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DETERMINATION OF ANTI-RABIES VIRUS ACTIVITIES OF CRUDE EXTRACTS FROM SOME TRADITIONALLY USED MEDICINAL PLANTS IN EAST WOLLEGA, ETHIOPIA

MSc THESIS

BY

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BISHOFTU, ETHIOPIA

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A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Veterinary microbiology

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STATEMENT OF THE AUTHOR

First, I affirm that this thesis is my unalloyed work and that all sources of material used for this thesis has been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for MSc degree at Addis Ababa university, Collage of Veterinary Medicine and Agriculture and is deposited at the university/ collage library to be made available to borrowers under rules of the library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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Name Signature

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Date of Submision:_____

DEDICATION

I dedicate this thesis manuscript to my beloved child Yonathan Demeke, for the nice time he enjoyed the new world on the achievement of my second degree.

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ABBRIVIATIONS

ABLV	Australian bat lyssa virus
ANOVA	Analysis of variance
ARAV	Aravan virus
BBB	Blood brain barrier
CCEEV	Cell culture embryonated egg based rabies vaccine
CDC	Center of disease control
cDNA	complementary Deoxyribo nucleic acid
CNS	Central nervous system
DUVV	Duvenhage virus
EBLV	European bat lyssa virus
EHNRI	Ethiopian health and nutrition research institute
ELISA	Enzyme linked immune sorben assay
EPA	Environmental protection agency
EPHI	Ethiopian public health institute
ERA	*Evelyn Rokitniki Abelseth*
FAT	Fluorescent antibody test
FITC	Fluorescent Isothio-cyanate
G	Glycoprotein
HCl	Hydro Chloric acid
IRKV	Irkut virus
KHUV	Khujand virus
L	Large protein
LBV	Lagos bat virus
LC8	Light chain eight
M	Matrix protein
MICLD50	Mouse intra-cerebral lethal dose fifty

ABBRIVIATIONS (*Continued***)**

mm	millimeter
MOKV	Mokola virus
MST	Mean survival time
MTCV	Modern tissue culture vaccine
N	Nucleo protein
NaOH	Sodium hydro-oxide
NCAM	Neural cell adhesion molecule
nm	nanometer
NTV	nurve tissue vaccine
OIE	Office of international des epizootics
P	Phospho-protein
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
RABV	rabies virus
RNA	Ribonucleic acid
RNP	Ribo nucleo protein complex
rpm	revolution per minute
SB	Highly pathogenic silver haired bat strain
SN	non pathogenic silver hair bat strain
USA	United State of America
VNA	Viral neutralization antibody
VRG	Vaccinia virus expressing rabies virus G
WHO	World health organization

ABSTRACT

An experimental study was conducted between November, 2016 and April, 2017 at Ethiopian public health institute to evaluate the anti-rabies activities of the leaf of *Justicia schimperiana*, root of *Phytolacca dodecandra*, root bark of *Croton macrostachyus* and their combination at 1:1:0.75 ratio, respectively. The plants are widely used traditionally for the treatment of rabies in humans and animals in East Wollega, Ethiopia. The plant parts were dried, grinded and crude extraction was conducted as per standard of Ethiopian Public Health Institute. Crude extracts were prepared from the powders of these plants using 80% hydro-ethanol and dried by condensation process. The anti-rabies activities of these plant extracts were tested in three different doses: 300, 2000 and 5000 mg/kg in mice and compared with positive control based on the difference in mean survival time of group of mice challenged with rabies virus (CVS-11). The result showed that *P.dodecandra*, *J. schimperiana* and combination of all the three plant extracts at 300 and 2000mg/kg dose levels and *C. macrostachyus* at 300 and 5000mg/kg doses didn't significantly($P>0.05$) increase the survival period of mice. However, at 5000 mg/kg dose level for *P.dodecandra* ($P=0.002$), *J. schimperiana* ($P=0.038$) and combination of all the three extracts ($P=0.021$) and at 2000mg/kg dose level for root bark of *C. macrostachyus* ($P=0.011$)*;* the plant extracts were significantly ($P<0.05$) increased the survival time of mice. The finding illustrates the existence of promising active compounds against rabies virus in extracts of roots of *P. dodecandra* and root bark of *C. macrostachyus* at indicated doses preferably to other extracts and needs further research to elucidate its active ingredients targeted to antiviral activity. The results also suggested good correlation between the *in vivo* anti-rabies virus activities and traditional therapeutic uses reported by traditional healers.

Key words: *Anti-rabies activity, Cytotoxicity, East Wollega, EPHI, Ethiopia, Hydroethanolic extracts, Mice, Traditional medicinal plants*

1. INTRODUCTION

Rabies is a fatal viral zoonosis, which causes encephalitis in warm-blooded animals and humans (Zulu *et al*., 2009). Rabies virus belongs to the order *Mononegavirales*, family *Rhabdoviridae*, and genus *lyssavirus*. Despite the invention and application of the first rabies vaccine by Louis Pasteur in 1885, human rabies, is still deadly disease globally (Hendekli, 2005). In the world, it has been estimated that about 55,000 people die from rabies each year, of which the highest death is in Asia and Africa due to the presence of endemic canine rabies and dogs remain the major animal reservoirs in such areas (Wunner & Briggs, 2010).

Canine rabies continued to be a serious problem in Africa, including Ethiopia (Pritchard and Dagnatchew, 2010). Although there are no formal studies, it is estimated that there is one owned dog per five household nationally (Deressa *et al*., 2010). The highest number of rabies cases was reported in cold season (June to September) in Ethiopia. This is most probably due to mass gathering and highest reproduction of dogs during the period which increases the contact between rabid and health dogs (Admasu and Mokonen, 2014). Low level of public awareness, lack of nationwide rabies (dog) surveillance, poor attention and resource allocation by the government are major constraints that hinder the control of rabies in Ethiopia. Ethiopia being one of the developing countries is highly endemic for rabies approximately 10,000 people were estimated to die of rabies annually which makes it to be one of the worst affected countries in the world (Fekadu, 1997).

The investigation of antiviral substances against rabies virus is not widely documented (Yamamoto *et al*., 1990; Lockhart *et al*., 1992; Pietropaolo *et al*., 1993; Marcheti *et al*., 1995), probably because of the well established prophylaxis. However, human rabies cases associated with solid organ transplants (Srinivasan *et al*., 2005) and after vaccination failure (Arya, 1989) demonstrate the need of a specific and efficient antiviral treatment for this disease. Recently, a

surprising reversal of a human rabies case occurred in the USA: a 15-year-old girl, after contacting an insectivorous bat, developed rabies and survived with an experimental protocol which included ribavirin, ketamine, amantadine and other anesthetic drugs (Willoughby *et al*., 2005). Once more, this fact encourages the search for agents with anti-rabies activity.

In most developing countries like Ethiopia nervous tissue vaccines are administered to the people after exposure to rabid animal. These vaccines have side effects with neuro-paralytic complications and should be replaced by modern cell culture vaccines (Hemachudha *et al*., 2002). Unlike other infective agents, viruses totally depend on the cell they infect for their multiplication and survival. Thus, a prospective antiviral agent may also damage the cell that houses the organism, and is therefore undesirable. It is for this main reason that many afflictions of viral origin still remain elusive to therapeutic and/or prophylactic means, though there have been very successful vaccines for the control of certain viral diseases. Therefore, the search for antiviral agents that are selectively virucidal remains a goal. In this regard, the potential of medicinal plants, especially those employed in indigenous medicine, is believed to be very significant in providing novel antiviral compounds or prototypes (Dawit *et al*., 2003). Rabies virus infection was reduced when infected cells were treated with South American plants and algal polysaccharides extracts (Muller *et al*., 2007; Mansour *et al*., 2011). In addition, phenolic compounds were also tested and showed some activity against rabies virus (Chavez *et al*. 2006).

The World Health Organization estimates that at least 80 % of the populations in most developing countries rely for their primary health care on traditional forms of health care (WHO, 1993). Recognizing this fact and the fact that modern health care system alone could not meet the health needs of the entire population of the world, the policy of urging its member states to promote and integrate traditional medicine into their national health care systems was launched by WHO in 1978 (WHO, 1996). The treatments recommended for people bitten by rabid animals mainly dogs have been recorded in many Ethiopian medical books since the early 17th century (Sterleyn, 1968 cited by Fekadu, 1982). The employment of a wide variety of supposed cures by early traditional practitioners illustrated the well-established character of the traditional pharmacopoeia in the country (Richard, 1990).

Most researchers in Africa have recognized the importance of recording traditional knowledge for further development, such as in Ethiopia (Teklehaymanot, 2007), Tanzania (Gessler, 1995) and South Africa (Grace, 2003). Traditional medicinal plants are also a rich source of inexpensive and novel biologically active compounds (Hostettmann, 2000), which are of great interest to both the developed and developing countries (McGaw, 2000). They have the ability to inhibit the replication cycle of various types of DNA or RNA viruses (Jassim and Naji, 2003). The evaluation of the efficacy, safety and dosage of traditional medicines is crucial, due to the reliance of the African population on plants as sources of medicines (Masika, 2002).

Ethiopia is a land of great topographical diversity, which is responsible for tropical, temperate and subtropical conditions. It is a land of multiplicity flora and fauna. This environmental mosaic accentuated the diversity of plant and animal life (Dawit and Ahadu, 1993). For centuries, most of the populations in Ethiopia, like other developing countries, have relied on a system of traditional medicine, which consists of both emperico-rational and magico-religious elements and at times a combination of both (Geyid *et al*., 2005). Tollosa (1997) has identified and documented 37 herbal plant species from central high lands of Ethiopia used as remedy for various animal diseases. In developing countries like Ethiopia, cost of treatment is an important determinant of the use of veterinary drugs. Moreover, they cannot deliver complete coverage in preventive and curative health care practices because of inadequate labor, logistic problems and an erratic supply of drugs. Consequently, the majority of those raising stock in rural areas are far from site of veterinary stations so that applying veterinary herbal medicine is the only option they have (Brhanemeskel *et al*., 2008).

In Ethiopia, traditional medicine practices are deep-rooted and continue to be widely used among both rural and urban population. This wide spread use of traditional medicine in the country could be attributed to cultural acceptability, efficacy against certain type of diseases, physical accessibility and economic affordability as compared to modern medicine (Tilahun and Mirutse, 2006).

Several traditional herbs have been formulated by traditional healers to treat human and animal rabies before occurrence of clinical signs. The fact that herbal medicines have been employed for

such a long time does not guarantee their efficacy and safety. Thus, antiviral activity evaluation of medicinal plants remains important to provide the society with new, effective and safe drugs. Traditional healers use traditional plants like *Phytolacca dodecandra* locally Endod; which belongs to family "Phytolaccaceae" a perennial plant for treatment of Rabies. Admasu *et al.* (2014) evaluated the efficacy of anti-rabies activities of hydroethanolic extract of roots and leaves of *P. dodecandra* in mice in which the leaves, of this plant showed some anti-rabies effect at higher doses. The hypothesis (H0) of this study was therefore; traditional medicinal preparations for *P. dodecandra*, *Justicia schimperiana*, *Croton macrostachyus* and their combination against rabies virus are not effective as traditional healers stipulate.

Therefore, this study was conducted with the following objectives:

- Evaluation of anti-rabies activities of crude extracts from roots of *Phytolacca dodecandra*, leaves of *Justicia schemperiana,* root bark of *Croton macrostchyus* and their combination in mice model.
- Generation of a highlight regarding the efficacy of the crude extracts of these medicinal plants against rabies virus.

2. LITERATURE REVIEW

2.1 Classification and taxonomy of rabies virus

The rabies virus genome consists of a single stranded, non-segmented, enveloped, negative sense RNA of approximately 12kb (Tordo *et al.,* 1986). It is under *Lyssavirus* genus (Wunner 2002), in the family of *Rhabdoviridae*, order *Mononegavirales* which is transmissible to all mammals (WHO, 1996).

Eleven distinct species can be distinguished within the genus *Lyssavirus* by cross-protection tests and molecular biological analysis (Baer, 1991; Bourhy *et al*., 1993), namely the classical rabies virus itself (RABV, genotype 1, serotype 1), Lagos bat virus (LBV, genotype 2, serotype 2), Mokola virus (MOKV, genotype 3, serotype 3), and Duvenhage virus (DUVV, genotype 4, serotype 4). The European bat lyssa viruses (EBLV), subdivided into two biotypes (EBLV1, genotype 5 and EBLV2, genotype 6) and the Australian bat lyssa virus (ABLV, genotype 7), isolated in Australia (Hooper *et al*., 1997), are also members of the *Lyssa virus* genus, but are not yet classified into serotypes. Also four putative viruses (Aravan, Khujand, Irkut and West Caucasian Bat Virus, Shimoni bat virus isolated in 2009) as well as Bokeloh bat lyssavirus (Freuling*et al.*, 2011) and the Ikoma *lyssavrus* (Marston *et al*., 2012) were discovered in 2011 and 2012 respectively. Viruses of serotypes 2–4, EBLV and ABLV are known as rabies-related viruses.

The *lyssaviruses* have been divided into two phylogroups with distinct pathogenicity and immunogenicity (Badrane *et al.*, 2001). For RABV, DUVV, EBLV and ABLV, conserved antigenic sites on the surface glycol-proteins allow cross-neutralization and cross-protective immunity to be elicited by rabies vaccination. A reduced protection with pre-exposure vaccination and with conventional rabies post-exposure prophylaxis was observed against IRKV, ARAV, and KHUV (Hanlon *et al.,* 2005) and all of the above-mentioned *lyssavirus* species were assigned to phylogroup 1. Little or no cross-protection against infection with the members of

phylogroup 2 (MOKV and LBV) is elicited by rabies vaccination and most anti-rabies virus anti-sera do not neutralise these *lyssaviruses* (Badrane *et al.*, 2001).

