg body weight. Whereas, the Log LD_{50} for the water extract was found to be 3.59 hence, the LD_{50} value was 3900 mg/kg (Table 5).

Table 5: Mice treated with *Croton macrostachyus* extracts, and responses in acute toxicity testing

Group	Dose (mg/kg)	Log Dose	Death n (%)	Probits
A2	1000, 2000, 3000	3, 3.30, 3.48	0 (0%)	2.76
	4000	3.60	4 (67%)	5.44
	5000	3.70	5 (83%)	5.95
B2	1000, 2000, 3000	3, 3.30, 3.48	0 (0%)	2.76
	4000	3.60	4 (67%)	5.44
	5000	3.70	5 (83%)	5.95
C2	1000, 2000, 3000	3, 3.30, 3.48	0 (0%)	2.76
	4000	3.60	4 (67%)	5.44
	5000	3.70	4 (67%)	5.44

Where, A2= group of mice treated with ethanol extracts of *Croton macrostachyus*, B2= mice treated with 80% methanol extracts of *Croton macrostachyus* and C2= mice treated with water extracts of *Croton macrostachyus*. A graph was plotted between probit vs. Log dose and LD50 values of extracts were confirmed by the graph which was the dose at probit 5.0, i.e. 50% mortality.

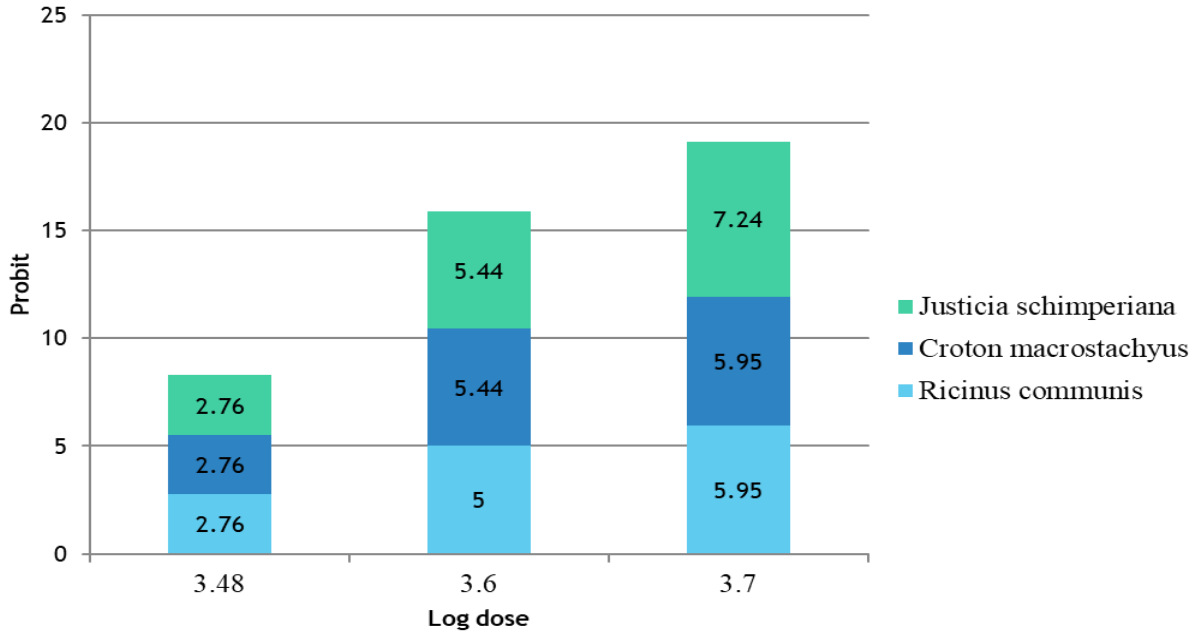

Figure 6: LD50 values of 80% methanol extracts from the three selected plants

4.3. Cytotoxicity determination of plant extracts in vero cell line

Evaluation on vero cell lines by using MTT assay showed that the 50% cytotoxic concentration (CC_{50}) values of the ethanol extracts from three plants; *Justicia schimperiana*, *Croton macrostachyus* and *Ricinus communis* were found to be above 12.8 mg/ml (Table 6).

