Evaluation of the Efficacy of Crude Extracts of Salix subserrata and Silene macroselen for the treatment of rabies in Ethiopia

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Abstract

A study was conducted between November, 2007 and April, 2008 to evaluate the anti-rabies activity of the leaf of Salix subserrata and root of Silene macroselen which are traditionally used for the treatment of rabies in Bereh-Aleltu Woreda, North Shewa, Central Ethiopia. The study involved oral administration of crude extracts of Salix subserrata and Silene macroselen to Pasteur Virus (PV) strain rabies virus infected mice which were assigned into three treatment and one negative control groups. Chloroform, methanol 80% and aqueous extracts of the study plants were prepared using maceration technique. Statistical analysis was performed by one-way analysis of variance tests coupled to least significant difference to compare result between treatment and positive control groups. Of the 6 crude extracts, chloroform and methanol 80% of Silene macroselen and chloroform and aqueous of Salix subserrata were found to increase the survival time of mice significantly ($P< 0.05$). Nevertheless, aqueous extract of Silene macroselen and methanol 80% extract of Salix subserrata did not exhibit a significant effect on the survival time. 1 day and 2 days treatment groups of the chloroform extract of S. macroselen indicated significant difference on the survival time from the positive control ($P< 0.05$). The chloroform and aqueous extracts of S. subserrata and the methanol 80 % extract of S. macroselen in their 1 day and 3 days treatment showed significant difference on survival time from the positive control group ($P< 0.05$). The results obtained from the present work suggest good correlation between traditional therapeutic uses and the in vivo anti-rabies activity. Further characterization of the active ingredients would reveal useful compounds.

Keywords: Anti-rabies, Ethno-pharmaceutical, Mice model, PV strain, Salix subserrata, Silene macroselen

Introduction

Rabies is a fatal viral zoonosis, which causes encephalitis in warm-blooded animals and humans. 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 a problem in the world (Hendekli, 2005).

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 neuroparalytic 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 Abebe et al., 2003).

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 Mekonnen, 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).

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 acceptabili-

ty, efficacy against certain type of diseases, physical accessibility and economic affordability as compared to modern medicine (Tilahun and Mirutse, 2007).

The practice of traditional medicine for the control of rabies in most parts of Ethiopia is based on the use of plant medicines for many years. Several traditional herbs have been formulated by traditional healers to treat human and animal rabies. The fact that herbal medicines have been employed for such a long time does not guarantee their efficacy and safety. Thus, pharmacological screening of medicinal plants remains important to provide the society with new, effective and safe drugs. The present study was, therefore, carried out to evaluate the anti-rabies activities of the claimed medicinal plants and encourage further research on the claimed plants if they are proven to have antiviral activity.

Materials and Methods

The leaves of Salix subserrata and the roots of Silene macroselen used by traditional healers were collected from Bereh-Aleltu Woreda, North Showa Zone of Oromia Regional State between December, 2007 and January, 2008. Botanical identification and authentication was done using the manual of the Department of Drug Research of the Ethiopian Health and Nutrition Research Institute (DDR-EHNRI), (Dawit Abebe et al., 2003).

Processing and Extraction

The collected plant materials were brought to the DDR-EHNRI and stayed overnight. The specimens were processed and extracted according to the procedures given by Asfaw Debella (2002). Briefly, garbling was performed following collection before drying and processing of the roots and leaves of Silene macroselen and Salix subserrata respectively. The plant materials were chopped into smaller pieces before drying. Then, they were dried indoors without exposure to sunlight for 1 month. The dried plant materials were grounded to various degrees of fineness depending on their botanical structures. The grounded plant materials were weighed by sensitive digital weighing balance (scientech balance) and successively extracted in chloroform, methanol 80% and aqueous solvents in Erlenmeyer flask.

In Vivo Anti-rabies Screening of Crude Extracts

Experimental Animals

Male and female Swiss albino mice weighing 20-25 g were used for the in vivo anti- rabies drug trial. All the animals used for this experiment were bred in a standard laboratory animal house of the Ethiopian Health and Nutrition Research Institute (EHNRI.) After the mice were obtained from the laboratory animal unit, they were housed in a metal cage. As described by Gebrie Endalk et al., 2005 with some modification. Six mice were used in each group and each animal was used only for one experiment.

Experimental Protocols (Table 2)

The animals were randomly assigned into three treatment groups and three controls (one positive control and two negative controls). Though mice were assigned randomly, each group contained only male or female.