2.2 Viral structure/ morphology

Rabies viruses have approximately 180 nm long and 75 nm wide (Drew, 2004) and its genetic information is organized in the form of a helical ribonucleoprotein complex (RNP), in which the linear RNA is tightly associated with the viral nucleoprotein (Figure 1). The genome of RABV encodes for only five proteins in the order: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the large protein (L, also termed RNA-dependent RNA polymerase, RdRp) (Albertini*et al.*, 2011) (Figure 2). The N plays a critical role in viral transcription and replication (Albertini *et al.*, 2002). The G forms approximately 400 trimeric spikes, which are tightly arranged on the surface of the virions. The G is a major determinant for RABV neuropathogenicity by binding specific receptor(s), entering the nervous system through the endosomal transport pathway via a low-pH-induced membrane fusion process (Gaudin, 2000). All transcription and replication events take place in the cytoplasm inside a specialized 'virus factory', the Negri body (Lahaye *et al*., 2009).

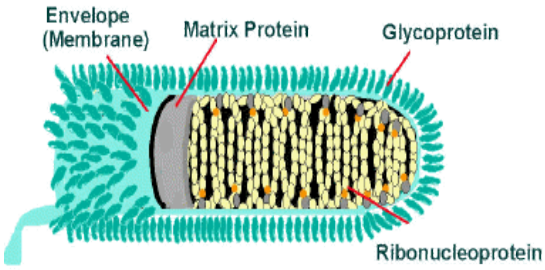

Figure 1: A bullet shape of rabies virus

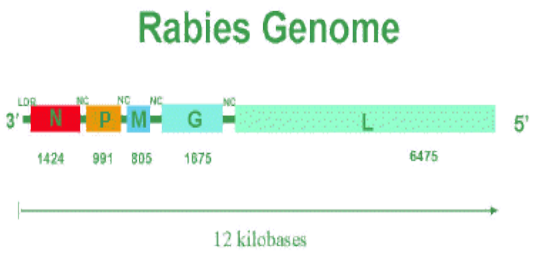

Fig 2: Genomic structure of rabies virus

Source:http://viralzone.expasy.org/all_by_species/2.html. Accessed on 16 September, 2016

2.3 Distribution and animal reservoir of rabies

Although all mammals are susceptible to RABV, only a few species are important as reservoirs for the disease. Dogs remain the most important reservoirs for rabies in the developing countries of Asia and Africa (Lackay *et al*., 2008). According to WHO, high death rate was experienced in India in 2004 and lowest in Cambodia and Magnolia (Raux, *et al*., 2000). In 2006-2007, more than 3,000 rabies cases were reported which reduced to 205 in the year 2008 in China (Iwasaki, 2002). In Sri Lanka, more than 95% of approximately 100 human rabies deaths occur each year due to the bites by unvaccinated dogs (Rozario, 2008). According to a study by the National Rabies control program of Pakistan, Punjab, Sindh and the Khyber Pakhtunkhwa as well as few districts of Naseerabad, Jafferabad, and Pishin in Baluchistan are categorized as the high risk areas for rabies (Smith and Rupprecht, 2008).

Rabies causes at least 24,000 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 the developed nations, dog rabies has been eliminated or controlled through enforced policy of animal mass vaccination during the past 70 years (Finnegan *et al*., 2002). The epidemiological and genetic analysis of many isolates showed that canine rabies remains in certain countries as well as borders of Europe. In 1946, a total of 8,384 indigenous rabies cases were reported among dogs and 33 cases in human in USA. After that there was noticeable decrease in rabies cases in USA. In 2006, none of human rabies case was reported in the country. However, wildlife rabies becomes a major concern. Canine rabies has been eliminated from Western Europe, Canada, Japan, Malaysia and a few Latin American countries; while Australia is free from carnivore rabies, and many Pacific island nations have always been free from rabies and related viruses. In these areas, human deaths from rabies are restricted to people exposed while living or travelling in areas endemic for canine rabies. About two deaths per year due to imported human rabies have been reported in Europe, North America and Japan (Malerczyk *et al*., 2011).

In North America, rabies is endemic in raccoons, foxes, coyotes and skunks (Finnegan *et al*., 2002) while fox rabies is endemic in Europe (Irsara *et al*., 1982). Wolves, jackals, and other wild animal species have also been reported as reservoirs in other regions (Sillerozubiri *et al*., 1996). Bats are probably the ultimate reservoirs for rabies virus (Constantine, 1979). In the Americas, a number of bat species carry distinct rabies virus strains (Krebs *et al*., 2000), whereas in Europe and Australia bats carry rabies-related *lyssa viruses* (Lackay *et al*., 2008).

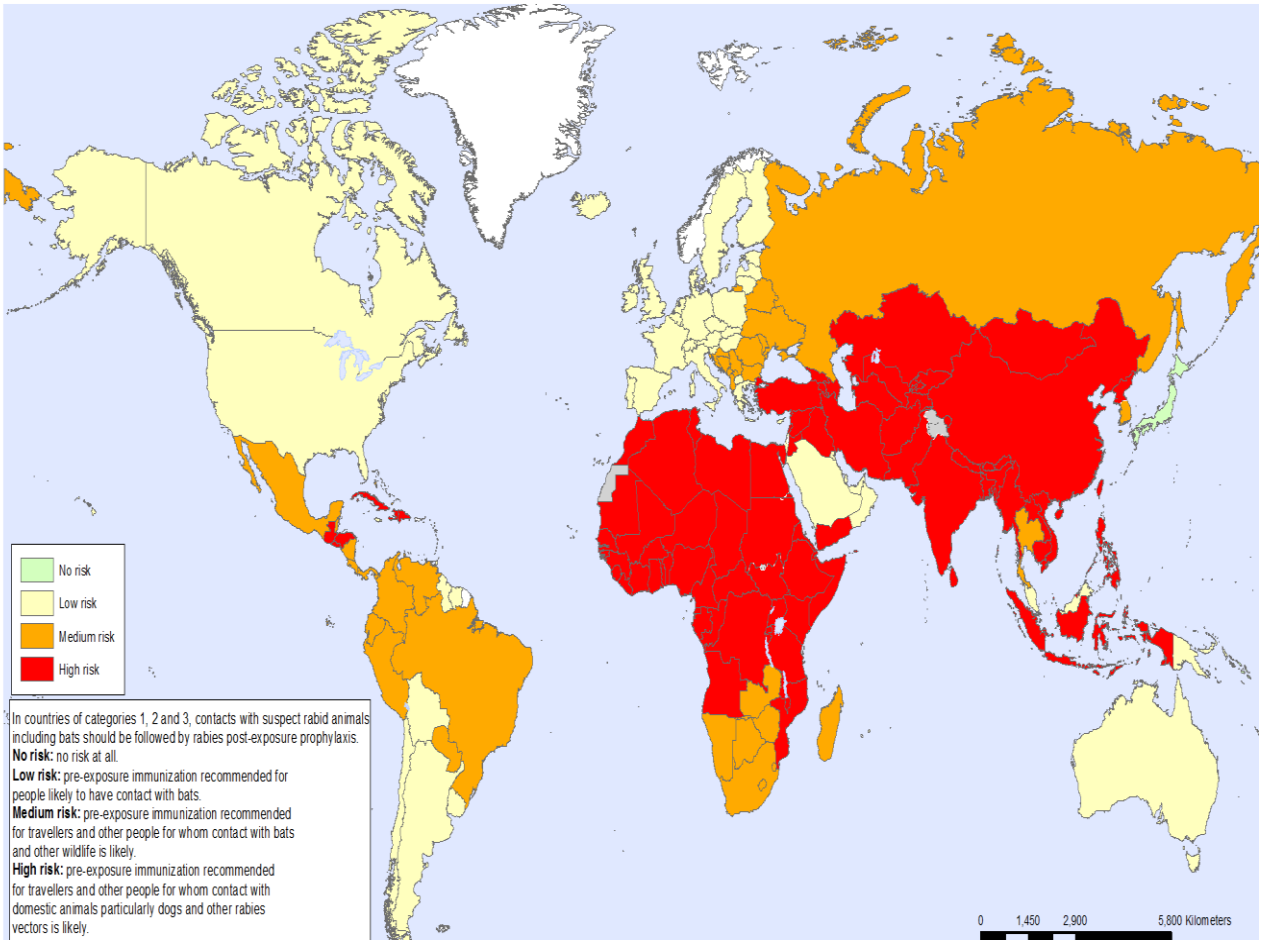

Figure 3: Distribution of rabies in the world, based on data of 2013

Source: WHO, 2013

Table 1: Rabies free countries and territories

Asia	America	Europe	Oceana	Africa
Bahrain	Antigua and Barmuda	Albania	Cook Islands	Cape Verde
Cyprus	Bahamas	E.Y.R.of Macedona	Fiji	Congo
Hong Kong	Barbados	Finland	French Polynesia	Libya
Japan	Belize	Gibraltar	Guam	Mauritius
Malaysia	Falkland	Greece	New Caledonia	Reunion
Maldives	Jamaica	Iceland	New Zealand	Seychelles
Qatar	Saintkitts and Nevis	Isle of Man	Papua New Guinea	
Singapore	Trinidad and Tobago	Malta	Solomon Islands	
Lakchyadeep, Andaman and Nicobar islands of India	Uruguay	Norway (except Svalbard and isl.)	Vanuatu	
			Portugal	
			Spain (except centa + Melill)	
			United Kingdom	

Source: Yousaf *et al*., 2012

2.4 Transmission

All warm-blooded species, including humans, may become infected with rabies virus and develop symptoms. Birds were first artificially infected with rabies in 1884; however, infected birds are largely if not wholly asymptomatic, and recover (Shannon *et al*., 1988). Other bird species have been known to develop rabies antibodies, a sign of infection, after feeding on rabies-infected mammals (Gough and Jorgenson, 1976)

The virus has also adapted to grow in cells of poikilothermic ("cold-blooded") vertebrates (Wong, 2009). Most animals can be infected by the virus and can transmit the disease to humans. Infected bats, monkeys, raccoons, foxes, skunks, cattle, wolves, coyotes, dogs, mongooses (normally yellow mongoose) (Taylor, 1993) and cats present the greatest risk to humans.

Rabies may also spread through exposure to infected bears, domestic farm animals, groundhogs, weasels, and other wild carnivores. Lagomorphs, such as hares and rabbits, and small rodents such as chipmunks, gerbils, guinea pigs, hamsters, mice, rats, and squirrels, are almost never found to be infected with rabies and are not known to transmit rabies to humans (CDC, 2010). Bites from mice, rats, or squirrels rarely require rabies prevention because these rodents are typically killed by any encounter with a larger, rabid animal, and would, therefore, not be carriers (Anderson & Frey, 2006). The Virginia opossum is resistant but not immune to rabies (McRuer and Jones, 2009).

The virus is usually present in the nerves and saliva of a symptomatic rabid animal (Merck manual, 2003). The route of infection is usually, but not always, by a bite. In many cases, the infected animal is exceptionally aggressive, may attack without provocation, and exhibits otherwise uncharacteristic behaviour (Turton, 2000). This is an example of a viral pathogen modifying the behaviour of its host to facilitate its transmission to other hosts.

Transmission between humans is extremely rare. A few cases have been recorded through transplant surgery (Srinivasan *et. al*., 2005). After a typical human infection by bite, the virus enters the peripheral nervous system. It then travels along the afferent nerves toward the central nervous system (Jackson and Wunner, 2002). During this phase, the virus cannot be easily detected within the host, and vaccination may still confer cell-mediated immunity to prevent symptomatic rabies. When the virus reaches the brain, it rapidly causes encephalitis, the prodromal phase, which is the beginning of the symptoms. Once the patient becomes

symptomatic, treatment is almost never effective and mortality is over 99%. Rabies may also inflame the spinal cord, producing transverse myelitis (Lynn *et al.,* 2012). As the virus is present in sperm or vaginal secretions, spread through sex may be possible (RabiesAlliance.org).

2.5 Rabies pathogenesis

Despite the long history of rabies, its pathogenic process remains poorly understood. Most of what we know about the disease process is acquired from investigations conducted in experimental animal models. The overall outcome of an exposure to RABV depends in part upon the rabies genotype (different strains and mutants) or variant involved, its pathogenicity (apoptogenicity, neuroinvasiveness), the dose of virus inoculated (severity of exposure), the route as well as the host species and its susceptibility to the particular pathogen together with innate and adaptive immune responses of the host (Franka *et al.,* 2009). However, various studies in animal models indicate that the pathogenic wild-type/street RABV and the fixed (laboratory-adapted) RABV evidently behave differently in each step of their life cycle in the host.

RABV G is the only surface protein of the virion and capable of inducing virus neutralizing antibodies (VNA) (Benmansour *et al*., 1991). The G protein plays an important role in rabies pathogenesis (Ito *et al*., 2001) by binding to neural receptor such as acetylcholine receptor (Lentz *et al*., 1982)and neural cell adhesion molecules (NCAM) (Thoulouze *et al*., 1998) contributing to the exclusive neurotropism and neuroinvasiveness of RABV (Thoulouze *et al*., 2000). Virus may enter muscles and replicate at the site of inoculation or enter directly into peripheral nerves without prior replication in non-neural tissues (Shankar *et al*., 1991). It is believed that once virus particles enter the peripheral nervous system and start to spread to the CNS, a fatal outcome of the disease is inevitable, though there are some reports of rabies survivors. RABV enters motor and/or sensory axons of peripheral nervous system and spreads to the CNS by retrograde fast axonal transport at a rate of approximately 50–200 mm/day (Tsiang *et al*., 1991).