Table 6: Effect of the ethanol extracts of three plants on vero cells

	Concentration (mg/ml)	Absorbance 490nm			% cell viability			% cytotoxicity		
		JS	CM	RC	JS	CM	RC	JS	CM	RC
A	12.8	0.28	0.27	0.25	62.2	60	55.5	37.8	40	44.5
B	6.4	0.29	0.28	0.33	64.4	62.2	73.3	35.6	37.8	26.7
C	3.2	0.31	0.34	0.35	68.9	75.5	77.8	31.1	24.5	22.2
D	1.6	0.31	0.37	0.36	68.9	82.2	80	31.1	17.8	20
E	0.8	0.32	0.39	0.42	71.1	86.7	93.3	28.9	13.3	6.7
F	0.4	0.36	0.42	0.44	80	93.3	97.8	20	6.7	2.2
G	Control cells	0.45	0.45	0.45	100	100	100	0	0	0

Where, JS= *Justicia schimperiana;* CM= *Croton macrostachyus*; RC= *Ricinus communis*

The 50% cytotoxic concentration (CC_{50}) value of the methanol extracts from *Justicia schimperiana* and *Croton macrostachyus* were found to be 9.6mg/ml and 8mg/ml whereas, *Ricinus communis* showed above 12.8 mg/ml (Table 7). In contradiction to the results from the present study (CC_{50}>12 mg/ml), Mukherjee *et al.,* 2017 observed the median cytotoxicity (CC_{50}) of methanol extract of *Ricinus communis* in BHK_21 cells at 9.79 mg/ml.

Table 7: Effect of the 80% methanol extracts of three plants on vero cells

	Concentration (mg/ml)	Absorbance 490nm			% cell viability			% cytotoxicity		
		JS	CM	RC	JS	CM	RC	JS	CM	RC
A	12.8	0.21	0.22	0.24	46.7	49	53.3	53.3	51	46.7
B	6.4	0.23	0.21	0.25	51	46.7	55.5	49	44.5	44.5
C	3.2	0.24	0.23	0.28	53.3	51	62.2	46.7	49	37.8
D	1.6	0.26	0.25	0.30	57.8	55.5	66.7	42.2	44.5	33.3
E	0.8	0.31	0.28	0.31	68.9	62.2	68.9	31.1	37.8	31.1
F	0.4	0.34	0.36	0.33	75.5	80	73.3	24.5	20	26.7
G	Control cells	0.45	0.45	0.45	100	100	100	0	0	0

JS= *Justicia schimperiana;* CM= *Croton macrostachyus*; RC= *Ricinus communis*

The 50% cytotoxic concentration (CC_{50}) values of the aqueous extracts from three plants were also found to be above 12.8mg/ml (Table 8).

Table 8: Effect of the water extracts of three plants on vero cells

	Concentration (mg/ml)	Absorbance 490nm			% cell viability			% cytotoxicity		
		JS	CM	RC	JS	CM	RC	JS	CM	RC
A	12.8	0.29	0.31	0.34	64.4	68.9	75.5	35.6	31.1	24.5
B	6.4	0.33	0.35	0.35	73.3	77.8	77.8	26.7	22.2	22.2
C	3.2	0.32	0.28	0.35	71.1	62.2	77.8	28.9	37.8	22.2
D	1.6	0.36	0.39	0.38	80	86.7	84.4	20	13.3	15.6
E	0.8	0.27	0.41	0.42	60	91.1	93.3	40	8.9	6.7
F	0.4	0.40	0.43	0.43	88.9	95.5	95.5	11.1	4.5	4.5
G	Control cells	0.45	0.45	0.45	100	100	100	0	0	0

JS= *Justicia schimperiana;* CM= *Croton macrostachyus*; RC= *Ricinus communis*

Generally, the percentage viability was found to be increasing with decreasing concentration of test extracts. The MTT assay results revealed that all extracts tested were non-cytotoxic and exhibited CC_{50} values above the cut-off point which is 30µg/ml. An extracts can be considered as non-cytotoxic if the CC_{50} is higher than 30µg/ml (Nondo *et al.,* 2015). The MTT assay is based on the reduction of MTT into formazan crystals by living cells. The reducing power is mainly provided by NAD(P)H which is derived from dehydrogenase activity in mitochondria, endoplasmic reticulum and plasma membrane (Stockert *et al.*, 2012).