Table 2: Experimental Design of the study

Group	No of mice/group	Administration/dose/freq
I	6	*PV 0.25ml, one time, 10- 3
II	6	80mg/kg extract, one time
III**	6	*PV 0.25ml + 80mg/kg extract for 1day
IV**	6	*PV 0.25ml + 80mg/kg extract for 2 days
V**	6	*PV 0.25ml + 80mg/kg extract for 3days
VI	6	Polysorbate-80, 1ml ,one time

* Pasteur Virus strain

**The treatment groups

Dilution of the Extracts

The crude extracts were diluted before administration to the mice. Organic extracts (chloroform and methanol 80% extracts) were diluted using a 4% Polysorbate-80 solvent. Aqueous extracts were diluted using sterile distilled water.

Rabies Virus Inoculation

PV strain rabies virus was obtained from Zoonoses Research Team of the -EHNRI. The stock PV strain rabies virus (10^{-1} rabbit brain suspension) was diluted using sterile distilled water to 10^{-3} suspension. Each mouse in the treatment

groups and positive control was injected 0.25ml of 10^{-3} rabbit brain suspension intramuscularly into gastronemicus muscle (Meslin, 1996).

Extract Administration

After virus inoculation, the mice were allowed to stay in their respective cages for about 1 hour so as to make them calm. Then, the extracts were orally administered to the mice in the treatment groups and negative control (Group II) (Table 2). Administration of the extract was done by using an intra-gastiric needle based on the animal's body weight in 1ml vehicle.

Determination of Mean Survival time

The animals were monitored daily for the sign of paralysis and mortality for about 50 days after inoculation of the virus. Death was recorded for each mouse in the treatment and control groups throughout the follow up period. The mean survival time (MST) for each group was calculated as (Birhanu Mengstie, 2008, unpublished).

$$MST = \frac{\text{Sum of survival time of each mice in a group}}{\text{Initial mice number in a group}}$$

Acute Toxicity Test

All extracts were examined for acute toxicity by administering an oral dose ranging from 0.5 mg/ml to 2mg/ml for 15 mice per extract. Mortality in each group within 24 hours was recorded. The animals were observed for about 7 days for any sign of delayed toxicity (Miller and Tainter, 1944).

Statistical Analysis

Data were analyzed using SPSS version 11.5 for windows software. Statistical analysis was undertaken by one-way analysis of variance (ANOVA) tests coupled to least significant difference (LSD) to compare results among different treatment groups and between treatment and control groups. Mean survival time were calculated by Microsoft-excel 2003 and expressed as mean ±SD for each treatment and control groups. The result was considered statistically significant at 95% confidence level and P-value<0.05.

Result

Parts of plants used by traditional healers to treat rabies are indicated in Table 1.

Table1: Parts of plants reported as used by traditional healers for treatment of rabies in Bereh-Aleltu woreda

Family	Species	Local name	Parts us used	Life form	Preparation	Route
Salicaceae	S. subserrata	Aleltu	Leaves	Tree	Liquid	Oral
Caryophyllaceae	S. macroselen	Wegert	Root	Herbs	Liquid	Oral

Effect of crude extracts on survival time

Table 2 and 3 show a significant ($P<0.05$) effect of crude extracts of S. macroselen and S. subserrata on survival time, respectively. Treatment with crude extracts significantly increases the survival time.

Table 2: Effect of crude extract of S. macroselen root on mean survival time

Extracts/dose	Group	Treatment frequency(day)	Survival time(day)
Chloroform	III	1	32±8.94
80mg/kg	IV	2	27.67±11.8
	V	3	22.8±13.17
PV only*	I	1	12±0.82
0.25ml			
80%MeOH	III	1	26.83±12.97
80mg/kg	IV	2	22.83±13.22
	V	3	24±12.05
PV only*	I	1	8.83±0.69
0.25ml			
Aqueous	III	1	22.67±13.36
80mg/kg	IV	2	19.17±11.96
	V	3	19.33±11.79
PV only*	I	1	9.67±0.94
0.25ml			

N=6; survival time=mean ± SD

*Included to compare with the treatment groups

Table 3: Effect of crude extract of S. subserrata leaves on mean survival time

Extracts/dose	Group	Treatment frequency(day)	Survival time(day)
Chloroform	III	1	50 ±0
80mg/kg	IV	2	6.5 ±1.89
	V	3	43 ± 15.65
PV only*	I	1	9 ±0.57
0.25ml			
80%MeOH	III	1	7.83 ±0.89
80mg/kg	IV	2	16.67 ±14.92
	V	3	10 ±0.57
PV only*	I	1	8.83 ±1.86
0.25ml			
Aqueous	III	1	31.83±9.31
80mg/kg	IV	2	24±12.12
	V	3	27.33±12.25
PV only*	I	1	11.83±1.34
0.25ml			

N=6; survival time=mean ± SD

* Included to compare with the treatment groups

Comparisons of treatment groups with positive control on survival time for each crude extract

Salix subserrata

Chloroform crude extract

As shown in table 4, 1day and 3 days treatment groups with the chloroform extract are significantly associated on the survival time. Therefore, 1 day and 3 days treatment exhibited anti- rabies activity.