The pathogenic RABV have evolved specific mechanisms to escape early immune system recognition in the periphery via limited replication, minimized G expression (Wang *et al*., 2005)

suppression of interferon response, anti-apoptotic stimulation, and transportation through neurons only. On the other hand, fixed RABV induces extensive inflammation by activating innate immune responses (Wang *et al*., 2005), induces apoptosis (Sarmento *et al.*, 2006), replicates to higher levels and express high levels of the G protein (Zhang *et al*., 2013). However, the mechanism adopted by the fixed RABVs to elicit immune responses and the wild-type RABVs to evade immune system is still not entirely clear. It has been shown that the innate immune responses and inflammation in the CNS is associated with BBB permeability enhancement (Fabis *et al*., 2008) in mice infected with fixed RABV but not in those infected with street RABV (Kuang *et al*., 2009). Current understanding on the striking difference between pathogenic and nonpathogenic rabies biology is summarized (Table 2).

Table 2: Striking difference between pathogenic and non-pathogenic RABV biology

Parameters	Non-pathogenic virus (fixed/lab-adapted)	Pathogenic virus (street/wild-type)	References
Cellular Tropism	Not exclusively neuronal	Highly neuronal	Thoulouze *et al.* (2000); Morimoto *et al*. (1999)
Glycoprotein Expression Levels	High	Low	Zhang *et al*. (2013); Morimoto *et al.* (1999)
Replication (titer)	High	Low	Thoulouze *et al.* (2000)
Apoptosis	High	Low	Morimoto *et al.* (1999); Yan *et al*. (2001)
Interferon sensitivity	Resistant	Highly sensitive	Niu *et al*. (2011)
Immune System	Activates innate/adaptive immunity	Evades innate/adaptive immunity	Shoji *et al*.(2004); Megret *et al*. (2005)
Blood-brain-barrier (BBB) permeability	Enhances	Little or no change	Phares *et al*. (2006); Faber *et al*. (2009)

Until recently, our knowledge of RABV pathogenesis was limited and largely based on descriptive studies with street RABV strains or experimental infection with attenuated laboratory-adapted strains. The advent of reverse genetics technology made it possible to identify viral

elements that determine the pathogenic phenotype of RABV and to obtain a better insight into the mechanisms involved in the pathogenesis of rabies (Dietzschold *et al*., 1983).

2.5.1 Viral elements involved in virus uptake

After receptor binding, rabies virus is internalized via adsorptive or receptor-mediated endocytosis (Marsh and Helenius, 1989). Then, the low pH environment within the endosomal compartment causes a conformational change in RABV G, which triggers the fusion of the viral membrane to the endosomal membrane, thereby releasing the RNP into the cytoplasm (Gaudin *et al.*, 1991). It has been demonstrated that the pathogenicity of tissue culture-adapted RABV strains like ERA correlates with the presence of antigenic determinant located in antigenic site III of the G protein (Dietzschold *et al*., 1983). An Arg→Gln mutation at position 333 in this antigenic site of the ERA G protein resulted in a sevenfold delay of internalization of the Gln333 RABV variant as compared with that of the wild-type.

An Asn_{194}→Lys_{194} mutation in RABV G, which accounted for the re-emergence of the pathogenic phenotype, was associated with a significant decrease in the internalization time (Faber *et al*., 2005). The time necessary for internalization of RABV virion was significantly increased and the pathogenicity was strongly reduced after exchange of the G gene of the highly pathogenic RABV strain SB, which was derived from a cDNA clone obtained from the silver-haired bat-associated RABV-18 strain (Faber *et al.*, 2004), with that of a highly attenuated SN strain, which was rescued from a cDNA clone of the SAD B19 RABV vaccine strain (Schnell *et al*., 1994). These support the notion that the kinetics of virus uptake, which is a function of RABV G, is a major factor that determines the pathogenicity of RABV.

2.5.2 Viral elements involved in virus spread & transport

A unique property of RABV is its ability to spread from cell-to-cell and it is transported intact within endosomes, but the significance of this transport for productive infection has not been examined (Iwata *et al*., 1999). The observation that the Gln333 ERA variant loses pH-dependent cell fusion activity *in vitro* (Dietzschold *et al*., 1985) and exhibits a strongly reduced ability to spread from cell to cell (Coulon *et al.*, 1989) suggests that RABV G also plays a pivotal role in cell-to-cell spread and thus viral transport, probably through its fusiogenic activity.

The finding that viral spread of the ERA G $Arg_{333} \rightarrow Gln_{333}$ mutant within the CNS is strongly reduced as compared with that of the wild-type (Dietzschold *et al*., 1985), also points to the function of intact RABV G in trans-synaptic spread. The strongest evidence for an essential role of RABV G in trans-synaptic transport, however, comes from intracranial infection of mice with a RABV G-deficient recombinant virus, which showed that the infection remained restricted to neurons at the inoculation site without any signs of spread to secondary neurons (Etessami *et al*., 2000).

In addition to RABV G, RABV M also plays a role in virus spread and thus in trans-synaptic transport. In this respect it was shown that spread of the chimeric RABV SN-BMBG variant, which contains both M and G from the highly pathogenic SB, was significantly higher than that of the chimeric SN-BG or SN-BM, which contain G and M from SB, respectively, suggesting that an optimal interaction of M with G might play an important role in virus cell-to-cell spread (Pulmanausahakul *et al.*, 2008). Since RABV M supports virus budding (Mebatsion *et al*., 1999), it is likely that the more efficient spread of the chimeric RABV SN-BMBG variant is due to optimal virus budding at the postsynaptic membrane. Peripheral infection of adult mice showed that removal of the LC8 binding domain from RABV P does not prevent virus entry into the CNS suggesting that the RABV protein is not directly involved in the retrograde axonal spread of RABV (Tan *et al.*, 2007).

2.5.3 Viral elements that control virus replication

Unlike many other viruses, such as influenza virus, the pathogenicity of RABVs correlates inversely with the rate of viral RNA synthesis and the production of infectious virus particles. The M of all *rhabdoviruses* is able to shut down viral gene expression by binding to the RNP, resulting in a highly condensed skeleton-like structure that is unable to support RNA synthesis (Mebatsion *et al*., 1999).

To identify other viral elements that control pathogenicity by regulating virus replication, 5′-end sequences of the highly pathogenic SB strain were exchanged in a stepwise manner with those of the highly attenuated vaccine strain SN, resulting in the recombinant viruses SB2 (trailer sequence [TS] + L), SB3 (TS + L + pseudogene [Ψ]), SB4 (TS + L + Ψ + G) and SB5 (TS + L + Ψ + G + M) (Faber *et al.*, 2004). Intramuscular infection with parental viruses SB and SN, and with the chimeric RABVs SB2, SB3, SB4 and SB5, revealed the highest mortality rates in mice infected with SB and no morbidity or mortality in mice infected with SN. Replacement of TS, L and Ψ in SB with the corresponding elements from SN resulted in a moderate decrease in morbidity and mortality, and additional exchange of G or G plus M strongly reduced or completely abolished virus pathogenicity. Additional evidence for an inverse correlation between pathogenicity and the rate of viral RNA synthesis and virus particle production was obtained from mice infected with chimeric recombinant viruses in which the G and M genes of the attenuated SN strain were exchanged with those of the highly pathogenic SB strain (Pulmanausahakul *et al.*, 2008). These experiments revealed a significant increase in the pathogenicity of the SN parental strain bearing RABV G from the pathogenic SB strain. These data indicate that both G and M play an important role in RABV pathogenesis by regulating virus replication. The finding that replacement of G or G plus M in SN by the G or G plus M of SB results in a moderate or strong decrease in viral RNA transcription and replication, respectively, while replacement of only the M in SN with the SB M results in a strong increase in viral RNA transcription and replication, indicating that RABV G also has an important regulatory function in viral RNA transcription/replication, either alone or via interaction with the M protein. The

mechanism by which the RABV G gene controls viral RNA synthesis is not known (Pulmanausahakul *et al.*, 2008).

To evade the immune response and to preserve integrity of the neuronal network, pathogenic RABV strains, but not attenuated strains, can regulate their growth rate. A lower replication level probably benefits the pathogenic RABV strains by conserving the structure of neurons that are used by these viruses to reach the CNS. Another explanation for the lower replication rate of pathogenic RABVs is that in order to evade early detection by the host immune system, the virus keeps the expression levels of its antigens at a minimum (Wang *et al*., 2005).

2.6 Clinical manifestation

The period between infection and the first symptoms (incubation period) is typically 1–3 months (Giesen, 2015). Incubation periods can be as short as four days and longer six years have been documented, depending on the location and severity of the contaminated wound and the amount of virus introduced (Giesen, 2015). Initial signs and symptoms of rabies are often nonspecific such as fever and headache (Giesen, 2015). As rabies progresses and causes inflammation of the brain and/or meninges, signs can include slight or partial paralysis, anxiety, confusion, abnormal behavior, aggressiveness, hydrophobia and hallucinations, progressing to coma and death (Cotran, 2005).

Death usually occurs 2 to 10 days after first symptoms. Survival is rare once symptoms have presented, (Giesen, 2015) even with the administration of proper and intensive care (Rupprecht, 2006). Jeanna Giese, who in 2004 was the first patient treated with the Milwaukee protocol, (Jordan, 2008) became the first person ever recorded to have survived rabies without receiving successful post-exposure prophylaxis. An intention-to-treat analysis has since found this protocol has a survival rate of about 8% (Willoughby, 2009).

Hydrophobia ("fear of water") is the historic name for rabies (Smallman, 2004). It refers to a set of symptoms in the later stages of an infection in which the person has difficulty swallowing, shows panic when presented with liquids to drink, and cannot quench his or her thirst. Any mammal infected with the virus may demonstrate hydrophobia (Smallman, 2004).

Hydrophobia is commonly associated with furious rabies, which affects 80% of the infected people. The remaining 20% may experience a paralytic form of rabies that is marked by muscle weakness, loss of sensation, and paralysis; this form of rabies does not usually cause fear of water (NHS, 2012).

Rabies is infectious to mammals having three stages. The first stage is a one- to three-day period characterized by behavioural changes and is known as the prodromal stage. The second is the excitative stage, which lasts three to four days. This stage is often known as "furious rabies" for the tendency of the affected animal to be hyper-reactive to external stimuli and bite at anything near. The third is the paralytic stage and is caused by damage to motor neurons. In-coordination is seen, owing to rear limb paralysis, and drooling and difficulty swallowing is caused by paralysis of facial and throat muscles. Death is usually caused by respiratory arrest (Ettinger and Feldman, 1995).

2.7 Diagnostic techniques

Several techniques such as; demonstration of Negri bodies by direct fluorescent antibody test (FAT), latex agglutination test, Virus isolation in new born mice, virus isolation in cell cultures, ELISA, Electron Microscopy and recently molecular methods have been used to detect rabies antigen details of which have been published in many review articles (Fook *et al.*, 2009). However, FAT is gold standard recommended by both WHO and OIE and the most widely used test for rabies diagnosis as it is highly sensitive, specific, cheap and gives reliable results

providing results within few hours in more than 95-99% of rabies cases (OIE, 2013). Despite the availability of vaccines to prevent this disease, it is still a significant public and veterinary health problem in many countries particularly in Asia and Africa (Meslin *et al.*, 1994; Knobel *et al*., 2005; WHO, 1999) as a result of lack of accurate data on the true impact of the disease and lack of political commitment for its control (Knobel *et al*., 2005).

2.7.1 Identification of the agent

Clinical observation may only lead to a suspicion of rabies because signs of the disease are not characteristic and may vary greatly from one animal to another (WHO, 1999). The only way to perform a reliable diagnosis of rabies is to identify the virus or some of its specific components using laboratory tests.

As rabies virus is rapidly inactivated, refrigerated diagnostic specimens should be sent to the laboratory by the fastest means available. Shipment conditions must be considered to be part of the 'rabies diagnostic chain'. In the brain, rabies virus is particularly abundant in the thalamus, pons and medulla. The hippocampus (Ammon's horn), cerebellum and different parts of the cerebrum have been reported to be negative in 3.9–11.1% of the positive brains. The structure of choice is the thalamus as it was positive in all cases. It is recommended that a pool of brain tissues that includes the brain stem should be collected and tested (Bingham and Van der merwe, 2002). To reach these parts of the brain, it is necessary to remove the entire organ after having opened the skull in a necropsy room. Under some conditions a simplified method of sampling through the occipital foramen or through the orbital cavity can be used (Montano, *et al.*, 1991).

Occipital foramen route for brain sampling: A 5 mm drinking straw or a 2 ml disposable plastic pipette is introduced into the occipital foramen in the direction of an eye. Samples can be collected from the rachidian bulb, the base of the cerebellum, hippocampus, cortex, and medulla oblongata. Bovine spongiform encephalopathy (BSE) should be considered in the differential diagnosis of most cattle that are considered to be 'rabies suspect' (Barrat and Balancou, 1988).

Retro-orbital route for brain sampling: In this technique (Montano, et al., 1991), a trocar is used to make a hole in the posterior wall of the eye socket, and a plastic pipette is then introduced through this hole. The sampled parts of the brain are the same as in the former technique, but they are taken in the opposite direction.

Shipment of samples: During the shipment of suspect material for diagnosis (animal heads, brain or other tissue samples), no risk of human contamination should arise. Brains must be placed in a leak-proof rigid container (animal heads will be wrapped in absorbent material) as prescribed in the International Air Transport Association (IATA) Dangerous Goods Regulations must be followed. When it is not possible to send refrigerated samples, other preservation techniques may be used. The choice of the preservative is closely linked to the tests to be used for diagnosis. Formalin inactivates the virus, thus the isolation tests cannot be used and diagnosis depends on using a modified and less sensitive direct fluorescent antibody test (FAT), immune histochemistry or histology (WHO, 1996).