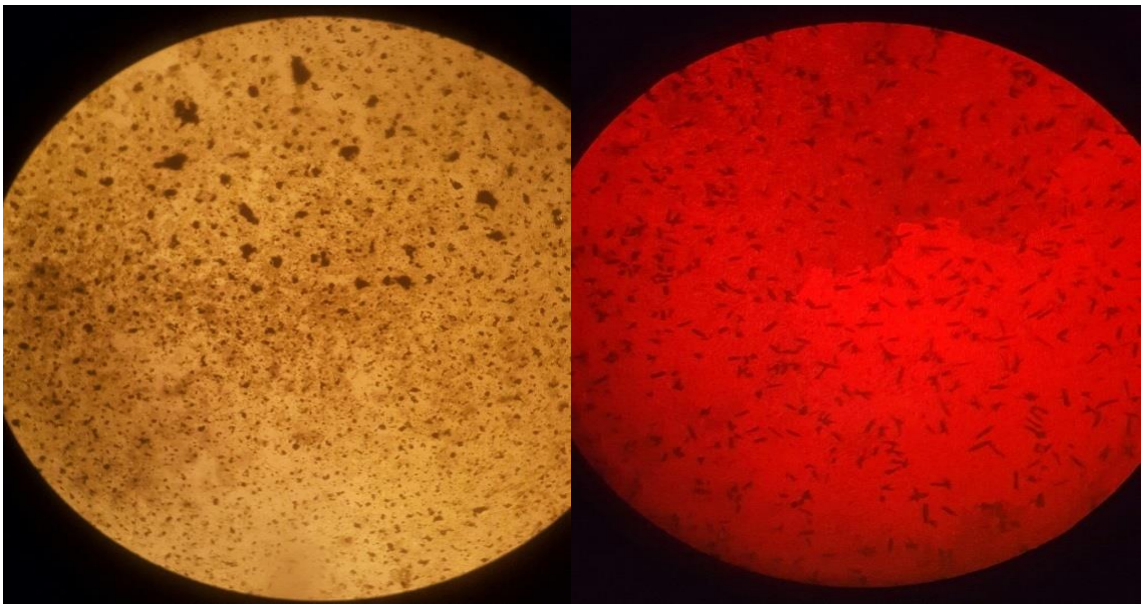

Figure 7: Microscopic photographs of vero cell lines treated with 80% methanolic extracts (6.4 mg/ml left and 12.8mg/ml right) of the leaf of *Justicia schimperiana*.

4.4. Determination of 50% end titer

Titration of rabies virus (PV) was performed in vero cell line as well as in Swiss Albino mice and the titer of 10^4 $TCID_{50}$/ml and $10^{5.6}$ LD_{50}/0.1ml of RV PV respectively, were obtained.

4.5. Determination of *in vivo* antirabies activity

Group of mice infected with rabies virus but not treated with any of plant extracts showed 0% survival rate and a mean survival period of 9.5days. However, oral treatment of mice infected with rabies virus with ethanol, methanol and water extracts from the leaves of *Justicia schimperiana* and *Ricinus communis* as well as the stem bark of *Croton macrostachyus* at a dose of 3000 mg/kg significantly ($p<0.05$) increased the mean survival time compared to those of negative control group (Table 9). Relatively higher mean survival time (22.16 days) was obtained when methanol extract of *Ricinus communis* was administered to mice at a dose of 3000 mg/kg. This indicates that the methanolic extract from the leaf of *Ricinus communis* has some more antirabies activity than others. The different extracts from the leaf of *Justicia schimperiana* ($p=0.018$), stem bark of *Croton macrostachyus* ($p=0.037$) and leaf of *Ricinus communis* ($p=0.031$) at 3000 mg/kg dose level, significantly ($p<0.05$) increased the survival period of mice.