Table 4: Multiple comparisons of positive control and treatment groups for chloroform extract of S. subserrata

GROUP (a)	GROUP (b)	Mean Difference (a- b)	Sig.
I	IIII	-41.0000*	.000
	IV	2.5000	.622
	V	-34.0000*	.000
III	I	41.0000*	.000
	IV	43.5000*	.000
	V	7.0000	.176
IV	I	-2.5000	.622
	III	-43.5000*	.000
	V	-36.5000*	.000
V	I	34.0000*	.000
	III	-7.0000	.176
	IV	36.5000*	.000

Where:
GROUP I=PV rabies virus
GROUP III=PV rabies virus + 1 day treatment
GROUP IV=PV rabies virus + 2 day treatment
GROUP V=PV rabies virus + 3 day treatment

* The mean difference is significant at the 0.05 level

i) Methanol 80% crude extract

Methanol 80 % extract did not show any statistical significant association on the survival time (table5).

Table 5: Multiple comparisons of positive control and treatment groups for methanol 80% extract of S. subserrata

GROUP (a)	GROUP (b)	Mean Difference (a-b)	Sig.
I	III	1.0000	.836
	IV	-7.8333	.116
	V	-1.1667	.809
III	I	-1.0000	.836
	IV	-8.8333	.079
	V	-2.1667	.655
IV	I	7.8333	.116
	III	8.8333	.079
	V	6.6667	.178
V	I	1.1667	.809
	III	2.1667	.655
	IV	-6.6667	.178

Where:
GROUP I=PV rabies virus
GROUP III=PV rabies virus + 1 day treatment
GROUP IV=PV rabies virus + 2 day treatment
GROUP V=PV rabies virus + 3 day treatment

The mean difference is significant at the 0.05 level.

i) Aqueous crude extract

As can be seen in table 6, there is a statistical significance difference on the survival time between the control and the treatment groups.

Table 6: Multiple comparisons of positive control and treatment groups for aqueous extract of S. subserrata

GROUP (a)	GROUP (b)	Mean Difference (a-b)	Sig.
I	III	-20.0000*	.004
	IV	-12.1667	.064
	V	-15.5000*	.021
III	I	20.0000*	.004
	IV	7.8333	.222
	V	4.5000	.477
IV	I	12.1667	.064
	III	-7.8333	.222
	V	-3.3333	.597
V	I	15.5000*	.021
	III	-4.5000	.477
	IV	3.3333	.597

Where:
GROUP I=PV rabies virus

GROUP III=PV rabies virus
+
1 day treatment

GROUP IV=PV rabies virus
+
2 day treatment

GROUP V=PV rabies virus
+
3 day treatment

* The mean difference is significant at the 0.05 level.

Silene macroselen

i) Chloroform crude extract

The third and fourth treatment groups have shown a reliable difference on survival time from the control group (table 7). Third group did show greater anti-rabies activity as compared to the fourth group.

Table 7: Multiple comparisons of positive control and treatment groups for chloroform extract of S. macroselen

GROUP(a)	GROUP(b)	Mean Difference (a-b)	Sig.
I	III	-20.0000*	.005
	IV	-15.6667*	.021
	V	-10.8333	.100
III	I	20.0000*	.005
	IV	4.3333	.498
	V	9.1667	.159
IV	I	15.6667*	.021
	III	-4.3333	.498
	V	4.8333	.450
V	I	10.8333	.100
	III	-9.1667	.159
	IV	-4.8333	.450

Where:
GROUP I=PV rabies virus

GROUP III=PV rabies virus
+
1 day treatment

GROUP IV=PV rabies virus
+
2 day treatment

GROUP V=PV rabies virus
+
3 day treatment

* The mean difference is significant at the 0.05 level.

ii) Methanol 80% crude extract

The survival time is significantly increased in 1day and 3 days treatment groups as indicated in table 8.

