International Journal of Basic and Applied Virology 8(2): 28-37, 2019
ISSN 2222-1298

DOI: 10.5829/idosi.ijbav.2019.28.37

Determination of Anti-Rabies Virus Activities of Crude Extracts from Some Traditionally Used Medicinal Plants in East Wollega, Ethiopia

[1]Demeke Zewde, [1]Fufa Dawo, [2]Brihanu Hurisa, [2]Abebe Mengesha and [2]Ashenif Tadele

[1]Addis Ababa University, College of Veterinary Medicine and Agriculture, Ethiopia
[2]Ethiopian Public Health Institute, Ethiopia

Abstract: An experimental study was conducted between November, 2016 to 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 ratios, respectively. The plants are widely used traditionally for the treatment of rabies in humans and animals in East Wollega, Ethiopia. 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 · East Wollega · Ethiopia · Hydroethanolic Extracts · *In vivo* · Traditional Medicinal Plants

INTRODUCTION

Rabies is a fatal viral zoonosis, which causes encephalitis in warm-blooded animals and humans [1]. 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 [2]. 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 [3].

Canine rabies continued to be a serious problem in Africa, including Ethiopia [4]. Although there are no formal studies, it is estimated that there is one owned dog per five household nationally [5]. 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 [6]. 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 [7].

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

Corresponding Author: Demeke Zewde, Addis Ababa University, College of Veterinary Medicine and Agriculture, Ethiopia.

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 [8]. Rabies virus infection was reduced when infected cells were treated with South American plants and algal polysaccharides extracts [9, 10]. In addition, phenolic compounds were also tested and showed some activity against rabies virus [11].

Recognizing 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 [12]. Traditional medicinal plants are a rich source of inexpensive and novel biologically active compounds [13], which are of great interest to both the developed and developing countries [14]. They have the ability to inhibit the replication cycle of various types of DNA or RNA viruses [15]. 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 [16].

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 [17]. The aim of this study was to evaluate anti-rabies activities of crude extracts from roots of *Phytolacca dodecandra, J. schimperiana, C. macrostachyus* and their compounds at 1:1:0.75 ratios respectively in mice model. Acute toxicity of the plant extracts also was determined.

MATERIALS AND METHODS

Collection of Medicinal Plants: 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 like [5, 7]. Botanical identification and authentication was carried out at national herbarium department of Addis Ababa University as shown in (Table 1).

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) 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 byDebella[18]. 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. 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. 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. 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 [19, 20].

Experimental Design: Acute toxicity test: For acute toxicity evaluation, five female albino mice per treatment and control groups were used. Initially body weights of all

Table 1: 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. *Justicia schemperiana*	*Euphorbiaceae*	Bisana	Root bark	001
(Hochst. ex Nees) T Anders.	*Acantiaceae*	Sensel	Leave	002
Phytolacca dodecandra L'Herit	*Phytolaccaceae*	Endod	Root	003

Table 2: 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 immediately during virus inoculation or extract administration.

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 [21]. A volume of 20mg/ml distilled water was used for oral preparation [22]. Mortality in each group within 24 hours was recorded and the animals were observed for about 14 days for any sign of delayed toxicity [23]. 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) [21] 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 challenged virus strain-11. Details of the experimental animal group classifications are described in (Table 2). 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 [22] for 30g mice.

Experimental Animals and Ethical Consideration: 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 Ethiopian public health institute. The experimental procedures and protocols used in this study were approved by animal welfare ethical committee of Addis Ababa university, collage of Veterinary Medicine and Agriculture with certificate ref. no. VM/ERC/29/06/09/2017 [24] and all mice subjected to the experiment were handled according to standard guidelines for the use and care of laboratory animals. 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*. [25]. Mice were randomly assigned to ten per group for evaluation of anti-rabies activity of plant extract as shown in (Table 4). The laboratory animals were provided with pelleted ration feed and water *ad libitum*. 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. 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 [26] 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.

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 [27, 28] for a single challenge which were determined according to methods of Reed and Muench [29]. 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. CVS-11 virus inoculation was conducted intra-cerebrally [30]. Hence, all groups of mice including the control group were inoculated with CVS-11 virus strain at day 0 after three days of acclimatization.