The presence of some antirabies activity of the extracts may be due to the presence of multiple classes of phytochemicals such as, alkaloids, flavonoids, phenols, steroids, tannins and terpenoids. From the literature survey it was found that flavonoids have wide range of biological properties such as anti-inflammatory, antibacterial, antiviral, anti-allergic, cytotoxic antitumor properties. Alkaloids and phenolic compounds along with hypoglycemic, antidiabetic properties also exhibit anti-inflammatory, antimicrobial and antioxidant effects (Bansode *et al.*, 2015). Terpenoids have been reported for its anti-inflammatory, anti-viral, anti-malarial, inhibition of cholesterol synthesis and anti-bacterial properties (Wadood *et al.*, 2013). Hence, the presence of pharmacologically useful substances in the leaves of *Justicia schimperiana* and *Ricinus communis* as well as stem bark of *Croton macrostachyus* confirm the claims and application of such part of the plants in local treatment of ailments like rabies.

For the confirmation of antirabies properties of the plant extracts, FAT were done by taking only one sample from each parameter per group of mice. One mouse from the negative control group died within four days of inoculation with PV but, didn't show any antigen against rabies virus which indicate the death was due to accidental rather than the effect of the virus. Death from samples taken from those at moribund state was due to the action of rabies virus PV strain because all the samples showed a viral antigen but, most of the samples from survivors indicate negative for antigen detection.

Table 9: Antirabies effect of plant extracts on mice survival time (n=6) at 3000mg/kg

Group	Treatment	Route of administration	Survival time/days (mean±SD)	FAT results		
				Death within four days	Moribund mice	Survivors/sacrificed mice
I	Distilled water	Os	24±2.529	ND	NM	_
II	PV	IC	9.5±0.438	_	+	NS
IIIA	*JS* ethanol extract	IC, Os	13.8±0.327	ND	+	+
IVA	*JS* methanol extract	IC, Os	14.3±0.413	ND	+	_
VA	*JS* water extract	IC, Os	13.3±0.413	ND	+	_
IIIB	*CM* ethanol extract	IC, Os	18±1.132	ND	+	+
IVB	*CM* methanol extract	IC, Os	21.3±0.413	ND	+	_
VB	*CM* water extract	IC, Os	21±1.78	ND	+	_
IIIC	*RC* ethanol extract	IC, Os	17.5±1.497	ND	+	_
IVC	*RC* methanol extract	IC, Os	22.16±2.287	ND	+	_
VC	*RC* water extract	IC, Os	20.16±1.468	ND	+	_

Where, I= Placebo administrated 1ml of dH2O; II=Negative control; *JS=Justicia schimperiana*; *CM= Croton macrostachyus*; *RC=Ricinus communis*; IC= Intracerebral; Os= Oral; ND= No death; NM= No moribund mice; NS= No survivor; - =No antigen detected and + = Antigen detected

4.6. The *in vitro* antirabies activity of plant extracts

The antirabies activity for the leaves of *Justicia schimperiana* and *Ricinus communis* and the stem bark of *Croton macrostachyus* against PV strain was summarized in (Table 10). Although, differences were observed between antirabies activities of the extracts, each of the extracts tested in the present study displayed antirabies activity against PV strain. These differences could be due to variations in the type and nature of solvents (extractants), which can determine quantity and diversity of phytochemicals to be extracted. Plant extracts from organic solvents (ethanol and methanol) gave more antirabies activity compared to water extract. It can be attributed to the presence of higher amounts of polyphenols as compared to aqueous extracts (Tiwari *et al*., 2011).