Table 8: Multiple comparisons of positive control and treatment groups for methanol 80% extract of S. macroselen

GROUP (a)	GROUP (b)	Mean Difference (I-J)	Sig.
I	III	-18.0000*	.018
	IV	-14.0000	.059
	V	-15.1667*	.042
III	I	18.0000*	.018
	IV	4.0000	.574
	V	2.8333	.690
IV	I	14.0000	.059
	III	-4.0000	.574
	V	-1.1667	.869
V	I	15.1667*	.042
	III	-2.8333	.690
	IV	1.1667	.869

Where:
GROUP I=PV rabies virus

GROUP III=PV rabies virus
+
1 day treatment

GROUP IV=PV rabies virus
+
2 day treatment

GROUP V=PV rabies virus
+
3 day treatment

* The mean difference is significant at the 0.05 level

iii) Aqueous crude extract

Any treatment group of the aqueous extract did not show a significant association on survival time as compared to the positive control (table 9).

Table 9: Multiple comparisons of positive control and treatment groups for aqueous extract of S. macroselen

GROUP (a)	GROUP (b)	Mean Difference (a-b)	Sig.
I	III	-13.0000	.070
	IV	-9.5000	.177
	V	-9.6667	.170
III	I	13.0000	.070
	IV	3.5000	.612
	V	3.3333	.629
IV	I	9.5000	.177
	III	-3.5000	.612
	V	-.1667	.981
V	I	9.6667	.170
	III	-3.3333	.629
	IV	.1667	.981

Where:
GROUP I=PV rabies virus

GROUP III=PV rabies virus
+
1 day treatment

GROUP IV=PV rabies virus
+
2 day treatment

GROUP V=PV rabies virus
+
3 day treatment

* The mean difference is significant at the 0.05 level.

Acute toxicity

No mortality was observed with the extract given up to 80mg/kg. No visible signs of delayed toxicity were also observed within 7 days in surviving mice.

General information about these plants is listed in Table 1.

Discussion

S. maroselene and S. subserrata have never been evaluated for anti-rabies activity in the laboratory before, and thus, the present study was the first to evaluate the above plants in mice model. Some extracts of the plants showed anti-rabies activity. Similarly, Muller and colleagues (2007) found leaves and flowers of Alamanda schottii to have anti-rabies activity. Four of the six crude extracts, namely chloroform and methanol 80 % extracts of S. macroselen and chloroform and aqueous extracts of S. subserrata, did show a significant anti-rabies activity. Methanol extracts of Alamanda schottii showed anti-rabies activity (Muller et al., 2007). However, the anti-viral activity resided only in aqueous rather than in ethanol extracts of Nepeta nepetella leaves (Abad et al., 2000). On the other hand, in the current study, neither the aqueous extract of

S. macroselen nor methanol 80% extract of S. subserrata did show significant anti-rabies activity.

The 1 day and 2 days treatment groups of the chloroform extract of S. macroselen did show a significant difference on their survival time from the control group than the 3 days treatment group. This might be attributed to the established fact that the percentage of population affected increases as the dose is raised (Goldan et al., 2005).

The chloroform and aqueous extracts of S. subserrata and the methanol 80 % extract of S. macroselen in their 1 day and 3 days treatment did show significant difference on survival time from the control group. Nevertheless, they did not show any statistical difference in their 2 days treatment groups. This might be due to the fact that all mice admitted to Group II were males. However, Gochfeld (2007) suggested that female rats required half the dose of barbiturates, compared to males, to induce sleep, and the duration of sleep was substantially longer in females given the same dose as males.

In the current investigation, anti-rabies activity of S. macroselen was detected from the chloroform and methanol 80% crude extracts. Though no identification of secondary metabolites responsible for this activity was done, the active compound(s) is/are more of non-polar. However, that of S. subserrata was observed from the chloroform and aqueous crude extracts, which indicates the responsible secondary metabolites are from both non-polar and polar compounds. Medicinal plants have a variety of chemical constituents, which have the ability to inhibit the replication cycle of various types of DNA or RNA viruses (Jassim and Naji, 2003).

In the investigated plants' crude extracts, aqueous of S. macroselen and methanol 80 % of S. subserrata did not show a significant anti-rabies activity. However, negative results do not mean absence of bioactive constituents nor that the plant is inactive. Active compound(s) may be present in insufficient quantities in the crude extracts to show activity with the dose levels employed (Taylor et al., 2001). Alternatively, if the active principle is present in high enough quantities, there could be other constituents exerting antagonistic effects or negating the positive effects of the bioactive agents (Jager et al., 1996).

Generally, the results obtained are in line with the traditional uses of the plants as crude anti-rabies drugs. However, traditional healers seldom use a single plant in their extracts. 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 view of the significant number of plant extracts that have yielded positive results in the present study, seems reasonable to conclude that there are probably numerous kinds of anti-rabies agents in these materials. It is also recommendable to make further investigation of the active ingredients to reveal more useful compounds.

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