Infectivity at room temperature may be extended for several days if brain material is kept in a mixture of 50% glycerol in phosphate buffered saline (PBS). Glycerol/PBS slows bacterial action and therefore protects against the chemical and biological effects of putrefaction. It does not protect against titer decline due to thermal conditions and therefore, because rabies is thermo-labile, the virus titer will decline during glycerol/PBS storage. Therefore, whenever possible samples in glycerol/saline should be kept refrigerated; as the virus is not inactivated by glycerol/PBS (WHO, 1996).

2.7.2 Immunochemical identification of rabies virus antigen

Fluorescent antibody test (FAT): The most widely used test for rabies diagnosis is the FAT, which is recommended by both WHO and OIE. This test may be used directly on a smear, and can also be used to confirm the presence of rabies antigen in cell culture or in brain tissue of mice that have been inoculated for diagnosis. The FAT gives reliable results on fresh specimens within a few hours in more than 95–99% of cases. The sensitivity of the FAT depends on the specimen

(the degree of autolysis and how comprehensively the brain is sampled,) on the type of *lyssavirus* and on the proficiency of the diagnostic staff. Sensitivity may be lower in samples from vaccinated animals due to localization of antigen, which is confined to the brainstem. For direct rabies diagnosis, smears prepared from a composite sample of brain tissue that includes the brain stem, are fixed in high-grade cold acetone and then stained with a drop of specific conjugate. Anti-rabies fluorescent conjugates may be prepared in the laboratory. In the FAT, the specific aggregates of nucleocapsid protein are identified by their fluorescence (Barrat, 1992 as shown in (fig.4 below).

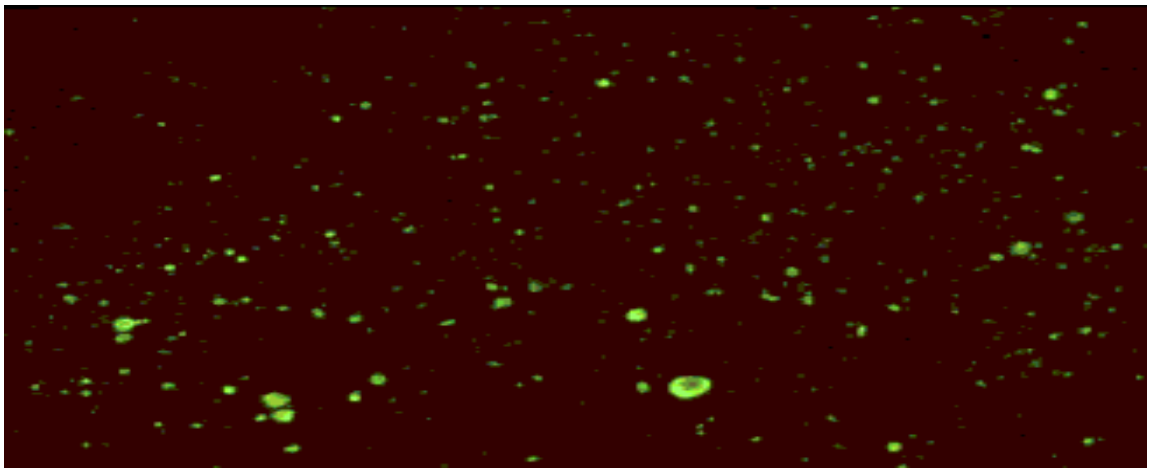

Figure 4: Microscope image of nervous tissue with positive direct FAT

Source: http://virology-online.com/general/rabiesdfa.gif. Accessed on 29 December , 2016

The FAT may be applied to glycerol-preserved specimens. If the specimen has been preserved in a formalin solution, the FAT may be used only after the specimen has been treated with a proteolytic enzyme (Barnard and Voges, 1982; Barrat, 1992). However, the FAT on formalin-fixed and digested samples is always less reliable and more cumbersome than when performed on fresh tissue (Barrat, 1992).

2.7.3 Detection of the replication of rabies virus after inoculation

These tests detect the infectivity of a tissue suspension in cell cultures or in laboratory animals. They should be used if the FAT gives an uncertain result or when the FAT is negative in the case of known exposure (WHO, 1996).

Cell culture test: A Neuroblastoma cell line, e.g. CCL-131 in the American Type Culture Collection (ATCC) 1, is used for routine diagnosis of rabies. The cells are grown in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS), incubated at 36°C with 5% CO_2. Its sensitivity has been compared with that of baby hamster kidney (BHK-21) cells (Rudd and Trimachi, 1987). This cell line is sensitive to street isolates without any adaptation step, but should be checked for susceptibility to locally predominant virus variants before use. Presence of rabies virus in the cells is revealed by the FAT. The result of the test is obtained after at least 18 hours (one replication cycle of virus in the cells); generally incubation continues for 48 hours or in some laboratories up to 4 days. This test is as sensitive as the mouse inoculation test. Once a cell culture unit exists in the laboratory, this test should replace the mouse inoculation test as it avoids the use of live animals, is less expensive and gives more rapid results. It is often advisable to carry out more than one type of test on each sample, particularly when there has been human exposure (Rudd and Trimachi, 1987).

Mouse inoculation test: Five-to-ten mice, 3–4 weeks old (12–14 g), or a litter of 2-day-old newborn mice, are inoculated intra cerebrally. The inoculum is the clarified supernatant of a 20% (w/v) homogenate of brain material (cortex, Ammon's horn, cerebellum, medulla oblongata) in an isotonic buffered solution containing antibiotics. To reduce animal pain, mice should be anaesthetized when inoculated. The young adult mice are observed daily for 28 days, and every dead mouse is examined for rabies using the FAT. For faster results in newborn mice, it is possible to check one baby mouse by FAT on days 5, 7, 9 and 11 post-inoculation. Any deaths occurring during the first 4 days are regarded as nonspecific (due to stress/bacterial infection etc.). This *in-vivo* test should be avoided when possible on animal welfare grounds. It is also expensive, particularly if SPF mice are used, and does not give rapid results (compared with *in-*

vitro inoculation tests), but when the test is positive, a large amount of virus can be isolated from a single mouse brain for strain identification purposes (Orciari *et al*., 2011).

2.7.4 Histological identification of characteristic cell lesions

Negri bodies correspond to the aggregation of viral proteins, but the classical staining techniques detect only an affinity of these structures for acidophilic stains. Immunohistochemical tests are the only histological test specific to rabies. An unfixed tissue smear may be stained by the Seller's method; diagnosis is then obtained in under 1 hour. Histological tests, such as Mann's test, are performed on fixed material after a paraffin-embedding step, and the result of the test is obtained within 3 days. These techniques have the advantage that the laboratory equipment needed to perform them is inexpensive and any need to keep specimens cold after fixation is avoided. These histological methods, especially the Seller's method, can no longer be recommended because they have very low sensitivity and should be abandoned (Drew, 2004*).*

2.7.5 Other identification tests

Enzyme-linked immune sorbent assay: Commercial kits are available for indirect ELISA that allow a qualitative detection of rabies antibodies in individual dog and cat serum samples following vaccination. In accordance with the WHO recommendations (WHO, 1985), 0.5 IU per ml rabies antibodies is the minimum measurable antibody titer considered to represent a level of immunity that correlates with the ability to protect against rabies infection. The ELISA provides a rapid (~ 4 hours) test that does not require handling of live rabies virus, to determine if vaccinated dogs and cats have sero-converted. The sensitivity and specificity of any kit used should be determined by comparison with virus neutralization methods. The ELISA is acceptable as a prescribed test for international movement of dogs or cats provided that a kit is used that has been validated and adopted on the OIE Register as fit for such purposes (Cliquet *et al*., 2000).

2.8 Control and prophylaxis of rabies

2.8.1 Rabies vaccines

In human: Since their development more than four decades ago, concentrated, purified cell culture and embryonated egg-based rabies vaccines (jointly referred to as CCEEVs) have proved to be safe and effective in preventing rabies. These vaccines are intended for both pre- and post-exposure prophylaxis and have been administered to millions of people worldwide (WHO, 2010). Prompt administration of CCEEVs after exposure combined with proper wound management and simultaneous administration of rabies immune-globulins is almost invariably effective in preventing rabies, even after high-risk exposure (WHO, 2010). Nerve tissue vaccines induce more severe adverse reactions and are less immunogenic than CCEEVs. Since 1984, WHO has recommended discontinuation of the production and use of nerve tissue vaccines and proclaimed their replacement by CCEEVs.

Sheep brain derived Fermi type of rabies vaccine is still being manufactured and utilized since 1944 for the majority of exposed patients in Ethiopian health and nutrition research institute; even though this vaccine has been discouraged by the WHO Ethiopian Health and Nutrition Research Institute (EHNRI, 2012). The high costs of tissue culture vaccine have been the main barrier to the replacement of Fermi type vaccine. Currently Ethiopian Public Health Institute (EPHI) is working to improve anti-rabies vaccine production by changing the nervous tissue vaccine (Fermi type) to cell culture based vaccine. The vaccine is based on the *Evelyn Rokitniki Abelseth* (ERA) fixed rabies virus seed strain obtained from CDC donation and propagated on Vero cell line. It is inactivated with 5% formalin and it is in liquid form (Hurisa *et al*., 2013). According to (EHNRI, 2012) annual consumption of rabies vaccine in Ethiopia is more than 36,000 doses for human and 12,000 doses for animals specifically dogs.

In animals: Inactivated rabies vaccines are currently used for routine vaccination of pet animals like dogs and cats, however, multiple immunizations have to be carried out to provide sufficient

immunity throughout the life of the animals (Wu *et al.*, 2011). In 2011, EHNRI has already produced over 100,000 doses of cell culture based vaccine for animal use. This bulk vaccine will be handed over to the National Veterinary Institute for quality assurance, packaging and distribution for veterinary use (EHNRI, 2012). Oral rabies vaccines (live attenuated or live-recombinant vaccines) have been successfully developed for wildlife and two of them are commercially available (Flamand *et al*., 1993). Vaccinia virus expressing RABV G (VRG) is found to be an effective oral immunogen for raccoons and foxes under laboratory settings and in the field (Kieny *et al*., 1984). Although VRG is safe and effective in vaccinated animals, its exposure to humans can induce skin inflammation and systemic vaccine infections (Rupprecht *et al*., 2001). SAG-2, derived from an attenuated Street Alabama Dufferin (SAD) strain, has been successfully used in Europe for oral immunization of foxes (Schumacher *et al.*, 1993). SAG-2 has also been shown to be safe, immunogenic and effective in dogs in field trails (MacInnes *et al*., 2001). However, the immunogenicity of SAG-2 is low and only low levels of VNA titers are detected in the immunized dogs (Hanlon *et al*., 2002).

2.8.2 Challenges encountered in rabies vaccine development

The greatest challenge in the industry of anti-rabies vaccine production for both humans and animals, especially in some developing countries, is little or non-availability of modern technologies in order to transit from production of nerve tissue vaccine (NTV) to the tissue culture (MTCV) (Ghosh, 2005) or sub-unit vaccines. For instance, due to presence of high content of myelin of the adult brain tissue in NTV there is a high incidence of neuroparalysis after usage of the NTV (Ghosh, 2005; Acha, 1981). NTV is not only paralyticogenic, it is less convenient, less immunogenic, more reactogenic, less tolerable and less acceptable. In addition, more number of doses is needed and the administration of which comprise comparatively a painful procedure. MTCV however is more antigenic, acceptable, well tolerated, and convenient with less reactogenicity (Ghosh, 2005).

Cost of vaccines is also another problem, as new vaccines require not less than $300 - $800 million to develop, and the companies doing the research and development must first recover this cost (Plotkin, 2005). To alleviate this problem therefore, support from governments and donor agencies to purchase the vaccines for developing countries is necessary (WHO, 2010). Another major challenge to be contended with in anti-rabies vaccine development and production in developing countries is absence of enforcement of registration and compulsory vaccination of dogs, free or subsidized vaccine/vaccination cost, regular mass vaccination campaigns and creation of awareness on rabies among the populace (Fagbami *et al*., 1981; WHO, 1984). These result to low patronage of vaccines, and adversely affect production turnover and impetus to embark on developmental research activities. Rabies Immunoglobulin (RIG) is commonly avoided by medical practitioners in Asian countries, leading to treatment failure in the fear of reactogenicity (Ghosh, 2005). Generally, there is a growing demand for vaccine safety worldwide. One of the advantages of the newer molecular technology is improved safety, although zero risk is not possible (Plotkin, 2005).

2.9 Application of some traditional herbal remedies against rabies

The use of plants as remedies to treat various forms of an ailment has been a common practice all over the world. According to the WHO report, nearly 70-90% of the world population depends on herbal medicine, which caters for most of African population (Akerele, 1993; Nair and Nathan, 1998). Pharmacological and phytochemical studies done with medicinal plants used traditionally in other countries have led either to isolation of novel structures for the manufacture of new drugs or templates that served for the production of synthetically improved therapeutic agents (Geyid *et al*., 2005).

The prohibitively expensive cost of modern drugs to treat various infections and the clinical importance of drug resistant pathogens, particularly in third world countries makes the search of alternative anti-infective agents from natural products more urgent (Shu, 1998). People in developed countries are also shifting back to herbal medicine because of non curability of viral infections and the serious side effects of the modern drugs (Nair and Nathan, 1998). Ethiopia is

one of the six regions of the world where about 60% of plant with healing potential are said to be indigenous in origin (Balcha, 2003).

Several traditional anti-rabies plants in different ethnic groups of Ethiopia were reported by different investigators for the treatment of rabies in animals and humans (Admasu and Mokonen, 2014).The efficacy for some of ethno – medical and ethno – veterinary plants against rabies were evaluated with modern pharmaceutical practices by few researches in the country.

Phytolacca dodecandra: locally called Endod belongs to family "Phytolaccaceae" is perennial plant used for treatment of rabies. Admasu *et al.* (2014) evaluated the efficacy of anti-rabies from the extracts of roots and leaves of *P.dodecandra* in mice showed minimal anti-rabies effect in leaves extract of the plant.

