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In vivo Antirabies Activity Evaluation of Hydroethanolic Extract of Roots and Leaves of *Phytolacca dodecandra*

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Abstract: The study was undertaken to evaluate antirabies activity of hydroethanolic extract of roots and leaves of *Phytolacca dodecandra* (L' Herit) (Phytolaccaceae), one of the widely used plants for traditional treatment of rabies in humans and animals in Ethiopia, by using mice model. The antirabies activity of both parts of plant extract in the doses of 300, 600 and 1000 mg/kg were compared with negative control based on the difference in survival rate and period (days) of group of mice challenged with rabies virus (CVS-11). The result showed that all doses of roots and 300 and 600 mg/kg doses of leaves of the plant extract didn't significantly ($P>0.05$) increase the survival period of mice compared to negative control group and significant ($P<0.05$) survival period decrement was obtained compared to both positive control groups. However, 1000 mg/kg dose of leaves of the plant extract was significantly ($P<0.05$) increased the survival period of mice as compared to their respective negative control group. The finding indicated the existence of some antirabies activity in extract of leaves of *P. dodecandra* at higher dose, for which further research is needed to elucidate its active ingredients.

Key words: Antirabies · *In vivo* · Hydroethanolic Extract · *Phytolacca dodecandra*

INTRODUCTION

Rabies is a fatal viral zoonotic disease which causes encephalitis in all warm-blooded animals including humans [1]. Though the first appearance of the disease was in the fourth century B.C, precise diagnosis was not possible before the first century B.C [2, 3]. The first human rabies vaccine was developed in 1885 by Louis Pasteur and since then, significant developments have been made in this field including progress in laboratory diagnosis, vaccination and rabies control in wild, domestic and farm animals [4].

Rabies kills an estimated 55,000 per year, mostly in Africa and Asia [5, 6]. Though there are significant numbers of rabies-related deaths in many developing countries, the economic impact of rabies is probably far greater than overt human mortality. Global economic cost of rabies is estimated to be more than $583 million which didn't include the trauma that deaths from rabies inflict on families and communities. Costs associated with rabid animals and those incurred from the moment a suspect rabid animal were high due to various remedies owning to the disease [7, 8]. Although rabies can be well controlled among domesticated animals by different types of useful and widely available vaccines, canine rabies continued to be a serious problem in Africa, including Ethiopia.

Traditional medicine (TM) includes herbal medicines composed of herbs, herbal materials, herbal preparations and finished herbal products (Contain as active ingredients of plant parts, or other plant materials) [9]. Most developing countries, especially those in Asia, Africa, Latin America and the Middle East, 70%–95% of their population rely on traditional medicines for treatment of different diseases [10]. Herbal medicines include the medicinal products of plant roots, leaves, barks, seeds, berries or flowers that can be used to promote health and treat diseases in humans and animals [11]. The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant which used as sources of medicines throughout history and continued to serve as the basis for many pharmaceuticals used today [12] However, their potential as the source of drugs is still unexplored [13].

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In Ethiopia TM have been using since time immemorial, with 90% of population dependent on TMs for the management diseases in both humans and animals [10]. The wide spread use of traditional medicine among both urban and rural population of Ethiopia could be attributed to cultural acceptability, physical accessibility and economic affordability as compared to modern medicine. Various traditional antirabies plants were reported in different indigenous people and parts of Ethiopia which were used for the treatment of rabies in humans and animals. Among the reported plants, *Phytolacca dodecandra* is widely used for the traditional treatment of rabies in both humans and animals in Ethiopia. However, a little work was done on evaluating the efficacy of these and other unidentified ethno-medicinal and ethono-veterinary plants used to treat rabies cases.