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-gastric needle (lavage) based on the animal's body weight at 20ml/kg vehicle [22]. 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.

Determination of Mortality Rate: Mortality rates as a result of rabies virus challenge were determined by clinical signs and direct fluorescent antibody test [31]. 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. The direct FAT used commercially available rabies anti-nucleocapsid antibodies (polyclonal antibody) tagged with fluorescein isothiocyanate (FITC) -dye (Rabies Conjugate Anti-nucleocapsid, BIORAD, South Africa) and the working dilution was prepared in accordance with the manufacturers recommendations [32].

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 Deanand Abelseth[33]. 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 [34] 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 Bourhy *et al*. [31]. 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°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 [35].

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 $P<0.05$ [36].

RESULTS

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 3).

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.

Table 3: 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

Table 4: 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

None of the mice were protected from rabies deaths from groups of mice treated with 80% hydro-ethanolic root extract of *P. dodecandra* at300mg/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 4).

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'tsignificantly ($P>0.05$) increase thesurvival rate of mice as compared to positive controlgroup as shown in (Table 5).

Table 5: Significance level of plant extracts against challenged rabies virus

Group	95% CI, for survival time	Survival time	*p* -value
A(300mg/kg)	(7.832, 17.279)	12.56(days)	0.102
A(2000mg/kg)	(8.672, 10.128)	9.4(days)	0.081
A(5000mg/kg)	(8.563, 21.437)	15(days)	0.038
B(300mg/kg)	(8.712, 9.888)	9.3(days)	0.077
B(2000mg/kg)	(8.200, 20.022)	14.11(days)	0.071
B(5000mg/kg)	(13.808, 26.858)	20.33(days)	0.002
C(300mg/kg)	(7.380, 16.620)	12(days)	0.148
C(2000mg/kg)	(11.246, 22.954)	17.1(days)	0.011
C(5000mg/kg)	(7.858, 17.892)	12.87(days)	0.080
D(300mg/kg)	(8.649, 10.601)	9.63(days)	0.060
D(2000mg/kg)	(8.602, 16.598)	12.6(days)	0.067
D(5000mg/kg)	(10.082, 20.318)	15.2(days)	0.021
E(Control)	(8.167, 9.033)	8.6(days)	

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

Table 6: Effect of crude extracts on body weight (paired t test) and survival time of mice

	Body weight in gram (g) (mean ± SD)			*p* -values for body weight			Survival time with p-value	
Group	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:Standarddeviation,A:*J.schemperiana*,B:*P.dodecandra, C:macrostachyus*,
D: their combination

Acute Toxicity Test: 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 6).

DISCUSSION

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 ($p>0.05$) in terms of survival time. The result suggests that the *in-vivo*oral median lethal dose (LD50) of the extracts of all plant partscould be greater than limit dose 5000 mg/kg which is in line with the report of Andualem *et al.* [37] who reported LD50 for *J. schimperiana* might be greater than 2000mg/kg.

The current result of root extracts for *P. dodecandra* disagrees with the reports of Admasu *et al.*[38] 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 [39]. 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 report of Admasu *et al*. [38] (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 report of Admasu *et al.*[38] 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 [38] and Coxsackie virus *in-vitro* system [40].

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 report of Geyid *et al.*[20] 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 [20]. Leaf extract of *J. schemperiana* can have active ingredients like phenolic compounds which were also tested and showed some activity against rabies virus [11].

C. macrostachyus was one of widely known and used traditional plant for the treatment of different bacterial, fungal and viral diseases in our society [20, 41]. 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 [42]. The free radical scavengers and hydroxyl group present in the chemical can be responsible for anti-viral activity [39] 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 [43, 44]. 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 mayexert antagonistic effects or negating the positive effects of the bioactive agents, if the active principle presents in high enough quantities [45].

There were other reports of plants that showedanti-rabies/antiviral activity tested *in vitro* and/or *in vivo*systems. Muller *et al.* [9] investigated methanol extract of leaves and flowers of *Alamanda schottii* with some anti-rabies activity tested *in vitro* system. Similarly, Abad *et al*. [46] reported that 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*. [47]. In addition, Deressa *et al*. [48] reported that crude hydro-methanolic and chloroform extracts from roots of *Silene macroselen* and chloroformand aqueous extracts of leaves of *Salix subserrata* showed significant improvement on the survival period ofexperimental 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 [49]. 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) [44].