Chloroform and methanol 80 % extracts of roots of *Silene macroselen* locally named Wegert and chloroform and aqueous extracts of leaves of *Salix subserrata* locally called Aleltu did show a significant anti-rabies activity (Deresa *et al*., 2010).

Croton macrostachyus: locally called Bisana belongs to family "*Euphorbiaceae"*. The plant has wide traditional medicinal values as it is used to treat rabies, epilepsy, cough, skin disease, dysentery, lung complaints, plain full eyes, toothache, etc. (Monica, 2005). In Ethiopia it has many uses like leaf extract is applied against itchy and root or stem bark is used to treat toothache and rabies (Indu, 2007).

Leaf of *Justicia schemperiana* locally named sensel or Dhummuugaa in Oromiffaa is used for treatment of different diseases like rabies, malaria, syphilis, leprosy, gonorrhoea and measles (Geyid, 2005). According to his report methanol extraction of the plant is preferable with concentration of 2000µg/ml, 1000µg/ml or above. Polyphenols, unsaturated sterols and saponin chemical compounds are present in the preparations which are supposed to have anti-microbial and anti-viral properties (Geyid, 2005).

2.10 Description of plants used in current study

*Justicia schemperiana***:** is a perennial herb or shrub with much branched stems, up to 0.8-3 m tall with slightly unpleasant smell. Leaves blade up to 15-24×8-12 cm, ovate-elliptic, broadest near the base, base cuneate to attenuate entire along the margins, apex acuminate, pubescent along veins on both surfaces, or rarely pubescent all over both surfaces; lateral veins 5-9 pairs; petiole up to 10-40 mm long; pubescent with non-glandular hairs to nearly glabrous" (Ensermu *et al*., 2006; Thulin, 2008).

Figure 5a: Photos of *J. schemperiana plant*

P.dodecandra: It is also known as 'soapberry' or 'endod' in Ethiopia, belongs to the family Phytolaccaceae. It is a perennial climbing plant growing rapidly in Ethiopian highlands (1,600 to 3,000 m above sea level) and native to Tropical Africa, Southern Africa, and Madagascar (Hanelt, 2001*).* It produces fruits twice a year from December to February and June to July (Lemma *et al*., 1979; Karunamoorthy *et al*., 2008). The powdered fruits/berries are commonly used in various parts of the country for cloth washing as when it is mixed with water it produces foaming detergent solution. The multi-potential bioactive plant *P. dodecandra* grows naturally in Ethiopian highlands and also cultivated for use in the snail control program (Misganaw *et al*., 2012).

Figure 5b: Photos of *Phytolacca dodecandra*

Croton macrostachyus: It belongs to the family Euphorbiaceae, a deciduous tree, crown rounded, light and open with slender trunk and spreading branches, reaching 25 m. It has a bark of pale grey, fairly smooth when young and longitudinally fissured when old. The leaves of *Croton macrostachyus* are large and heart shaped. It is commonly found on forest edges along rivers, around lakes, woodlands, wooded grasslands, and along roadsides. It grows mostly on soils of volcanic origin and it is native to Ethiopia, Eritrea, Kenya, Nigeria, Tanzania, west of Guinea, South Angola, Zambia, Malawi, Mozambique and Uganda. In Ethiopia it occurs in Dry, Moist and Wet Weynadega and Dega as well as in upper altitudes of Dry Kolla agro climatic zones in Tigray, Gondar, Gojam, Wollo, Wollega, Bale, Shewa, Illubabor, Kefa, Sidamo and Hararge regions at 1,100-2,500m above sea level. Apart from its medicinal uses, the tree is also used as firewood, timber, forage (young leaves) and as mulch. Means of propagation include, sowing of the seeds or the use of seedlings (Gilbert, 1989; Azene Bekele *et al*., 1993).

Figure 5c: *Croton macrostachyus* tree

3. MATERIALS AND METHODS

3.1 Area description

Plant parts collection was undertaken from Gida Ayana District, East Wollega Ethiopia between November and December, 2016. The area has altitude that ranges from1700 to 2200meters above sea level (masl). According to basic data of Gida Ayana Wereda Agricultural and Rural Development Office GAWARDO (2009) about 12,265.27 ha of land is plain (flat), 101,325.77 ha is hill slope, 6,319.5 ha is gentle gorge, 7,322.55 ha are swamps and 1,830.64 ha are other land forms. About 20%, 60% and 20% of the soils in Gida Ayana District was sandy, clay-loam and clay respectively. There are two types of agro-climatic zones in the district: *Kola* about 51% and *Weyina Degaabout* 49% of the total land area. It is about 412 km far from Addis Ababa having the mean annual rainfall of between 1487 – 2119 mm. The average annual temperature is about 18.9^0C (ENMSA, 2006).The forest resources of the area will be grouped in 4 categories, namely, natural closed forests, woodlands, bush lands and on-farm trees. Agriculture is the main occupation of the population at the area. The agricultural activities are mainly mixed type with livestock and crop production under taken side by side (GAWARDO, 2009).

3.2 Collection of medicinal plants

Botanical identification and authentication was carried out at national herbarium department of Addis Ababa University as shown in (table 3).

Table 3: Botanical identification of medicinal plants used in this study

Name of the plant	Family	Local name	Parts to be used	Voucher number

Croton macrostachyus Del.	*Euphorbiaceae*	Bisana	Root bark	001
Justicia schemperiana (Hochst. ex Nees) T Anders.	*Acantiaceae*	Sensel	Leave	002
Phytolacca dodecandra L'Herit	*Phytolaccaceae*	Endod	Root	003

All other laboratory activities including evaluations of the plants were performed at EPHI. These plant species could be selected on the basis of the available information from traditional healers, ethno medical information as well as anti-rabies activity study results of crude hydro-alcoholic 80% extracts of various indigenous plants against rabies (Admasu *et al*., 2014; Deresa *et al*., 2010).

3.3 Pre extraction preparation, processing and extraction

The collected plant parts were brought to the EPHI and stayed overnight. Briefly, garbling was performed following collection before drying and processing the plants. The plant materials were chopped into smaller pieces in the house and dried indoors without exposure to sunlight for a month. The dried plant materials were grounded to various degrees of fineness using a grinding mill (Hamburg 76 Germany) (Appendix 1A) depending on their botanical structures. Plant materials were weighed by sensitive digital balance and extracted in 80% ethanol maceration method and concentrated according to the procedures given by Debella (2002). Briefly, 100 g of powdered plant were soaked in 500 ml of 80% ethanol in Erlenmeyer flask of one liter and /or two liters capacity. The flask containing dissolved plant materials and 80% ethanol mixture is plugged with cotton wool and kept on a rotary shaker at 120-190 rpm for 24-48 hours (Appendix 1B). The samples were then strained using a tea strainer to remove solids. The resulting solutions were again filtered using Whatman's filter paper number 1 to obtain a solution free of solids (Appendix 1C). Additionally, the same solvent is added to residue and filtered two more times. The solution is then concentrated in a rotary evaporator to remove the solvent or ethanol (Appendix 1D). The trace solvent is evaporated on water bath at +40°C. Finally, yield of extracts were stored at +4 °C in airtight container throughout the study period (Holetz *et al*., 2002; Geyid *et al*., 2005). The extracts were designed to be given at a dose of 300mg/kg, 2000mg/kg and

5000mg/kg according to Organization of Economic Corporation and Development's guideline (OECD, 2001**)** for *in-vivo* experiment both individually and compounded at the proportion of 1:1:0.75 ratios for *P.dodecandra*, *J. schemperiana* and *C. macrostachyus* respectively (information obtained from traditional healers).

3.4 Study method

Anti-rabies viral activity of the selected medicinal plants was evaluated on mice survival period compared to positive control group. Dosage was setup based on the acute toxicity of laboratory animal's manifest i.e. the highest dose which showed lowest toxicity would be selected. Cytotoxicity of crude extracts also performed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay in Vero cell line.

3.5 Experimental Design

In vitro cytotoxicity assay: The assessment of cytotoxicity is usually performed by cell viability assays, such as the dye uptake by non- viable cells, or by alterations in cell functions, and these endpoints have been established for many cell types (Eisenbrand *et al*., 2002). The *in vitro* cytotoxicity assay of the plant extracts in this study were performed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay in Vero cell line (Meslin *et al*., 1996). Briefly, Vero cells were trypsinized and cultured onto 96-well plate at the density of 10^4cells/well. Once the monolayer is formed after 24 h, different concentrations (1, 5 and 10mg/ml) of extracts with 50 µl/well can be serially diluted in DMEM (without phenol red) and added to each culture wells in triplicate and incubated at 37°C with 5% CO_2 for 24 hr. After incubation, the medium with plant extracts were removed and 5 mg/ml MTT (20 µl) salt was added to each 96-well containing the Vero cells and incubated for 3 hr at 37°C. After incubation the crystal formed (purple formazan) (Vistica, 1991) was solubilized by adding dimethyl sulfoxide (DMSO 50 µl) to each well. The purple color formed at this step was read at absorbance of 490 nm by an Enzyme linked immune-sorbent assay (ELISA) reader (Mosmann, 1983) and the color intensity of the final

solution formed is directly proportional to cell viability. Results were calculated according to the formula given by Reed and Muench, (1938) using percentage growth inhibition.

$$\% \text{ Growth Inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD ofcontrol group}} \times 100\right)$$

Acute Toxicity Test: For acute toxicity evaluation, five female albino mice per treatment and control groups were used. Initially body weights of all mice were measured. Then, crude extracts were examined for acute toxicity by administering with an oral dose of 50mg/kg, 300mg/kg, 2000mg/kg and 5000mg/kg (OECD, 2001). A volume of 20mg/ml distilled water was used for oral preparation (Erhirhie *et al*., 2014). Mortality in each group within 24 hours was recorded and the animals were observed for about 14 days for any sign of delayed toxicity (Miller and Tainted, 1944). Any body weight change was again examined after 7^{th} and 14^{th} day of extract administration.

In-vivo anti-rabies assay: A total of 130 laboratory albino mice were randomly assigned into treatment and control groups. The treatment groups were categorized into three sub-groups for each dose of four parts of plant extracts. Accordingly, three dose levels (300, 2000 and 5000 mg/kg) (OECD, 2001) for each part of plant extracts were employed. One placebo positive control group of mice were administered with only distilled water instead of plant extracts but challenged with CVS-11. Details of the experimental animal group classifications are described in (Table 4). Administration of the extracts and distilled water was done by using an intra-gastric needle (lavage) based on the animal body weight in 0.6 ml vehicle i.e. 20ml/kg (Erhirhie *et al*., 2014) for 30g mice.

Table 4: Classification of experimental mice into different groups

Medicinal plant	Plant part used	No. mice per group	Dose (mg/kg) for 3 days	Route of administration	Group code
J. schemperiana	Leave	9*	300	PO	A300
J. schemperiana	Leave	10	2000	PO	A2000
J. schemperiana	Leave	8*	5000	PO	A5000
P.dodecandra	Root	10	300	PO	B300
P.dodecandra	Root	9*	2000	PO	B2000
P.dodecandra	Root	9*	5000	PO	B5000
C.macrostachyus	Root bark	9*	300	PO	C300
C.macrostachyus	Root bark	10	2000	PO	C2000
C.macrostachyus	Root bark	8*	5000	PO	C5000
compound**	mix	8*	300	PO	D300
compound**	mix	10	2000	PO	D2000
compound**	mix	10	5000	PO	D5000
control	non	10	0.6ml dH_2O	PO	E

PO= Per Os, dH_2O= distilled water

*Any number below 10 mice per group was due to accidental death of mice during virus inoculation or extract administration.

**1:1:0.75 ratios compounded for *J. schemperiana, P. dodecandra and C. macrostachyus* respectively

3.6 Experimental animals and their management

The experimental study was carried out on Swiss albino mice of 4-7 weeks old and 20-35 g in weight. All laboratory mice used for this experiment were male and bred in a standard laboratory animal house of EPHI. The experiment was approved by EPHI and all mice subjected to the experiment were handled according to standard guidelines for the use and care of laboratory animals (Wandeler, 1997). After mice were obtained from the laboratory animal unit, they were housed in a littered clean metal/plastic cage and in 12 hrs light /dark cycle with litter changed every three days as described by Gebrie *et al.* (2005). Mice were randomly assigned to ten per group for evaluation of anti-rabies activity of plant extract as shown in (table 4) and (Appendix 2A). The laboratory animals were provided with pelleted ration feed (Mice cubes) and cleans water *ad libitum* (Appendix 2B). They were left under controlled conditions at least for three days to acclimatize before conducting any experimental procedure and the mice were used for only one experiment. All experimental mice were treated under similar feeding management conditions. The investigator and all personnel managing rabies virus-inoculated mice were vaccinated with commercially available pre-exposure anti-rabies vaccine Verorab (PVRV, Sanofipasteur, France) according to WHO guidelines (WHO, 2005) as pre-exposure prophylaxis. The vaccine was administered three times per person on 0, 7 and 21or 28 days intramuscularly at deltoid area of the arm.

3.7 Virus Strain and its Inoculation

Titer of CVS-11 rabies virus was prepared from rabies infected suckling mouse brains and the virus (Atlanta, Georgia, USA) was diluted to 10^{-3}suspension in phosphate-buffered saline (PBS) solution to contain 50MICLD50/0.03 ml (OIE, 2008; Stokes *et al*., 2012) for a single challenge which were determined according to methods of Reed and Muench (WHO, 1954). Protocols for this experiment followed the guidelines on care and wellbeing of research animals and standard protocol observed in accordance with the Good Laboratory Practice (GLP) Regulations in rabies (Wandeler, 1997). CVS-11 virus inoculation was conducted intra-cerebrally (Wunderli, *et al*., 2006). Hence, all groups of mice including the control group were inoculated with CVS-11 virus strain at day 0 after three days of acclimatization as indicated in (appendix 2C).