Methanol extracts from all plant samples at a concentration of 8mg/ml showed a 100% RV-PV inhibition. The half maximal inhibitory concentration (IC_{50}) values ranged from 3mg/ml to 6mg/ml with the selectivity indices (SI) of each tested material above 2.13. The aqueous extracts of all tested samples and the ethanol extract of *Justicia schimperiana* showed relatively the highest IC_{50} values. The results exhibit that smaller the IC_{50} value higher the antirabies activity. Ethanol extracts of *Croton macrostachyus* stem bark and *Ricinus communis* leaf as well as methanol extract of *Ricinus communis* shows highest SI value against RV-PV with the value of >4.27. The selectivity indices ($SI = CC_{50}/IC_{50}$) of *Ricinus communis* (leaf) methanol extract (SI>4.27) was found to be higher than the methanol extracts of *Justicia schimperiana* (SI=3.2) and *Croton macrostachyus* (SI=2.7). SI values less than one considered as weak, greater than one moderate, and greater than three considered as good antiviral activities (Bagla *et al*., 2012).

The IC_{50} value and the selective index (SI) of ethanol, methanol and aqueous extracts of *Justicia schimperiana* (leaf) were calculated and found to be 6mg/ml (SI>2.13), 3mg/ml (SI=3.2), and 6mg/ml (SI>2.13) respectively. The methanol extract exhibited smaller IC_{50} value when compared to the other extracts. Hence, the methanol extracts of *Justicia schimperiana* leaf contain antirabies active compounds with good activity whereas; its ethanolic and aqueous extracts had shown a moderate antirabies activity against PV strain.

Croton macrostachyus stem bark showed 50% inhibition of RV-PV at 3mg/ml(ethanol and methanol extracts) and 6mg/ml(aqueous extracts).The ethanol extract of *Croton macrostachyus* stem bark contain antirabies active compounds with good activity (SI>4.27) whereas, the methanol (SI=2.7) and aqueous (SI>2.13) extracts had shown a moderate antirabies activity against PV strain.

The IC_{50} values of ethanol, methanol and aqueous extracts of *Ricinus communis* (leaf) was found to be 3 mg/ml (SI> 4.27), 3mg/ml (SI> 4.27) and 6 mg/ml (SI>2.13) respectively. This indicates that methanol and ethanol extracts had a good antirabies activity against PV strain while, aqueous extract with moderate activity. However, Mukherjee *et al*., (2017), reported the methanol extract of *Ricinus communis* exhibited the most effective activity against rabies with an IC_{50} value of 19.70 µg/ml (SI=496.95), which is many times less than the value determined in the present study (IC_{50}=3 mg/ml and SI> 4.27). This may be due to the antirabies effect of the extract depends on the type of cell line, the virus strain and the methodology used to evaluate antirabies activity of the extracts.

Table 10: Cytotoxicity (CC_{50}), inhibition concentration (IC_{50}) and selectivity index (SI) of plant extracts

Plant name	Solvent	CC_{50}	IC_{50}	SI
Justicia schimperiana	Ethanol	>12.8 mg/ml	6 mg/ml	> 2.13
	Methanol	9.6 mg/ml	3 mg/ml	3.2
	Aqueous	>12.8 mg/ml	6 mg/ml	> 2.13
Croton macrostachyus	Ethanol	>12.8 mg/ml	3 mg/ml	> 4.27
	Methanol	8 mg/ml	3 mg/ml	2.7
	Aqueous	>12.8 mg/ml	6 mg/ml	> 2.13
Ricinus communis	Ethanol	>12.8 mg/ml	3 mg/ml	> 4.27
	Methanol	>12.8 mg/ml	3 mg/ml	> 4.27
	Aqueous	>12.8 mg/ml	6 mg/ml	> 2.13

SI: Selectivity index, CC_{50} value was divided by the IC_{50} value

The figure below shows the Green bodies (Negri bodies) in brain tissue observed by using fluorescence microscope confirming the rabies infection.