Despite the wide availability of different types of useful vaccines used for rabies treatment, a continuing search for new compounds having antirabies agent remains crucial. Nearly 25% of modern medicines are derived from plants first used traditionally [9]. Traditional herbal rabies treatment is among the traditional medicine used by people in different parts of Ethiopia for the treatment of rabies. In spite of this, the effectiveness and safety of traditionally used antirabies herbal medicine in Ethiopia is not well demonstrated and understood than that of conventional antirabies vaccines. Though there is a lack of published evidences on overall problems posed by these traditionally used antirabies herbal remedies, adverse fatal side effects and cases of rabies deaths after traditionally treated were the most problems reported by some health centres/institutes in Ethiopia including Ethiopian Health Research Institute (EHNRI) owning to the non-standardization of constituents, quality and efficacy of these traditionally used antirabies herbal remedies. On the other hand, people and traditional rabies healers in Ethiopia claim that these traditional plants can cure both animals and humans that are exposed to rabies. Moreover, a new plant-derived antirabies compound investigation is of paramount importance not only in combating rabies in humans and animals but also helps in the progress of biomedical research.

About twelve traditional antirabies plants in different indigenous people from different parts of Ethiopia were reported by different investigators for the treatment of rabies in humans and animals. However, efficacy of these plants against rabies were not evaluated with modern pharmaceutical practices except findings of Deressa and his colleagues [14] who evaluated the efficacy of antirabies activities of crude extract of *Salix subserrata* and *Silene macroselen* plants in mice which improved the survival period (Days) of experimental mice compared to control group of mice. *P. dodecandra* is extensively reported traditionally used plant for the treatment of both humans and animals rabies among different ethnic groups of Ethiopia [15- 20]. However, no study has been made in evaluation of antirabies activity of extracts of roots and leaves of *P. dodecandra* both *in vivo* and *in vitro* systems. Therefore, the objectives of the study to evaluate antirabies activities of hydroethanolic extract of roots and leaves of *P. dodecandra* in compared with negative control group of Swiss albino mice.

MATERIALS AND METHODS

Plant Material Preparation and Extraction: Roots and leaves of *P. dodecandra* used by traditional healers were collected from natural habitat and were cut into pieces, washed thoroughly, air-dried (Dried indoors without exposure to sunlight), reduced to small pieces and preserved in a deep freezer until extraction of the plant material. Plant materials were weighed by sensitive digital balance and extracted in 80% ethanol by maceration method and concentrated according to the procedures given by Debella [21]. Briefly, 100 g of powdered plant materials were soaked in 1000 ml of 80% ethanol in Erlenmeyer flask of one liter capacity. The flask containing dissolved plant materials and 80% ethanol mixture was plugged with cotton wool and kept on a rotary shaker at 120-190 rpm for 24 hours. After 24 hours the supernatant was filtered with Whatman (No.1) filter paper and concentrated via rotary evaporator. Then trace solvent was evaporated on water bath of +40°C and under vacuum to dryness. Additional solvent was added to residue and filtered two more times. Finally, yield of extracts were stored at -4°C in airtight container through out the study period.

Experimental Animals and Their Management: The experimental study was carried out on both male and female Swiss albino mice of 4-7 weeks old and 20-35 g in weight. All laboratory animals used for this experiment were bred in a standard laboratory animal house of Ethiopian Health and Nutrition Research Institute (EHNRI). The experiment was approved by EHNRI and all animals subjected to the experiment were handled according to standard guidelines for the use and care of laboratory animals. After animals were obtained from the

laboratory animal unit, they were housed in a littered clean metal cage and in 12 hours light /dark cycle with litter changed every three days. Mice were randomly assigned to six per group and kept in one cage containing same sex for antirabies activity evaluation of both parts of plant extract. The animals were provided with pelleted ration (Mice cubes) and clean water *ad libitum*. The animals were left under controlled conditions at least for three days to acclimatize before conducting any experimental procedure and each animal was used only for one experiment. All experimental mice were treated under similar feeding management. The investigator and all personnel managing rabies virus-inoculated animals were administered with commercially available pre-exposure antirabies vaccine Verorab® (PVRV, Sanofipasteur, France), according to WHO guidelines of antirabies pre-exposure prophylaxis, three doses of vaccine injection on days 0, 7 and 21 intramuscularly at deltoid area of the arm.