3.8 Extract Administration

After virus inoculation, the mice were allowed to stay in their respective cages for about 1 hr so as to make them calm. Then, plant extracts were orally administered to the treatment group while the control group received only distilled water. Administration of the extract and distilled water was done using an intra-gastiric needle (lavage) based on the animal's body weight at 20ml/kg vehicle (Erhirhie *et al*., 2014).The mice were administered with dose of 300, 2000 and 5000 mg/kg of crude extracts per plant parts individually and using a mix of the three plants for three consecutive days after an hour of CVS challenge.

3.9 Determination of mortality rate

Mortality rates as a result of rabies virus challenge were determined by clinical signs and direct fluorescent antibody test (Bourhy *et al*., 1989). All mice were maintained and followed for consecutive 30 days after virus challenge and they were observed daily after challenged with rabies virus for signs of rabies (Roughening fur, tremors, in-coordination, paralysis and prostration) and any signs of rabies recorded each day on the mouse history cards (Appendix 3). The direct FAT used commercially available rabies anti-nucleocapsid antibodies (polyclonal antibody) tagged with fluorescein isothio-cyanate (FITC) -dye (Rabies Conjugate Anti-nucleocapsid, BIORAD, South Africa) and the working dilution was prepared in accordance with the manufacturers recommendations (Flamand, 1980).

Confirmatory diagnosis of rabies through direct FAT was conducted using brain samples of mice that were obtained by opening the skulls of mice according to procedure specified by Dean and Albelseth (1973). Briefly, the heads were held firmly in a vice fitted on the operation table with the rostral end of the head and the tail of mice pined. A midline incision was made on the dorsal

surface of the head using scalpel and blade. The skull was then exposed by dissecting away the skin, apo-neurosis and temporal muscles and reflecting them laterally. Then the brain tissues were exposed by cutting top of the skull (Calvarium) by scissor. The brain sample consists of cerebellum, hippocampus and brain stem (WHO, 1992) and any available brain tissues were taken and an impression smears made for direct FAT. Standardized protocol for the direct FAT was carried out in accordance with the procedures described by Kissling (1975). Briefly, impression smears on slide were prepared from brain tissues and air dried for 15-20 minutes at room temperature. The smears were fixed in acetone for about one hour at -20°C. Brain impression smears were stained with FITC-labelled anti rabies conjugate and incubated for 30 minutes at 37^{o}C. Soak in PBS to decrease any nonspecifically binding substances. Rinse with distilled water and air dried. Finally, after mounting media was applied, the slides were investigated under 40X objective of fluorescence microscope. Accordingly, 29 brain tissues of mice were collected randomly from mice died within four days of virus inoculation and extract administration, from those showed clinical sign for rabies and appeared on moribund stage and from survivors to detect characteristic green fluorescence associated with rabies antigen (Lembo, 2006).

3.10 Data management and analysis

Data were analyzed using SPSS version 24 for windows software (Armonk, NY: IBM Corp.). Statistical analysis was undertaken by one-way analysis of variance (ANOVA) tests coupled to survival analysis to compare results of treatment and control groups. Mean survival time were calculated and expressed as mean ±SD for each treatment and control groups. Paired t test statistic was applied for body weight change (if any) of mice in acute toxicity determination. The result was considered statistically significant at 95% confidence level and $P<0.05$ (Bross, 1971).

3.11 Ethical clearance

The experimental procedures and protocols used in this study were approved by Ethical Committee of Addis Ababa university, Collage of Veterinary Medicine and Agriculture with

certificate ref. no. VM/ERC/29/06/09/2017 (OECD, 2002). The animals were housed in plastic and/or metal cages in an air conditioned environment with 10 rats in each cage.

4. RESULTS

4.1 Yields from plant materials

Percentage yields obtained from 80% hydro-ethanol extraction of all plant parts per100g powder were 7.12, 6.73 and 6.26 grams, respectively for *J. schemperiana, P. dodecandra and C. macrostachyus* as shown in (table 5).

Table 5: Yields of plant extract per100g of coarsely grounded powder

Plant name	Parts used	Solvent	Yield (%)
Justicia schemperiana	Leave	80% Ethanol 800ml	7.12
Phytolacca dodecandra	Root	80% Ethanol 800ml	6.73
Croton macrostachyus	Root bark	80% Ethanol 800ml	6.26

4.2. Cytotoxicity determination of crude extracts

The Vero cell line (10^4 cells / well) were seeded in 96-well tissue culture plate for MTT based cytotoxicity assay as indicated in. The median Cytotoxic concentration of *P.dodecandra* was found to be 10 mg/ml at 10^{-2} dilution rate with percentage growth inhibition of (% GI=50.24). Whereas for *C. macrostachyus* it was at a dose of 10mg/kg at about 10^{-1}dilution rate having a percentage growth inhibition of (% GI=51.47) as shown in (figure 6C). However, the median cytotoxic concentration for *J. schemperiana* and for their overall combination were above 10mg/ml dose level as indicated in figure (6A, B, C).

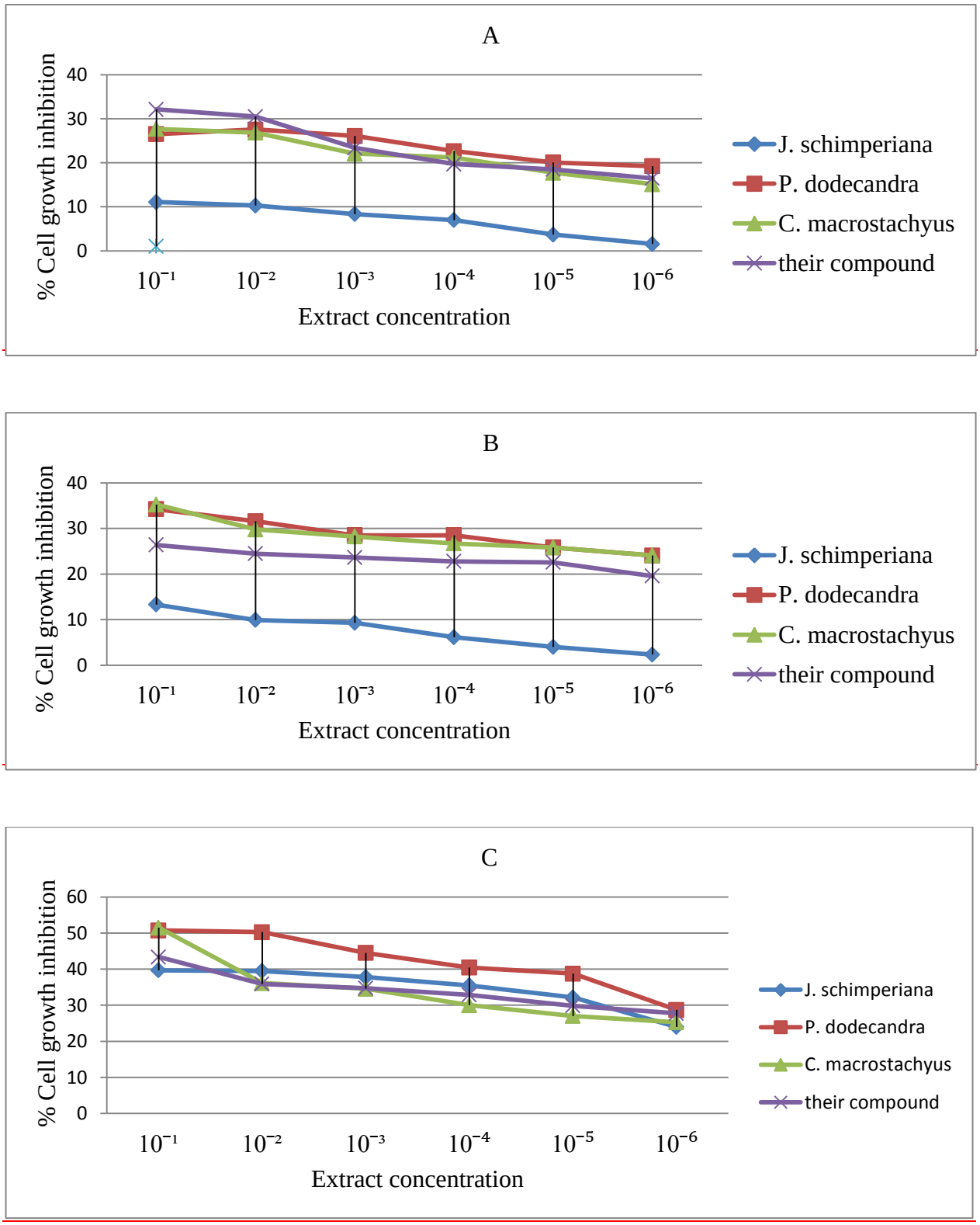

Figure 6: *In vitro* cytotoxicity evaluation of crude extracts at 1mg/ml (A), 5mg/ml (B) and 10mg/ml (C)

4.3. Results obtained from fluorescent antibody test

Of 29 total samples tested for dFAT 15 specimens (51.7%) were positive for rabies virus antigen and 14 (48.3%) were negative which infer the groups in general (table 6). All mice died within four days of inoculation and treatment didn't show any antigen against rabies virus which indicate the death was due to accidental rather than the effect of the virus. However, death from samples taken from those at moribund state was due to the action of challenging virus standard and showed a viral antigen 100% (13/13). Samples from survivors indicate negative for antigen detection except for *Croton macrostachyus* at 300mg/kg and from combination at 2000mg/kg where at these dose levels some viral antigens were detected though the mice were alive.

Table 6: Fluorescent antibody test results from mice brain sample

Group of mice	Only one sample was taken from each parameter per group of mice		
	Results from Death within four days	Results from Moribund mice	Results from Survivors/sacrificed mice
A300	-	++	-
A2000	SN	+++	SN
A5000	-	++	-
B300	SN	+++	SN
B2000	-	++	-
B5000	-	+	-
C300	-	+++	+
C2000	SN	+	-
C5000	-	+++	-
D300	-	+++	SN
D2000	SN	+++	+
D5000	SN	++	-
E	SN	+++	SN

Note: SN=Sample not taken either due to no death within four days or no survivors present in respective groups

(-) showed no antigen detected, (+) a slight detection of viral antigen, (++) a moderate antigen detected and (+++) high concentration of antigen detected.

4.4. Percentage survival and mean survival time of mice against rabies virus

Percentage survival and mean survival period of group of mice infected and treated with all parts of plant extracts and infected but not treated with all parts of plant extract were identified as findings of the study. Group of mice infected with rabies virus but not treated with any of plant extracts (positive controls) showed 0% (0/10) percentage survival and 8.6 days mean survival time.

None of the mice were protected from rabies deaths from groups of mice treated with 80% hydroethanolic root extract of *P. dodecandra* at 300mg/kg with mean survival period of 9.3 days. At 2000mg/kg two mice 22.2% (2/8) were survived with mean survival time of 14.11 days. However, at highest dose (5000mg/kg) the extract showed better percentage survival 44.44% (4/9) and mean survival period of mice (20.33 days) when compared to all other plant extracts and control group.

The crude extract for leaf of *J. schimperiana* showed a percentage survival of 11.1% (1/9) and mean survival period of 12.56 days at 300mg/kg. But the extract didn't save the life of mice when treated at 2000mg/kg with a mean survival time of 9.4 days. However, at 5000mg/kg dose level the plant showed a 22.2% (2/8) and 15days percentage survival and mean survival periods, respectively.

At 300mg/kg and 5000mg/kg; *C. macrostachyus* protected only one mouse per each dose level with 11.1% (1/9) and 12.5% (1/8) percentage survival respectively. The mean survival time of mice for these dose levels were 12 and 12.87 days consecutively. At 2000mg/kg the extract indicated a better survival of mice next to a 5000mg/kg for *p. dodecandra*. In doing so, the percentage survival of *C. macrostachyus* at 2000mg/kg was observed 30% (3/10) with 17.1 days mean survival time.

The combination of all extracts at 1:1:0.75 ratios for *J. schimperiana*, *P. dodecandra* and *C. macrostachyus* respectively, also showed a minimal life saving at 2000 and 5000mg/kg with only 10% (1/10) and 20% (2/10) percentage survival with 12.6 and 15.2 days mean survival time consecutively.

Relatively higher percentage survival and mean survival time of mice against challenged rabies virus were obtained for *P. dodecandra* and *C. macrostachyus* at 5000mg/kg and 2000mg/kg correspondingly as shown in (table 7).