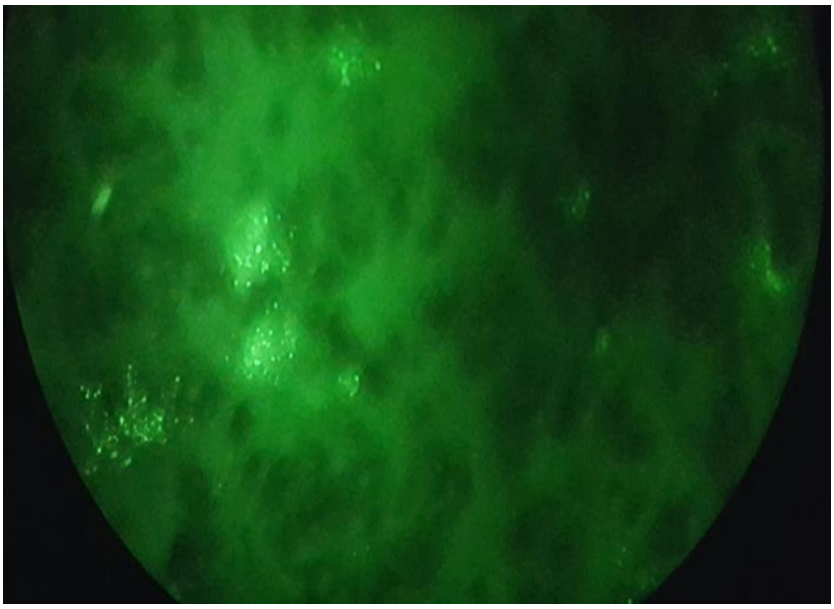

Figure 8: Negri bodies in mice brain tissue infected with rabies virus

5. Conclusions and Recommendations

5.1. Conclusions

The phytochemical analysis showed that the ethanol, methanol and water extracts of the leaves of *Justicia schimperiana* and *Ricinus communis* as well as the stem bark *Croton macrostachyus* contains a mixture of phytochemicals as alkaloids, flavonoids, phenols, steroids, tannins and terpenoids but lack saponins. All the extracts are slightly toxic in animal model but non cytotoxic in vero cell lines. All the Plant extracts had a moderate to good antirabies activity against PV strain. The methanol plant extracts gave more antirabies activity compared to ethanol and water extracts in mice.

5.2. Recommendations

- Further studies are necessary for isolation, identification and characterization of biologically active substances that are more affordable, effective and safer alternative to Imovax Rabies, Rab Avert rabies vaccine and others.
- Toxicity tests should be examined in major vital organs by histopathological examination to setup the starting dose for antirabies activity.
- The antirabies activity of plant extracts was conducted by direct fluorescent antibody test (DFA) method. However, according to the CDC, the DFA "can only be interpreted by laboratory workers with special skills, extensive training, and a specific type of microscope. Autolysed tissue samples can reduce the sensitivity of this test and often are unsuitable for confirming the presence of rabies antigen. This makes the test hard to use and access in resource-poor areas. RT-PCR is a suitable alternative for FAT in rabies diagnosis as the cost incurred per diagnostic sample is lesser than that of FAT (Babu *et al.*, 2012). Therefore, it is recommended to carry out antirabies tests by PCR-based tests as it is simpler and more precise than direct fluorescent antibody testing, and could be considered in place of the current standard test, the DFA.

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7. Appendices

Appendix 1: Drying the plant samples

Appendix 2: Weighting and shaking samples at 130 rpm

Appendix 3: Filtration and condensation process

Appendix 4: phytochemical screening

Appendix 5: Animal (mice) management throughout experimentation period

Appendix 6: Postmortem examination of mice brain

Appendix 7: Titration of Rabies virus in vivo by intracerebral inoculation

Microbiological safety cabinet, sterile pipettes (10ml and 1ml), a tray with ice flasks, rabies virus suspension (PV strain, stored at –80°C), mice (Swiss albino) 3-4 weeks old, cages for housing mice, one ml syringe and needle for I/C inoculation, test tubes, sterile PBS pH 7.4 and sterile bovine (Calf) serum, were used in the *in vivo* rabies virus titration test.

Procedure:

Working in microbiological safety cabinet, the diluting fluid which is PBS containing 2% serum was prepared and 9ml in test tubes labeled 10^{-1} to 10^{-7} were dispensed and the test tubes in rack were kept immersed in plenty of ice. To make 10 fold (log) dilutions of the virus material, 1ml of virus in 9ml of diluent was diluted to get the initial dilution i.e. 10-1. Subsequently 1ml of previous virus dilution was transferred to next dilution by using at each step a fresh pipette, to achieve serial tenfold dilutions. 0.03 ml of each virus dilution was inoculated intracerebrally into mice, starting from the highest dilution (in this case 10^{-7}). At least 6 mice per dilution were used and transferred these into cages appropriately labeled. The mice were observed for 14 days. Any death occurring within first 5 days should be considered non-specific (due to stress/bacterial infection etc). Observe for specific signs and symptoms of rabies. Note down total number of specific deaths in each dilution and calculate the virus titre by using Spearman-Karber formula.