Virus Strain and its Inoculation: Titre of CVS-11 rabies virus was prepared from rabies infected suckling mouse brains and the virus (Atlanta, Georgia, USA) was diluted by phosphate-buffered saline (PBS) solution to contain 50-200 mouse intramasseter 50% lethal dose ($MIMLD_{50}$) per 0.03 ml for a single challenge which were determined according to methods of Reed and Muench [22] before this experiment was conducted. Protocols for this experiment followed the guidelines on care and well being of research animals and standard protocol observed in accordance with the Good Laboratory Practice (GLP) Regulations in rabies [23]. CVS-11 virus inoculation via intramasseter muscle was performed based on the procedure Wunderli and his colleagues [24]. Hence, all groups of mice were inoculated with CVS-11 virus strain at day 0. Treatment groups of mice were administered with dose of 300, 600 and 1000 mg extracts of both roots and leaves of *P. dodecandra* for seven consecutive days after an hour of CVS challenge.

Experimental Design: The animals were randomly assigned into two main groups: treatment group and negative control groups. Treatment groups were classified into three sub-groups for each dose of both parts of plant extract; accordingly three dose levels (300, 600 and 1000 mg/kg) for each part of plant extract were employed. One placebo negative control group of mice were administered dH_2O instead of both parts of plant extracts but challenged with CVS-11. Experimental animal group classifications are described in Table 1. Administration of the extracts and distilled water (dH_2O) was done by using an intra-gastric needle based on the animal body weight in 1 ml vehicle.

Determination of Mortality Rate: Mortality rates of rabies were determined by clinical signs and/or direct fluorescent antibody test (FAT). All mice were maintained and followed for consecutive 30 days after virus challenge and they were observed daily after infection with rabies virus for signs of rabies (Roughing fur, tremors, incoordination, paralysis and prostration) and any signs of rabies recorded each day on the mouse history cards. Direct FAT which stained with commercially available rabies anti-nucleocapsid antibodies (Monoclonal 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 [25].

Confirmatory diagnosis of rabies through direct FAT conducted by opening of the skulls and collection of the brain of mice, followed according to procedure specified by Dean and Albelseth [26] 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, aponeurosis 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 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 [27]. 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 one hour to overnight at -20°C. Brain impression smears were stained with FITC-labelled antirabies conjugate and incubated for 30 minutes at 37°C. Finally, after excessive conjugate was drained from the slides and mounting media was applied, the slides were investigated under 40X objective of fluorescence microscope to detect characteristic green fluorescence associated with rabies antigen [28]. All mice dead from rabies and tissues processed containing rabies virus were disposed as medical waste and all samples for rabies diagnosis were performed using appropriate biosafety practices to avoid direct contact with potentially rabies virus-infected tissues or fluids.

Table 1: Classification of experimental mice into different groups with their respective descriptions

Description of administered substance/dose/frequency/route	Number of mice/group	Group assigned
300 mg/kg of *P.dodecandra* roots extract for 7 days, PO	10	A
600 mg/kg of *P.dodecandra* roots extract for 7 days, PO	10	B
1000 mg/kg of *P.dodecandra* roots extract for 7 days, PO	11	C
300 mg/kg of *P.dodecandra* leaves extract for 7 days, PO	11	A'
600 mg/kg of *P.dodecandra* leaves extract for 7 days, PO	11	B'
1000 mg/kg of *P.dodecandra* leaves extract for 7 days, PO	12*	C'
Placebo administration of 1ml of dH_2O, PO	12	D

dH_2O= distilled water, PO= Per Os.

*Any number below 12 mice per group was due to accidental deaths of mice during virus inoculation oral administration.

Statistical Analysis: Data were analyzed using SPSS version 20 by using different statistical tools to determine survival rate and mean survival period (Days) in each group of mice. Student's t test was used to evaluate the significance of observed differences between groups of mice in the mean survival period (Days) and Chi-square test with Fisher's exact test was employed to compare number of survivors (Survival rate) in different groups of mice. All statistical evaluations were two-tailed and P-values < 0.05 were considered as significant [29].

RESULTS

Percentage survival and mean survival period of group of mice infected and treated with both parts of plant extract and infected but treated with both 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 showed 0% survival rate and 9.08 days survival time and none of the mice were protected from rabies deaths from groups of mice treated with hydroethanolic extract of roots of *P. dodecandra* at 300 mg/kg and 1000 mg/kg doses and only 1 (10%) of mice were protected when same plant part administered at 600 mg/kg dose level. The mean survival time of mice treated with roots of *P. dodecandra* extract at 300, 600 and 1000 mg/kg were 9.9, 12.2 and 10.36 days, respectively (Table 2). Almost approaching results were obtained between groups of mice treated with all doses of an extract of roots of the plant and negative control group of mice in their percentage survival and mean survival period.