Table 7: Effects of plant extracts on percentage survival and mean survival period of mice

Group	Survival n (%)	Death n (%)	Survival time (days) (Mean ± SD)	Mean difference(T-C')
A(300mg/kg)	1 (11.1%)	8 (88.9%)	12.56± 7.23	3.96
A(2000mg/kg)	0 (0%)	10 (100%)	9.4±1.17	0.8
A(5000mg/kg)	2 (25%)	6 (75%)	15±9.29	6.4
B(300mg/kg)	0 (0%)	10 (100%)	9.3±0.949	0.7
B(2000mg/kg)	2 (22.2%)	7 (77.8%)	14.11±9.05	5.51
B(5000mg/kg)	4 (44.4%)	5 (55.6%)	20.33±9.99	11.73
C(300mg/kg)	1 (11.1%)	8 (88.9%)	12±7.07	3.4
C(2000mg/kg)	3 (30%)	7 (70%)	17.1±9.45	8.5
C(5000mg/kg)	1 (12.5%)	7 (87.5%)	12.87±7.24	4.27
D(300mg/kg)	0 (0%)	8 (100%)	9.63±1.41	0.76
D(2000mg/kg)	1 (10%)	9 (90%)	12.6±6.45	4
D(5000mg/kg)	2 (20%)	8 (80%)	15.2±8.26	6.6
E(Control)	0 (0%)	10 (100%)	8.6±0.7	

Note; n: number of mice, SD: Standard deviation, A: *J. schemperiana*, B: *P. dodecandra*,

C: *C.macrostachyus*, D: their combination, T: Treatment group, C: Control group

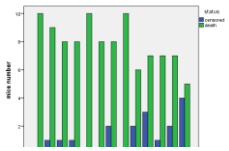

Figure 7: Association of mice status and dose using a chi-square test

4.5 Significance level of extracts used for treatment of mice challenged with rabies virus

Most of plant extracts significantly (P<0.05) increase the survival time of mice as compared to positive control group at higher doses. Root extract of *P. dodecandra* signifies a promising anti-viral activity against rabies virus at 5000mg/kg (P=0.002) when compared to all other extracts. The rest dose levels for *P. dodecandra* didn't revealed significance. Leaf extract of *J. schemperiana* (P=0.038) and the combination of all (P=0.021) also significantly increased survival period (days) of mice (P<0.05) as compared to control group at 5000mg/kg. However, extracts of *C. macrostachyus* showed significance at 2000mg/kg (P=0.011) which was a second relevant outcome in this study. The rest extracts with respective doses didn't significantly (P>0.05) increase the survival rate of mice as compared to positive control group as shown in (Table 8).

Table 8: Significance level of plant extracts against challenged rabies virus

Group	95% CI, for survival time	F	*p* -value
A(300mg/kg)	(7.832, 17.279)	2.981	0.102
A(2000mg/kg)	(8.672, 10.128)	3.429	0.081
A(5000mg/kg)	(8.563, 21.437)	5.077	0.038
B(300mg/kg)	(8.712, 9.888)	3.528	0.077
B(2000mg/kg)	(8.200, 20.022)	3.710	0.071
B(5000mg/kg)	(13.808, 26.858)	13.816	0.002
C(300mg/kg)	(7.380, 16.620)	2.302	0.148
C(2000mg/kg)	(11.246, 22.954)	8.055	0.011
C(5000mg/kg)	(7.858, 17.892)	3.500	0.080
D(300mg/kg)	(8.649, 10.601)	4.088	0.060
D(2000mg/kg)	(8.602, 16.598)	3.801	0.067
D(5000mg/kg)	(10.082, 20.318)	6.344	0.021
E(Control)	(8.167, 9.033)		

A: *J. schemperiana*, B: *P. dodecandra*, C: *C. macrostachyus*, D: combination**,** F: F statistic

4.6. Acute toxicity test

The test was conducted according to guideline of OECD (2001) which was examined by up and down procedure EPA (2002) starting at a dose of 50mg/kg for each plant types and ended at the limit dose 5000mg/kg (Bruce,1985) for estimating the median lethal dose (LD50). None of the extracts show significant values in terms of survival time when compared to negative control group. The LD50 estimation can be above the limit dose. However, there was a significant difference for body weight changes i.e. as survival period increases the body weight of mice also raised up as indicated in (table 9).

Table 9: Effect of crude extracts on body weight (paired t test) and survival time of mice.

Group	Body weight in gram (g) (mean ± SD)			*p* -values (2-tailed) for body weight			Survival time with p-value	
	Day 0	Day 7	Day 14	Day 0 and 7	Day 0 and14	Day 7 and14	Survival time	*p* -value
A(50mg/kg)	25.00±3.536	32.27±3.837	30.28±4.680	.000	.001	. 017	14.00±.000	.347
A(300mg/kg)	25.60±2.302	31.94±4.533	26.30±3.550	.007	.351	. 001	14.00±.000	.347
A(2000mg/kg)	24.60±3.435	26.14±14.743	28.36±15.978	.789	.562	.037	11.60±5.367	.846
A(5000mg/kg)	25.20±2.280	30.02±1.704	32.94±1.721	.000	.000	.004	14.00±.000	.347
B(50mg/kg)	27.20±4.764	33.04±4.908	35.50±5.755	.000	.000	.008	14.00±.000	.347
B(300mg/kg)	24.20±2.280	25.44±14.482	21.92±13.224	.836	.673	.133	11.80±4.919	.892
B(2000mg/kg)	20.40±2.074	13.40±7.795	11.92±11.687	.081	.124	.696	10.80±5.215	.647
B(5000mg/kg)	27.00±2.915	27.53±4.574	32.54±2.865	.708	.002	. 007	14.00±.000	.347
C(50mg/kg)	23.80±3.194	22.10±4.867	12.52±11.775	.211	.048	.052	12.40±2.302	.926
C(300mg/kg)	26.80±4.970	29.43±4.970	24.46±13.952	.085	.592	.160	13.60±.894	.469
C(2000mg/kg)	25.80±3.633	31.94±2.492	34.56±4.865	.002	.008	.102	14.00±.000	.347
C(5000mg/kg)	24.60±3.435	16.56±15.119	17.90±16.379	.204	.316	.124	9.80±5.848	.471
D(50mg/kg)	28.60±4.219	32.94±3.651	36.16±5.101	.002	.000	.001	14.00±.000	.347
D(300mg/kg)	24.60±2.702	26.06±14.644	26.10±14.760	.799	.796	.926	11.60±5.367	.846
D(2000mg/kg)	26.44±2.027	31.14±3.444	35.00±3.949	.002	.001	.000	14.00±.000	.347
D(5000mg/kg)	25.00±2.828	26.87±4.422	24.14±14.153	.082	.882	. 614	13.80±.447	.406
E(Control)	23.40±3.912	21.90±12.622	24.04±14.289	.750	.904	.090	12.20±4.025	

SD: Standard deviation, A:*J. schemperiana*, B: *P. dodecandra C: C. macrostachyus***,** D: their combination

5. DISCUSSION

Cytotoxicity assays are essential for the initial phases of antiviral drug development because they define the concentrations to be used, avoiding cell damage and assuring selectivity for the virus *in vitro*. In this study, *in vitro* cytotoxicity test of crude extracts of the study plants were conducted in Vero cell line using MTT assay (Meslin *et al*., 1996). The MTT assay is probably one of the most widely used colorimetric indicators of cell viability, and it assesses mitochondrial cellular function based on the enzymatic reduction of the tetrazolium salt by the mitochondrial dehydrogenases in viable cells (Mosmann, 1983).

Accordingly, root extract of *P. dodecandra* and root bark extract of *C. macrostachyus* were established a median cytotoxicity with percentage cell growth inhibition at 10 mg/ml at 10^{-2} (% GI=50.24) and10^{-1} (%GI=51.47) dilution rates respectively as shown in (figure 9C). This could be due to the presence of hydroxyl groups in bioactive compound called flavonoids which are directly related to the increase of intracellular oxygen reactive species, possibly leading to cell damage, although the structure–cytotoxicity relationship of compounds with this structural characteristic in some cell types remains unclear (Matsuo *et al*., 2005). It is important to take into account the fact that some substances can selectively affect the enzymes responsible for MTT reduction, resulting in an overestimation of cytotoxicity (Smee *et al*., 2002).

However, leaves of *J. schimperiana* and their combination didn't show cytotoxicities in vero cells, at the concentrations employed. Similar results were observed when cytotoxicities of alkyl-esters of Gallic acid compounds were established using Vero cells (Savi *et al*., 2005), which might be due to unavailability of toxic substances that able to affect morphological and functional unit of cells at the concentrations engaged.

There were other reports for *in vitro* cytotoxic activity of soxhlet and cold extracts of *Datura metel* (fruit and seed) was performed in Vero cell line using MTT assay. The CC50 of *Datura* fruit (water and methanol) soxhlet extracts was found to be 5 mg/ml and 4 mg/ml, respectively, and of cold extracts (water and methanol) was found to be 7 mg/ml and 3 mg/ml,

respectively (Soumen, *et al*., 2016). Whereas the CC50 *Datura* seed (water and methanol) soxhlet extracts were found to be 7.5 mg/ml and 5 mg/ml, respectively, and of cold extracts (water and methanol) was found to be 3.5 mg/ml and 5.5 mg/ml, respectively (Soumen, *et al*., 2016).

Although medicinal plants are assumed to be safe, many of them can be potentially toxic (Ajaiyeoba *et al.,* 2006). Mice observed for acute toxicity determination of plant extracts in the present study revealed no significant values at all doses and all types of plant extracts ($p>0.05$) as shown in (table 8) in terms of survival time. The result suggests that the *in-vivo* oral median lethal dose (LD50) of the extracts of all plant parts could be greater than limit dose 5000 mg/kg which is in line with the reports of Andualem and his colleagues (2016) who reported LD50 for *J. schimperiana* might be greater than 2000mg/kg. This encourages the safety use of plant extracts for anti-rabies activity evaluation in mice model. Despite the fact that no significant level obtained on survival period of laboratory animals there was a change in body weight at initial day of treatment and seventh and last day of observations. However, the body weight change was not occurred negatively as illustrated in (table 8). As the time of observation prolonged almost all mice were increased in weight with good body condition except cannibalism to each other in some groups.

Oral treatment of mice infected with rabies virus at higher doses of an extract for *J. schemperiana, P.dodecandra* and their combination at (5000 mg/kg) which were administered for three consecutive days including root bark of *C. macrostachyus* at 2000 mg/kg significantly increased the mean survival time ($P<0.05$) compared to a positive control group.

The current result of root extracts for *P. dodecandra* disagree with the reports of Admasu *et al*, (2014) who stated "root extract of *P. dodecandra* was not dose dependent and did not show significance at 300, 600 and 1000mg/kg." But the prolonging survival time (MST=20.33 days) effect of this extracts at higher doses (5000mg/kg) ($p=0.002$) showed as the extract were dose dependent. This might be due to a flavonoid chemical found in the plant which was believed to be responsible for the detected anti-viral activity (Madhusudana *et al*., 2004b). The difference could be attributed to differences of dosage used for antiviral activity evaluation primarily and location difference from where the plant parts were collected.

However, mice treated with root of *P. dodecandra* at 300mg/kg (p=0.077) was in line with reports of Admasu *et al.* (2014) (p=0.232) where both of them didn't show significant results. Although *P. dodecandra* at (2000mg/kg) save the life of two mice with (MST=14.11days), it didn't show significance (p=0.071) at 95% confidence interval. The study revealed that root extract of *P. dodecandra* was dose dependent when examined at higher doses beyond 1000mg/kg unlike the reports of Admasu *et al*., (2014) which is restricted at up to dose of 1000mg/kg. Other previous studies showed that the leave extract of *P. dodecandra* has a moderate *in-vivo* activity against rabies (Admasu, *et al*., 2014) and coxsackie virus *in-vitro* system (Zimudzi, 2007).

Leaf extract of *J. schemperiana* also showed anti-rabies activity at higher dose (5000mg/kg) (p=0.038) and (MST=15days) which is in line with the reports of Geyid, *et al*., (2005) who reported *in-vitro* anti-rabies and antimicrobial activity of the plant species. Different chemical compounds present in the preparations like polyphenols, unsaturated sterols and saponin are supposed to have anti-microbial and anti-viral properties (Geyid, 2005). Leaf extract of *J. schemperiana* can have active ingredients like phenolic compounds which were also tested and showed some activity against rabies virus (Chavez *et al*. 2006).

C. macrostachyus was one of widely known and used traditional plant for the treatment of different bacterial, fungal and viral disease in our society (Amuamuta *et al*., 2014; Geyid *et al*., 2005). The plant showed a good result in keeping mice from death next to root extract of *P. dodecandra*. At 2000mg/kg crude extract from root bark of *C. macrostachyus* showed significant value (P=0.011). It is the only plant extract in this experiment which saved the life of mice from all predetermined dose levels i.e. one mouse from each 300mg/kg (P=0.148) and 5000mg/kg (P=0.080) doses. The extract looks to have anti-viral activity against rabies because of the presence of flavonoid chemical which is a primary antioxidant or free radicals scavengers (Bhandary *et al*., 2012). The free radical scavengers and hydroxyl group present in the chemical can be responsible for anti-viral activity (Madhusudana *et al*., 2004b) similar to *P. dodecandra*.

In many cases, the therapeutic benefits are attributed to the consumption of plant mixtures in which different plant parts are prepared and/or consumed in combination or in sequence (Etikin, 1986; Taylor *et al*., 2001). In this study also, crude extracts from leaves of *J. schimperiana*, roots of *P. dodecandra* and root bark of *C. macrostachyus* were mixed at 1:1:0.75 ratios (information obtained from traditional healers) and observed for anti-viral activity. Only at 5000mg/kg the extract combination increases the survival time of mice 15.2 days (P=0.021). Most traditional healers use combination of different plant preparations to treat rabies victims and this may exert antagonistic effects or negating the positive effects of the bioactive agents, if the active principle presents in high enough quantities (Jager *et al*., 1996) which needs isolation and evaluation of chemical constituents in each plant species.

There were other reports of plants that showed anti-rabies/antiviral activity tested *in vitro* and/or *in vivo* systems. Muller and his colleagues (2007) investigated methanol extract of leaves and flowers of *Alamanda schottii* with some anti-rabies activity tested *in vitro* system. Similarly, Abad and his colleagues (2000) reported aqueous extract from leaves of *Nepeta nepetella* also has antiviral activity. The potential for *in vitro* anti-rabies activity of *Datura metel* (seed) extracts were also reported by Soumen, *et al*., (2016). In addition, Deressa and his colleagues (2010) reported that crude hydro-methanolic and chloroform extracts from roots of *Silene macroselen* and chloroform and aqueous extracts of leaves of *Salix subserrata* showed significant improvement on the survival period of experimental mice compared to control group.