Table 1: Calculation of virus titre in mice using the Spearman Karber method

Dose	Mice						
	Inoculated	Died	Survived	Cumulative dead	Cumulative alive	Cumulative mortality ratio	Cumulative % mortality
10^{-1}	6	6	0	31	0	31/31	100
10^{-2}	6	6	0	25	0	25/25	100
10^{-3}	6	6	0	19	0	19/19	100
10^{-4}	6	6	0	13	0	13/13	100
10^{-5}	6	4	2	7	2	7/9	78
10^{-6}	6	2	4	3	6	3/9	33
10^{-7}	6	1	5	1	11	1/12	8

The LD_{50} lies between the LD_{78} (10^{-5}) and the LD_{33}(10^{-6})

Spearman-Karber method

Log10 50% end point dilution = - (x0 - d/2 + d ∑ ri/ni)

X0 = log10 of the reciprocal of the highest dilution (lowest concentration) at which all animals are positive;

d = log10 of the dilution factor;

ni = number of animals used in each individual dilution (after discounting accidental deaths);

ri= number of positive animals (out of ni).

Summation is started at dilution x0.

To determine the LD_{50}, first calculate the proportion distance (PD):

$$PD = \frac{50\% - (\%\ mortalitybelowLD50)}{(\%\ mortalityaboveLD50) - (\%\ mortalitybelowLD50)}$$

$$PD = \frac{50\% - 33\%}{78\% - 33\%} = 0.38$$

X0 = 4; d = 1; log10 of 50% endpoint dilution = - [4 - ½ + 1 (13/6)] = -5.6;

50% end point dilution = $10^{-5.6}$; the titre of the virus = $10^{5.6}$ LD50/0.1ml

Appendix 8: Calculation of median lethal dose (LD_{50}) of plant extracts in Swiss Albino mice

Median LD_{50} is usually an initial screening step in the assessment and evaluation of the toxic characteristic of a substance. It is the amount (dose) of an extract, which produce death in 50% of the population of test animals to which it is administered by any of the methods such as oral, dermal inhalation or intravenous. Determination of this test examines the relationship between dose and the most extreme response-death. The more potent or toxic the chemical (extract), lower the LD_{50} and the smaller the dose needed to cause death. LD_{50} figures are frequently used as a general indicator of a substance's acute toxicity (Chandra *et al.*, 2014). The percentage of mice that died at each dose was transformed to probit using Finney's method (Table 2).