In groups of mice treated with hydroethanolic extract of leaves of *P. dodecandra* at 300, 600 and 1000 mg/kg doses, 0 (0%), 1(9.1%) and 4 (33.3%) mice were protected from rabies deaths, respectively. The mean survival time of group of mice treated with leaves of *P. dodecandra* extract at 300, 600 and 1000 mg/kg include 10.81, 8.18 and 22.83 days, respectively (Table 2). Relatively higher percentage survival (33.3%) and mean survival period (22.83 days) were obtained when an extract of leaves of *P.dodecandra* administered at 1000 mg/kg unlike the effect of all doses of root part of the plant extract.

The results depicted all doses of both parts of the plant extracts didn't significantly (P>0.05) increase the survival rate of mice as compared to negative control group of mice. The result also showed that all doses of roots of the plant extract and 300 mg/kg and 600 mg/kg of leaves extract didn't significantly increase the survival period (days) (P>0.05) of mice compared to negative control group of mice. However, the leaves part of the plant extract at dose of 1000 mg/kg was significantly increased survival period (days) of mice (P<0.05) compared to negative control groups (Table 3).

DISCUSSION

Both parts of plant extract did not significantly increase the percentage survival of treatment group of mice as compared to negative control group. The non-effectiveness of all doses of roots and 300 and 600 mg/kg doses of leaves of the plant was may be due to the absence of compounds (If present they exist in trace amount) that inhibit the propagation and pathogenesis of rabies virus in tested mice. Contrary, the role of antirabies vaccines is to produce antibodies against the virus that restrict infection from mice inoculated with rabies virus (CVS-11) [24, 30]. Oral treatment of mice infected with rabies virus with higher dose (1000 mg/kg) of leaves of *P. dodecandra* which is administered for consecutive seven days increased significantly the mean survival time (P<0.05) compared to those of negative control group which indicates that hydroethanolic extract of leaves of *P. dodecandra* has some antirabies activity. The prolonging survival time effect of leaves extract of *P. dodecandra* was dose dependent (The higher dose showed higher survival time than lower doses) while the effect of roots of the plant extract didn't depend on dose (All three doses of root extract didn't significantly increased the survival time and didn't save mice from death).

Table 2: Effects of roots and leaves of *P. dodecandra* on percentage survival and mean survival period of mice challenged with rabies virus

Group	Survival n (%)	Death n (%)	Survival time (days) (Mean±SD)
A (300 mg/kg)	0 (0%)	10 (100%)	9.90±1.45
B (600 mg/kg)	1 (10%)	9 (90%)	12.20±6.43
C (1000 mg/kg)	0 (%)	11 (100%)	10.36±1.50
A' (300 mg/kg)	0 (0%)	12 (100%)	10.81±6.42
B' (600 mg/kg)	1 (9.1%)	10 (90.9%)	8.18±0.98
C' (1000 mg/kg)	4 (33.3%)	8 (66.7%)	22.83±6.42
D (Placebo)	0 (0%)	12 (100%)	9.08±1.62

SD= Standard deviation, n= number of mice

Table 3: Survival time and survival rate comparison of treatment groups mice with negative control group of mice

Group	Survival time Mean Difference (days)	P-value†	P-value‡
A (300 mg/kg)	-0.82	0.232	nd
B (600 mg/kg)	-3.17	0.119	0.455
C (1000 mg/kg)	-1.28	0.063	nd
A' (300 mg/kg)	-1.73	0.374	0.478
B' (600 mg/kg)	0.90	0.126	nd
C' (1000 mg/kg)	-13.75	0.000	0.093

†P-value for comparison of survival period with negative control group (D) obtained by Student's t test; ‡P-value for comparison of survival rate with negative control group (D) obtained by Chi-square with Fisher's exact test; A, B & C (300, 600 & 1000 mg/kg roots, respectively); A', B' & C' (300, 600 & 1000 mg/kg leaves, respectively), nd-not done