Generally, in the current study most of plant extracts evaluated for anti-rabies activity showed a moderate improvement of mice challenged with rabies virus at higher doses compared to lower doses and control group. This might be attributed to the established fact that the percentage of population affected (viral load) increases as the dose is raised (Goldan *et al*., 2005). On the other hand, as the dose decreased the active ingredient present in the plant parts would be reduced to the level of not inhibiting the activity of viruses. Crude extracts in this study showed a potential anti-rabies activity at higher doses which might be attributed to hold active compounds that affect the propagation and pathogeneses of rabies virus *in vivo* than lower doses (didn't have active ingredients or present in trace amount) (Taylor *et al*., 2001).

6. CONCLUSION AND RECOMMENDATIONS

Hydro-ethanolic (20-80%) extracts of leaves of *J. schemperiana*, roots of *P. dodecandra*, root bark of *C. macrostachyus* and their combination at 1:1:0.75 ratios respectively, increases the survival time of mice as compared to positive control group at higher doses i.e. 5000mg/kg and 2000mg/kg for *C. macrostachyus.* Crude extracts from *P. dodecandra* and *C. macrostachyus* revealed a potential anti-rabies activity compared to all other extract trials in mice model. The finding indicated that there might be the presence of moderate active ingredients that able to affect the propagation and pathogeneses of rabies virus *in vivo* at higher doses when monitored on survival period of mice compared to control group. This ensures rejection of a predetermined null hypothesis.

In line with above concluding remarks, the following recommendations will be forwarded:

- Further fractionation and Isolation of active compounds that exhibit anti-rabies activity using improved techniques is highly recommended

- The standardization of the methods of plant extraction for an *in vitro* testing is highly recommended to make the search for anti viral agents more systematic and interpretation of the results easier.

- In the study area most traditional healers use mixture of plant parts to treat rabies victims and this needs further bio-active chemical identification to reduce occurrence of antagonism or unexpected reaction (Jager *et al*., 1996).

- Sub acute and chronic toxicity tests on the crude extracts and isolated fractions should be examined to setup the starting dose for anti rabies activity.

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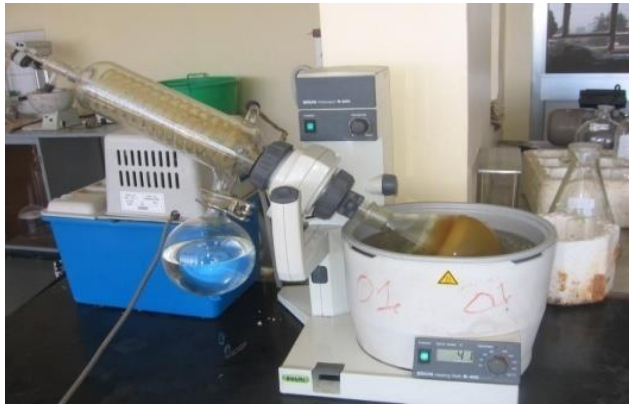
8. APPENDICES

Appendix 1: Process sing and 80% hydroethanolic extraction of medicinal plants.

A: Grinding mill machine

B: Rotary shaker adjusted at 140rpm

C: Samples filtered in Erlenmeyer flasks



D: Buchi rota vapor (on condensation process)

Appendix 2: Photos of mice found in their respective cages and at the time of challenged virus inoculation and extract administration

A: A group of ten mice kept per cage

B: A group of mice found in the treatment group

C: Virus inoculation

D: Extract administration

Appendix 3: Daily mice history recording formats for anti-rabies activity evaluation

Group	Dose	Initial mice number	Accidental death	Sign for rabies	FAT test	Survival time(days)		Status	Remark
						death	censor		

Appendix 4: Daily mice history recording formats for acute toxicity evaluation of extracts

Group	Dose	Initial mice number	Body weight(g) at day0	Accidental death(if any)	Sign for toxicity	Body weight(g) at day7	Body weight(g) at day14	Survival time(days)		Status	Remark
								death	censor		

Appendix 5: FAT Procedures

1. Smear mice brain sample on microscopic slide
2. Fix the smears in acetone at -20 ^{o}c for about 1hr
3. Air dried for about 5-10 minutes
4. Add, on each smear, a sufficient quantity of clarified conjugate (i.e. 0.1ml) reconstituted a vial with 3ml distilled water and centrifuge at 1500rpm for 5 minutes for clarification
5. Incubate at 37^{o}c for 30 minutes in a moist chamber
6. Soak in PBS to decrease any nonspecifically binding substances
7. Rinse with distilled water and air dried
8. Examine slides under a fluorescence microscope

   Source: Bourhy *et al.*, (1989)

Appendix 6: Procedure for cytotoxicity test

1. 50µl of cells (10^{-4} cells/well) were seeded in 96 well micro-plate in triplicates for each plant types and incubated for 24 hr (37^{0}c, air humidified 5% CO_2)
2. 50µl of media is added at the same time
3. 50µl of different concentration of extracts with tenfold serial dilutions were added except for negative control and blank wells and incubated for further 48hr.
4. For determination of either cell survival or growth inhibition each well was incubated with 20µl of MTT solution (5mg/ml) for 3hr.
5. The yellow crystal formazan of MTT salt was changed into purple color by the action of active metabolic cells.
6. This non solubilized substance was solublized with 50µl of dimethyl sulfoxide and slightly pipettes up and down to dissolve the crystal easily.
7. The purple color formed was measured by an ELISA reader at an absorbance of 490nm wave length and calculated the following formula.

$$\% \text{ Growth Inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD ofcontrol group}} x\ 100\right)$$

Source: Reed and Muench, (1938)

Appendix 7a: ELISA plate reader results for cytotoxicity assay at a dose of 1mg/ml in triplicate forms

RawData{Wavelength:490.0}

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.462	0.458	0.454	0.333	0.348	0.351	0.305	0.343	0.358	0.326	0.352	0.324
B	0.462	0.467	0.456	0.325	0.355	0.338	0.312	0.348	0.355	0.328	0.367	0.331
C	0.466	0.487	0.464	0.335	0.352	0.352	0.354	0.36	0.367	0.375	0.364	0.324
D	0.481	0.49	0.467	0.346	0.376	0.364	0.354	0.364	0.374	0.389	0.388	0.408
E	0.506	0.503	0.48	0.362	0.376	0.383	0.384	0.375	0.381	0.395	0.394	0.415
F	0.511	0.503	0.487	0.375	0.384	0.394	0.392	0.386	0.397	0.404	0.416	0.412
G	0.535	0.523	0.488	0.465	0.466	0.473	0.45	0.468	0.469	0.477	0.516	0.484
H	0.042	0.43	0.041	0.042	0.038	0.046	0.04	0.041	0.042	0.045	0.039	0.044

Appendix 7b: ELISA plate reader results for cytotoxicity assay at a dose of 5mg/ml in triplicate forms

Raw Data{Wavelength:490.0}

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.416	0.406	0.411	0.335	0.287	0.271	0.275	0.308	0.305	0.356	0.359	0.322
B	0.453	0.417	0.413	0.357	0.296	0.276	0.293	0.364	0.305	0.375	0.355	0.335
C	0.452	0.423	0.415	0.358	0.307	0.307	0.304	0.374	0.306	0.375	0.361	0.342
D	0.491	0.422	0.422	0.363	0.303	0.306	0.308	0.382	0.314	0.381	0.365	0.344
E	0.507	0.431	0.429	0.384	0.311	0.313	0.32	0.383	0.314	0.387	0.343	0.364
F	0.506	0.439	0.443	0.387	0.318	0.326	0.327	0.391	0.322	0.381	0.369	0.385
G	0.511	0.437	0.475	0.425	0.491	0.444	0.426	0.486	0.459	0.458	0.488	0.464
H	0.039	0.037	0.042	0.041	0.042	0.039	0.042	0.038	0.046	0.045	0.039	0.044

Appendix 7c: ELISA plate reader absorption results for cytotoxicity assay at a dose of 10mg/ml in triplicate forms

Raw Data{Wavelength:490.0}

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.287	0.274	0.307	0.195	0.232	0.191	0.198	0.216	0.232	0.218	0.274	0.234
B	0.29	0.272	0.31	0.201	0.204	0.22	0.337	0.246	0.265	0.262	0.293	0.27
C	0.296	0.277	0.321	0.239	0.221	0.236	0.354	0.254	0.262	0.292	0.276	0.273
D	0.301	0.294	0.333	0.26	0.305	0.183	0.349	0.297	0.284	0.292	0.285	0.286
E	0.34	0.311	0.323	0.272	0.294	0.201	0.345	0.33	0.294	0.302	0.308	0.294
F	0.368	0.352	0.373	0.281	0.332	0.282	0.349	0.328	0.317	0.311	0.318	0.3
G	0.432	0.444	0.465	0.406	0.414	0.433	0.465	0.422	0.441	0.422	0.426	0.438
H	0.043	0.041	0.042	0.041	0.038	0.043	0.04	0.042	0.041	0.041	0.04	0.041

Note: 1, 2 and 3=triplicate for *J. schemperiana*, 4, 5 and 6= for *P. dodecandra*, 7, 8 and 9=for *C. macrostachyus*, 10, 11 and 12 for their compound.

A=10^{-1}, B=10^{-2}, C=10^{-3}, D=10^{-4}, E=10^{-5}, F=10^{-6} dilution rates, G= negative control and H= blank well

Appendix 8: *Paired t-test* result of body weight of mice on day 0, 7 and 14 after administration of different crude plant extracts.

Group	Mean body weight of mice (g)			t-value		df	Sig.	
	Day 0	Day 7	Day 14	day0 - day7	day0 - day14		day0 - day7	day0 - day14
A(50mg/kg)	25.00	32.27	30.28	-14.428	-8.795	4	.011	.002
A(300mg/kg)	25.60	31.94	26.3	-5.106	-1.054	4	.056	.009
A(2000mg/kg)	24.60	26.14	28.36	-.286	-.632	4	.083	.090
A(5000mg/kg)	25.20	30.02	32.94	-13.247	-17.456	4	.010	.030
B(50mg/kg)	27.20	33.04	35.5	-38.847	-15.054	4	.000	.001
B(300mg/kg)	24.20	25.44	21.92	-.221	.455	4	.053	.034
B(2000mg/kg)	20.40	13.40	11.92	2.318	1.944	4	.284	.016
B(5000mg/kg)	27.00	27.53	32.54	-.402	-7.528	4	.122	.076
C(50mg/kg)	23.8	22.1	12.52	1.486	2.811	4	.049	.033
C(300mg/kg)	26.8	29.43	24.46	-2.275	.581	4	.000	.000
C(2000mg/kg)	25.8	31.94	34.56	-6.832	-3.73348	4	.069	.306
C(5000mg/kg)	24.6	16.56	17.9	1.516	1.145	4	.010	.007
D(50mg/kg)	28.6	32.94	36.16	-10.898	-9.019	4	.002	.019
D(300mg/kg)	24.6	26.06	26.1	-.272	-.276	4	.005	.004
D(2000mg/kg)	26.4	31.14	35	-7.683	-10.214	4	.000	.000
D(5000mg/kg)	25	26.87	24.14	-2.308	.158	4	.006	.151
E(Control)	23.4	21.9	24.04	.342	-.128	4	.111	.070

A=*Justicia schemperiana*, B=*Phytolacca dodecandra*, C=*Croton macrostachyus*, D=their compound at 1:1:0.75 ratio for A, B and C respectively, E=negative control, df =degree of freedom, Sig. = Significance

Appendix 9: ANOVA results on mean survival time of mice's infected with challenged virus strain and treated with different types of plant extracts

Group	Source of variation	SS	DF	MS	F	Sig
A(300mg/kg)	B/G	74.115	1	74.115	2.981	.102
	W/G	422.622	17	24.860		
A(2000mg/kg)	B/G	3.200	1	3.20	3.429	.081
	W/G	16.800	18	.933		
A(5000mg/kg)	B/G	182.239	1	182.239	5.077	.038
	W/G	610.182	17	35.893		
B(300mg/kg)	B/G	2.450	1	2.450	3.528	.077
	W/G	12.500	18	.694		
B(2000mg/kg)	B/G	143.869	1	143.869	3.710	.071
	W/G	659.289	17	38.782		
B(5000mg/kg)	B/G	652.126	1	652.126	13.816	.002
	W/G	802.400	17	47.200		
C(300mg/kg)	B/G	54.758	1	54.758	2.302	.148
	W/G	404.400	17	23.788		
C(2000mg/kg)	B/G	361.250	1	361.250	8.055	.011
	W/G	807.300	18	44.850		
C(5000mg/kg)	B/G	81.225	1	81.225	3.500	.080
	W/G	371.275	16	23.205		
D(300mg/kg)	B/G	4.669	1	4.669	4.088	.060
	W/G	18.275	16	1.142		
D(2000mg/kg)	B/G	80.000	1	80.000	3.801	.067
	W/G	378.800	18	21.044		
D(5000mg/kg)	B/G	217.800	1	217.800	6.344	.021
	W/G	618.000	18	34.333		

B/G=between groups, W/G=within groups, SS=Sum square, MS= Mean square, DF=degree of freedom, F= F statistic, Sig. =Significance

Appendix 10: Plant identification certificate

THE NATIONAL HERBARIUM
ADDIS ABABA UNIVESITY – FACULTY OF SCIENCE

Date: 25/11/2016

List of specimens collected by Mr. Demeke Zewude, AAU

Voucher No	Local name	Scientific/Botanical name	Family
001	bisana	Croton macrostachyus Del.	Euphorbiaceae
002	sensel	Justicia schimperiana (Hochst. ex Nees) T Anders.	Acanthaceae
003	endod	Phytolacca dodecandra L 'Herit.	Phytolaccaceae

The National Herbarium (ETH)
Addis Ababa, Ethiopia

Appendix 11: Ethical clearance certificate