CONCLUSIONS

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

ACKNOWLEDGEMENTS

We are very much grateful to Asfaw Meresa, Jemal Mohamod, Sintayehu Abdena, Geremew Tassew and Dereje Nigussie for their positive cooperation and technical assistance during the laboratory work. We would like to extend our thanks to Ethiopian public health institute for its invaluable cooperation for this study.

Funding: The cost of this research work was covered by the researcher own and helps of Ethiopian public health institute.

Availability of Data and Materials: The data sets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate: Ethical approval and consent for this study was obtained from Addis AbabaUniversity College of Veterinary Medicine and Agriculture Minutes of AnimalResearch Ethics and Review committee (ref. no.VM/ERC/29/06/09/2017). Verbalconsent was also obtained from the laboratory animal department unit.

Consent for Publication: Not applicable.

Competing Interests: The authors declare that they have no competing interests.

REFERENCES

1. Zulu, G.C., C.T. Sabeta and L.H. Nel, 2009. Molecular epidemiology of rabies focus on domestic dogs (Canisfamiliaris) and black-backed jackals (Canismesomelas) from northern South Africa. Virus Res., 140: 71-78. Make references like this style.
2. Hendekli, C.M., 2005. Brief review Current therapies in rabies. J. Archvirol., 150: 1047-1057.
3. Wunner, W.H. and D.J. Briggs, 2010. Rabies in the 21 century. PLoSNegl Trop. Dis., 4(3): 591.
4. Pritchard, J. and Z. Dagnatchew, 2010. Animal welfare policy and Legislative Gap Analysis. Addis Ababa, Ethiopia.
5. Deressa, A., A. Ali, M. Beyene, B. Newaye Selassie and E. Yimer, 2010. The status of rabies in Ethiopia: A retrospective record review. Ethio. J. Health Dev., 24: 127-132.
6. Admasu, P. and Y. Mekonnen, 2014. Rabies and its Folk Drugs Remedies in Ethiopia: A Review. Int. J. Bas and App. Viro., 3: 22-27.
7. Fekadu, M., 1997. Human rabies surveillance and control in Ethiopia. In: proceeding of the southern and eastern African rabies group meeting 1997 March 4-6; Nairobi, Kenya.
8. Dawit, A. and A. Ahadu, 1993. Veterinary practice and medicinal plants. In: Medicinal plants and Enigmatic Health practice of Northern Ethiopia. ILCA. Addis Ababa, Ethiopia, pp: 419-432.
9. Muller, V., J.H. Chavez, F.H. Reginatto, Zucolotto, S.M. Niero, R.D. Nararro, R.A. Yunes, E.P. Schenkel, C.R. Barardi, C.R. Zanetti and C.M. Simoes, 2007. Evaluation of Antiviral Activity of South American Plant Extracts Against Herpes Simplex Virus Type 1 and Rabies Virus. Phytotherapy Research, 21: 970-974.
10. Mansour, H.A., S.A. Shoman and M.H. Kdodier, 2011. Antiviral effect of edaphic cyanophyteson rabies and herpes-1 viruses. ActaBiol Hung, 62(2): 194-203.
11. Chavez, J.H., P.C. Leal, R.A. Yunes, R.J. Nunes, C.R. Barardi, A.R. Pinto, C.M. Simoes and C.R. Zanetti, 2006. Evaluation of antiviral activity of phenolic compounds and derivatives against rabies virus. Vet Microbiol., 116(1-3): 53-9.
12. WHO, 1996. Traditional Medicinal Program. WHO manual for traditional medicine program policy and activities.World Health Organization (WHO), Geneva, Switzerland, pp: 96-102.
13. Hostettmann, K., A. Marston, K. Ndjoko and J.L. Wolfender, 2000. The potential of African plants as source of drugs. Current Organic Chemistry, 4: 973-1010. Make references like this style.
14. McGaw, L.J., A.K. Jägerand J.Van Staden, 2000. Antibacterial, anthelmintic and anti-amoebic activity in South African medicinal plants. J. of Ethno Pharm.72: 247-263.
15. Jassim, S.A. and M.A. Naji, 2003. A Review Novel antiviral agent: a medicinal plant perspective. J. Appl. microbiol., 95: 412 - 427.