An indication of the presence of some antirabies activity of hydroethanolic extract of leaves of *P. dodecandra* may be due to the presence ribosomal inhibiting protein (RIP) known as dodecandrin which have been proven having antiviral activities [31]. The prolonging survival time effect of leaves extract of *P. dodecandra* on tested mice may also be due to other secondary metabolites of the plant parts or their cumulative effects. The higher dose of leaves extract of the plant part may contain compounds that affect the propagation and pathogeneses of rabies virus *in vivo* than lower doses. Other previous studies showed that the leaves extract of *P. dodecandra* has moderate activity against coxsackie virus *in vitro* system [31]. Tadeg and his colleagues [32] also reported hydroalcoholic extracts of the aerial parts of *P. dodecandra* showed significant activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Histoplasma capsulatum* var. *farciminosum*, which causes epizootic lymphangitis.

There were other reports of plants that showed antirabies/antiviral activity tested *in vitro* and/or *in vivo* systems. Muller and his colleagues [33] investigated methanolic extract of leaves and flowers of *Alamanda schottii* with some antirabies activity tested *in vitro* system. Similarly, Abad and his colleagues [34] reported aqueous extract of leaves *Nepeta nepetella* also has antiviral activity. On the other hand, Deressa and his colleagues [14] reported that crude hydromethanolic and chloroform extracts of roots *S. macroselen* and chloroform and aqueous extracts of leaves of *S. subserrata* showed significant improvement on the survival period of experimental mice compared to negative control group of mice.

CONCLUSION

Both hydroethanolic extract of roots and leaves of *P. dodecandra* did not significantly increase the survival percentage of mice as compared to negative control groups. However, leaves extract of the plant has shown some antirabies activity at 1000 mg/kg dose level when monitored on their survival period (Days) compared to negative control group of mice. The finding indicated that there was an existence of some antirabies activity in extract of leaves of *P.dodecandra* at higher dose level. Therefore, further investigation of the active ingredients of extract of leaves of *P. dodecandra* having antirabies activity should be elucidated to reveal more useful compounds.

REFERENCES

1. Takayama, N., 2008. Rabies: A Preventable but Incurable Disease. Journal of Infectious Chemotherapy, 14: 8-14.
2. Blancou, J., 2004. Rabies in Europe and the Mediterranean Basin: from antiquity to the 19th century. In Historical Perspectives of Rabies in Europe and the Mediterranean Basin, Eds., King, A.A., A.R. Fooks, M. Aubert and A.I. Wandeler, OIE, Geneva, Switzerland, pp: 15-24.