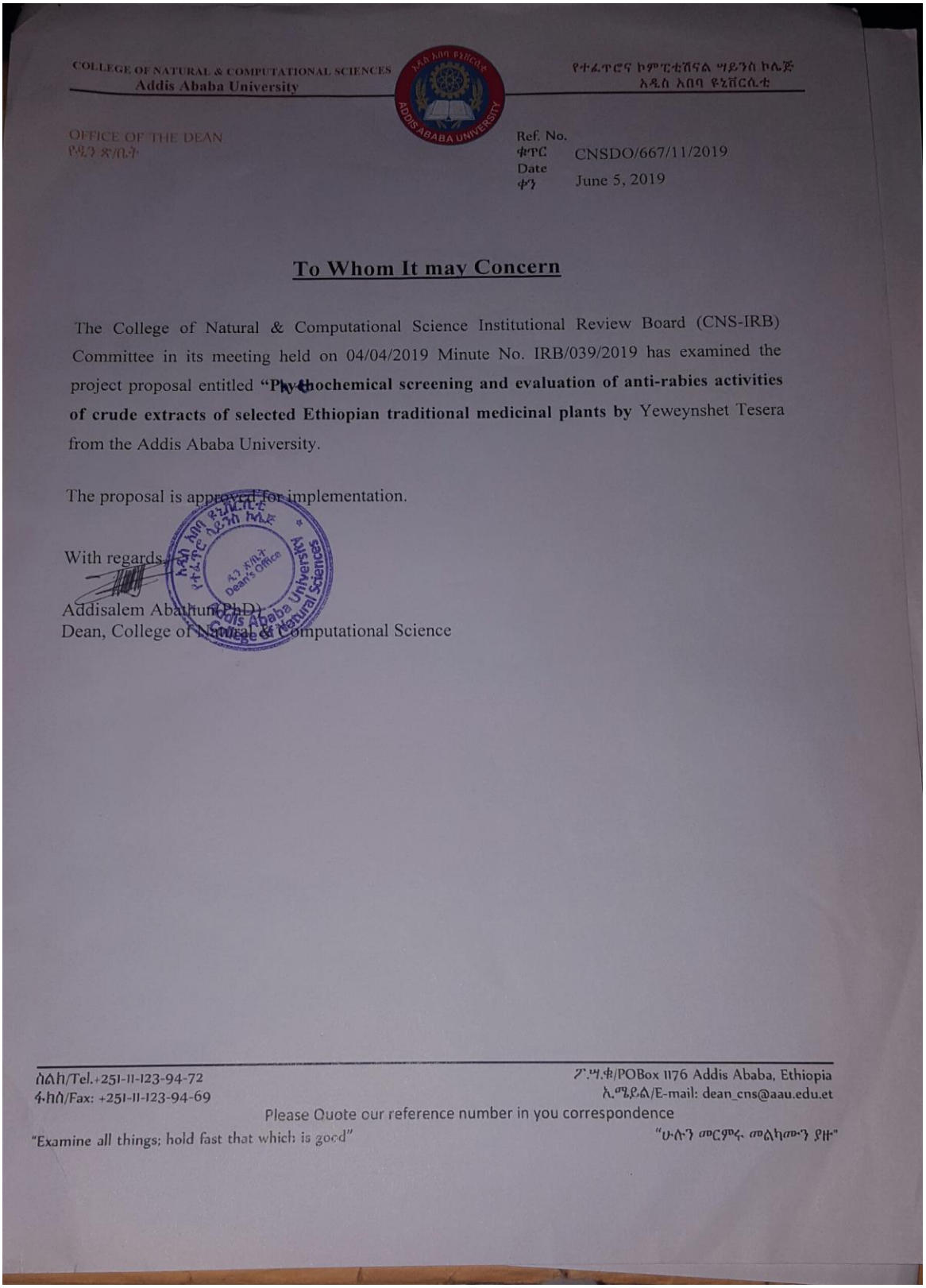
Table 2: Transformation of percentage mortalities to probits

%	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33

Source: (Yadav and Trivedi,2016)

According to the methods of Finney and Miller-Tainter, Probits values for mortality of 0% and 100% at n=6 was found to be 2.76 and 7.24 respectively.

Appendix 9: Ethical clearance certificate



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Date
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To Whom It may Concern

The College of Natural & Computational Science Institutional Review Board (CNS-IRB) Committee in its meeting held on 04/04/2019 Minute No. IRB/039/2019 has examined the project proposal entitled **"Phytochemical screening and evaluation of anti-rabies activities of crude extracts of selected Ethiopian traditional medicinal plants by** Yeweynshet Tesera from the Addis Ababa University.

The proposal is approved for implementation.

With regards,

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"Examine all things; hold fast that which is good"

"ሁሉን መርምሩ መልካሙን ያዙ"

Advisors´ Approval Sheet

This is to certify that the thesis entitled “Phytochemical Screening, Acute Toxicity and Anti-Rabies Activities of Extracts of Selected Ethiopian Traditional Medicinal Plants” submitted in partial fulfillment of the requirements for the Degree of Master of Science in Applied Microbiology and has been carried out by Yeweynshet Tesera Tewabe, under my supervision. Therefore, I recommend that the student has fulfilled the requirements and hence hereby can submit the thesis proposal to the Department.

Asnake Desalegn (PhD) _____ _____

Name of Advisor Signature Date