16. Masika, P.J. and A.J. Afolayan, 2002. Antimicrobial activity of some plants used for the treatment of livestock disease in the Eastern Cape, South Africa. J. Ethno Pharm., 83: 129-134.
17. Tilahun, T. and G. Mirutse, 2006. Ethnobotanical study of medicinal plants used by people in Zegie Peninsula, Northwestern Ethiopia. J. Ethnobiol. Ethnomed, 3: 12.
18. Debella, A., 2002. Manual for phytochemical screening of medicinal plants. Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia, pp: 1-83.
19. Holetz, F.B., G.L. Pessini, N.R. Sanches, A.G. Cortez, C.V. Nakamura and B.D. Filho, 2002. Screening some plants used in Brazilian folk medicine for the treatment of infectiousdiseases. MemInstOswaldo Cruz. Rio de Janeiro, 97(7): 1027-1031.
20. Geyid, A., D. Abebe, A. Debella, Z. Mekinene, F. Abera, F. Teka, T. Kebede, K. Urga, K. Yersaw, T. Biza, B. Hailemariam and M. Guta, 2005. Screening of some medicinal plants of Ethiopia for their antimicrobial properties and chemical profiles. J. Ethno Pharmaco., 97: 421-427.
21. OECD, 2001. Organization of economic corporation and development's guideline for testing of chemicals. pp: 4/14.
22. Erhirhie, E.O., N.E. Ekene and D.L. Ajaghaku, 2014. Guidelines on dosage calculation and stock solution preparation in experimental animals' studies. Journal of Natural Sciences Research, 4: 18.
23. Miller, L.C. and M.L. Tainter, 1944. Estimation of the ED50 and its errors by means of logarithmic-probit graph paper. Biol. Med., 57: 261-264.
24. OECD, 2002. Guidelines for the Testing of Chemicals / Section 4: Health Effects Test No. 423: Acute Oral toxicity - Acute Toxic Class Method. Organization for Economic Cooperation and Development, Paris, France.
25. Gebrie, E., E. Mekonnen, A. Debella and L. Zerihun, 2005. Phytochemical Screening and Pharmacological Evaluations for the antifertility effect of the methanolic root extract of Rumexsteudelii. J. Ethnopharmecol., 96: 139-143.
26. WHO, 2013. Control of neglected tropical diseases (NTD). Map production: Health statistics and information systems (HIS).
27. OIE, 2008. Manual of diagnostic tests and vaccines for terrestrial animals. CHAPTER 2.1.13.Rabieshttp:// www.oie.int/ fileadmin/ Home/ fr/ Health_standards/tahm/2.01.13_RABIES.pd
28. Stokes, W., R. Mcfarland, J. Kulpa-eddy, D. Gatewood, R. Levis, M. Halder, G. Pulle, H. Kojima, W. Casey, A. Gaydamaka, T. Miller, K. Brown, C. Lewis, J.M. Chapsal, L. Bruckner, S. Gairola, E. Kamphuis, C.E. Rupprecht, P. Wunderli, L. Mcelhinney, F.D.E. Mattia, K. Gamoh, R. Hill, D. Reed, V. Doelling, N. Johnson, D. Allen, L. Rinckel and B. Jones, 2012. Report on the international workshop on alternative methods for human and veterinary rabies vaccine testing: State of the science and planning the way forward. Biologicals, 40: 369-381.
29. WHO, 1954. Laboratory Techniques in Rabies. WHO, Geneva, Switzerland.
30. Wunderli, P.S., D.W. Dreesen, T.J. Miller and Baer, 2006. The Rabies Peripheral Challenge Test: More Accurate Determination of Vaccine Potency. Vaccine, 24: 7115-7123.
31. Bourhy, H., P.E. Rollin, J. Vincent and P. Sureau, 1989. Comparative field evaluation of Fluorescent-Antibody Test, Virus isolation from tissue culture and enzyme immunodiagnosis for rapid laboratory diagnosis of rabies. J. Clin Microbiol., 27(3): 519-523.
32. Flamand, A., T.J. Wilttor and H. Koprowsk, 1980. Use of Hybridoma Monoclonal Antibodies in the Detection of Antigenc Differences between Rabies and Rabies-Related Virus Proteins. I. The Nucleocapsid Protein. II. The Glycoprotein. J. Gen Viro., 48: 97-109.
33. Dean, D.J. and M.K. Abelseth, 1973. The fluorescent antibody test. In laboratory techniques in rabies, Eds., Kaplan, M.M. and H. Koprowski, WHO, Geneva, Switzerland, pp: 73-84.
34. WHO, 1992. World Health Organization Expert Committee on Rabies. 8th report. WHO Technical Report Series, no. 824. Geneva: World Health Organization, pp: 7-8.
35. Lembo, T., M. Niezgoda, A. Velasco-Villa, S. Cleaveland, E. Ernest and C.E. Rupprecht, 2006. Evaluation of a Direct Rapid Immunohistochemical Test for Rabies Diagnosis. Emerging Infectious Disease, 12(2): 310-313.
36. Bross, I.D.J., 1971. "Critical Levels, Statistical Language and Scientific Inference," In foundations of statistical inference, eds., Godambe, V.P. and Sprott, Toronto: Holt, Rinehart and Winston Canada, Ltd.