3. Neville, J., 2004. Rabies in the ancient world. In historical perspectives of rabies in Europe and the Mediterranean Basin, Eds., King, A.A., A.R. Fooks, M. Aubert and A.I. Wandeler, OIE, Geneva, Switzerland, pp: 1-13.
4. Muller, W.W., J. Cox and T. Müller, 2004. Rabies in Germany, Denmark and Austria. In historical perspective of rabies in Europe and the Mediterranean Basin, Eds., King, A.A., A.R. Fooks, M. Aubert and A.I. Wandeler, OIE/WHO, Geneva, Switzerland, pp: 79-89.
5. Knobel, D.L., S. Cleaveland, P.G. Coleman, E.M. Fèvre, M.I. Meltzer, M.E. Miranda, A., Shaw, J. Zinsstag and F.X. Meslin, 2005. Reevaluating the burden of rabies in Africa and Asian Bulletin WHO, 83: 360-368.
6. WHO, 2005. WHO expert consultation on rabies, 2004: first report. WHO Tech. Rep. Series, 931: 1-121. Geneva, Switzerland.
7. Meltzer, M.I. and C.E. Rupprecht, 1998. A Review of the Economics of the Prevention and Control of Rabies. Part 2: Rabies in Dogs, Livestock and Wildlife. Pharmacoeconomics, 14: 481-498.
8. Awoyomi, O., I.G. Adeyemi and F.S. Awoyomi, 2007. Socioeconomic factors associated with non-vaccination of dogs against rabies in Ibadan, Nigeria. Nigerian Veterinary Journal, 28: 59-63.
9. WHO, 2002. Traditional Medicine Strategy 2002–2005. WHO, Geneva, Switzerland.
10. WHO, 2011. The World Medicines Situation 2011. Traditional Medicines: Global Situation Issues and Challenges. WHO, Geneva, Switzerland.
11. Li, S., Q. Han, C. Qiao, J. Song, C.L. Cheng and H. Xu, 2008. Chemical Markers for the Quality Control of Herbal Medicines: An Overview. China Medicine, 28: 7-23.
12. Lopez, V., 2011. Are Traditional Medicinal Plants and Ethnobotany Still Valuable Approaches in Pharmaceutical Research? Boletin Latinoamericano y de Caribe de Plantas Medicinales y Aromaticas, 10: 3-10.
13. Karthishwaran, K. and S. Mirunalini, 2010. Therapeutic Potential of *Pergularia daemia* (Forsk.): The Ayurvedic Wonder. International Journal of Pharmacognosy, 6: 836-843.
14. 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. Ethiopian Veterinary Journal, 14: 1-16.
15. Teklehaymanot, T. and M. Giday, 2007. Ethnobotanical Study of Medicinal Plants Used by People in Zegie Peninsula, Northwestern Ethiopia. Journal of Ethnobiology and Ethnomedicine, 3: 12.
16. Giday, M., Z. Asfaw and Z. Woldu, 2009. Medicinal Plants of the Meinit Ethnic Group of Ethiopia: An Ethnobotanical Study. Journal of Ethnopharmacology, 124: 513-521.
17. Giday, M., Z. Asfaw and Z. Woldu, 2010. Ethnomedicinal Study of Plants Used by Sheko Ethnic Group of Ethiopia. Journal of Ethnopharmacology, 132: 75-85.
18. Giday, M., Z. Asfaw, Z. Woldu and T. Teklehaymanot, 2010. Medicinal plant knowledge of the Bench Ethnic Group of Ethiopia: An Ethnobotanical Investigation. Journal of Ethnobiology and Ethnomedicine, 5: 34.
19. Yirga, G., 2010. Assessment of Indigenous Knowledge of Medicinal Plants in Central Zone of Tigray, Northern Ethiopia. American Journal of Plant Science, 4: 006-011.
20. Amenu, E., 2007. Use and Management of Medicinal Plants by Indiginenous People of Ejaji Area (Chelya Woreda) West Shoa Ethiopia: An Ethnobotonical Approach. M..Sc. Thesis, Addis Ababa University, Addia Ababa, Ethiopia.
21. Debella, A., 2002. Manual for Phytochemical Screening of Medicinal Plants. EHNRI, Addis Ababa, Ethiopia, pp: 1-84.
22. WHO, 1954. Laboratory Techniques in Rabies. WHO, Geneva, Switzerland.
23. Wandeler, 1997. Laboratory techniques in rabies. Canadian Veterinary Journal, 38: 457-458.
24. Wunderli, P.S., D.W. Dreesen, T.J. Miller and G.M. Baer, 2006. The Rabies Peripheral Challenge Test: More Accurate Determination of Vaccine Potency. Vaccine, 24: 7115-7123.
25. 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. Journal of General Virology, 48: 97-109.
26. 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.
27. Kissling, R.E., 1975. The fluorescent antibody test in rabies. In The natural history of rabies, Ed., Kissling, R.E., Academic Press, NY, pp: 401-416.

28. 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.
29. 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 of Canada, Ltd.
30. Hooper, D.C., K. Morimoto, M. Bette, M. Weihe, H. Koprowski and B. Dietshchold, 1998. Collaboration of Antibody and Inflammation in the Clearance of Rabies Virus from the CNS. Journal of Virology, 72: 3711-3719.
31. 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 / Ressources végétales de l'Afrique tropicale), Wageningen, Netherlands.
32. Tadeg, H., E. Mohammed, K. Asres and T. Gebre-Mariam, 2005. Antimicrobial Activities of Some Selected Traditional Ethiopian Medicinal Plants Used in the Treatment of Skin Disorders. Journal of Ethnopharmacology, 100: 168-175.
33. Muller, V., J.H. Chavez, F.H. Reginatto, S.M. Zucolotto, R. Niero, 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.
34. 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.