37. Andualem, T., M. Eyasu and G. Shewatatek, 2016. Hypoglycemic and antihyperglycemic activity of aqueous extract of Justicia schimperiana leaves in normal and streptozotocin-induced diabetic mice., 7(02): 107-113.
38. Admasu, P., A. Deressa, A. Mengistu, G. Gebrewold and T. Fayera, 2014. *In vivo* Evaluation of Hydroethonolic Extract of Roots and Phytolacca dodecandra. Global Veterinaria, 12(1): 12-18.
39. Madhusudana, S.N., R. Shamsundar and S. Seetharaman, 2004b. *In vitro* inactivation of the rabies virus by ascorbic acid. Int. J. Infect. Dis., 8: 21-25.
40. Zimudzi, C., 2007. Phytolacca dodecandra L'Herit. In record from Protabase, Eds., Schmelzer, G.H. and A. Gurib- Fakim, PROTA (Plant Resources of Tropical Africa, Wageningen, Netherlands.
41. Amuamuta, A., Z. Mekonnen and E. Gebeyehu, 2014. Therapeutic Usage and Phytochemical Screening Study on some Selected Indigenous. Eur. J. App. Sci, 6(4): 83-90.
42. Bhandary, K., K. Sucheth, V. Bhat, K. Sharmilaand and B. Bekal, 2012. Preliminary screening of various extracts of Punicagranatum peel, whole fruit and seeds. Nitte Univ. J. Health Scie, 2: 34-38.
43. Etikin, N.L., 1986. Multidisciplinary perspectives in Interpretation of Plants used in Indigenous Medicine and Diet, Redgrave publishing company, New York, USA, pp: 2-29.
44. Taylor, J.L., T. Rabe, L.J. McGaw, A.K. Jager and J. Van Staden, 2001. Towards the scientific validation of traditional medicinal plants. Plant Gro. Regul., 34: 23-37.
45. Jager, A.K., A. Hutchings and J.van Staden, 1996. Screening of Zulu medicinal plants for protaglandin-synthesis in-hibitors. J. Ethno Pharmacol., 52: 95-100.
46. Abad, M.J., A. Guerra, P. Bermejo, A. Irurzun and L. Carrasco, 2000. Search for Antiviral Activity in Higher Plant Extracts. Phytotherapy Research, 14: 604-607.
47. Soumen, R., M. Sandeepan, P. Sandip and C. Abhay, 2016. Evaluation of *In vitro* Antiviral Activity of Datura metel Linn. Against Rabies Virus.Pharmacognosy Res., 8(4): 265-269.
48. Deressa, A., K. Hussen, D. Abebe and D. Gera, 2010. Evaluation of the Efficacy of Crude Exracts of. Salix subserrata and Silene macroselen for the Treatment of Rabies in Ethiopia. Ethio Vet. J., 14: 1-16.
49. Goldan, D.E., A.H. Tashjian, E.J. Armstrong, T.M. Galanter, A.W. Armstrog, R. Arnaout and H.S. Rose, 2005. Principles of Pharmacology. The Pathophysiologic Basis of Drug Therapy. Lippincott Willians and Wilkins. New York. USA, pp: 1-856.