

Full Length Research Paper

***In ovo* antiviral activity of *Synadenium glaucescens* (pax) crude extracts on Newcastle disease virus**

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Investigation on the effect of root bark and wood, stem bark and wood, leaves and sap of *Synadenium glaucescens* extracts against Newcastle disease (ND) virus was done using an *in ovo* assay. Viable 9 days embryonated chicken eggs were arranged into 25 treatment groups (n = 5). Groups 1 to 21 were challenged with a 13C/SUA virulent strain of ND virus treated with extract at concentration of 0.2 mg/ml. Un-inoculated group saved as negative control and groups inoculated with virus and diluent saved as positive controls. Haemagglutination test was used to quantify the amount for ND virus units. Embryo survival and embryo weight were significantly higher ($P \leq 0.05$) in groups treated with *S. glaucescens* extracts than the positive control. The root bark demonstrated significantly higher antiviral activities ($P \leq 0.05$). Furthermore, treatments with ethanolic extract SE1 resulted into 100% embryo survival, 91.2% mean embryo weight and reduced viral load by 99.2%. The minimum dose of SE1 with the highest efficacy was 0.2 mg/ml. The percent mean embryo weight and haemagglutination test demonstrated negative correlation ($R^2 = 0.94$). These findings validate the ethnoveterinary potential of *S. glaucescens* and the feasibility of its use for treatment and control of ND.

Key words: Ethnoveterinary, Euphorbiaceae, poultry viral diseases, Newcastle disease, *Synadenium glaucescens*.

INTRODUCTION

The use of plant extracts for control of Newcastle disease (ND) in rural Tanzania is not uncommon (Buza and Mwamuhehe, 2001). *Synadenium glaucescens* (euphorbiaceae) has been deployed by communities in Tanzania for ethnomedical (Chhabra et al., 1984) and ethnoveterinary purposes (Wickama et al., 2006; Mabiki et al., 2011). The water extract of the leaves and stems of *S. glaucescens* have demonstrated antimolluscicidal activity (Kloos et al., 1987) and weak inhibition of electrically induced contractions of the guinea-pig ileum (Rukunga et al., 1990). Newcastle, which is one of the diseases claimed to be treated and controlled by this

plant (Wickama et al., 2006; Mabiki et al., 2011), is among the serious challenges for development of poultry industry in Tanzania under rural setting (Yongolo et al., 2002; Komba et al., 2012). Though studies on efficacy of plant extracts against viruses is growing and *S. glaucescens* is increasingly being reported for its ethnoveterinary potential, there are limited controlled laboratory studies of the extracts on ND virus (NDV). Therefore, the aim of this study was to assess the effectiveness of different extracts of *S. glaucescens* on NDV. The findings of this study provide valuable information on the usefulness of the plant against ND.

Table 1. The coding of extracts.

Plant part (PP)	PP Code	Extract codes			
		Laboratory sequential extraction			Traditional extraction (T)
		Petroleum ether (P)	Dichloromethane (D)	Ethanol (E)	
Root Bark	1	SP1	SD1	SE1	ST1
Root wood	2	SP2	SD2	SE2	ST2
Stem Bark	3	SP3	SD3	SE3	ST3
Stem Wood	4	SP4	SD4	SE4	ST4
Leaves	5	SP5	SD5	SE5	ST5
Sap	6	na	na	na	ST6

S: *Synadenium*; na: not applicable

Table 2. Grouping and treatment allocation for the *in ovo* assay.

Group (G): n = 5	Treatment
G1 to G15	NDV + extract + DMSO ; 15 different extracts (SP/SD/SE 1-5)
G16 to G21	NDV + extract + DMSO; 6 different extracts (ST 1-6)
G22	NDV alone (V+)
G23	NDV + DMSO (V+)
G24	Egg + DMSO + Sample (VS+)
G25	Untreated embryonated chicken egg, ECE (V-)

MATERIALS AND METHODS

Plant collection and processing

Aided by local informants and botanist, the fresh plants were collected in Njombe region, Njombe district in Southern Highlands of Tanzania. The samples were identified and confirmed by a botanist and voucher specimen was stored in the herbarium at the Botany Department, College of Natural and Applied Sciences of the University of Dar es Salaam (UDSM) in Tanzania with specimen's number HOS/FM 3672. The roots, stems and leaves of *S. glaucescens* were cleaned and mechanically separated to get five parts; the root bark, root wood, stem bark, stem wood, and leaves. The parts were used as fresh or air dried and pulverized to a particle size of 1 mm for use. The sap was used as fresh.

Extraction of samples

In the laboratory sequential extraction (LSE), the dry samples were soaked sequentially in petroleum spirit, dichloromethane and then ethanol twice each for 72 h for each solvent. After filtration, the extracts were dried using rotary evaporator to obtain 15 crude extracts. Extracts for use were dissolved in dimethyl sulphoxide (DMSO) to make a concentration of 0.2 mg/ml. Traditional method of extraction (TE) was adopted from traditional practitioners and users of the plant. 100 g of fresh samples were mixed with 2 L of water and was boiled while allowing evaporation, till the volume of the solution was reduced to 100 ml. The sap was used after four fold dilution. The extracts from both methods were coded as indicated in Table 1. The extracts were stored at 4 °C before being used for antiviral tests.

Antiviral screening

The test organism 13C/SUA NDV strain, supplied by the Bacterio-

logy and Mycology Laboratory, Department of Veterinary Microbiology and Parasitology, Sokoine University of Agriculture was used. *In ovo* assay following the procedure by Sally (1998) and Senne (2002) with slight modification was used to test the antiviral potential of *S. glaucescens* extracts. Embryonated chicken eggs (ECE) of 9 days old were checked for viability by candling before being used. The ECE were randomized into 25 groups (n = 5), allocated as shown in Table 2. A hole was made through egg shell just above the air sac to allow vertical inoculation of 0.1 ml of the inoculum. The inoculum was prepared by mixing 0.1 ml virus suspension and 0.9 ml crude extract (0.2 mg/ml). After inoculation, the inoculated site was sealed with paraffin wax, and then the eggs were kept at 4 °C (refrigerated) for 1 h. The eggs were then incubated at 37 °C for 5 days with the air sac uppermost. Un-inoculated eggs (V-), eggs with virus suspension only (V+) and eggs injected with DMSO and virus (VS+) served as controls for all experiments.

The challenged ECE were observed daily for death of embryo for 4 days. After 5 days, the eggs were chilled and growing embryos were observed for growth and weight change. The assessments of antiviral activity were based on survival of the embryo; percentage mean embryo weight (%MEW) (Equation 1) and the viral load in the allantoic fluid during harvesting.

$$\text{MEW (\%)} = \frac{\text{MEW of the embryo harvested from treated ECE}}{\text{MEW of embryos from untreated ECE (V-)}} \times 100$$

Dose dependent study

The dose dependent study was done using the ethanolic extract of the root bark which demonstrated the highest antiviral activity against NDV. The extract concentration ranging from 0.05 to 0.3 mg/ml was used. The assessments of antiviral activity were based on the weights of the embryo, their survival and physical observation.

Haemagglutination (HA) test of the allantoic fluid collected from ECE challenged with the inoculums

HA test was used to quantify the amount of NDV using two fold dilutions in a V-shaped 96 well micro titre plates. One percent chicken red blood cells were two fold diluted, and then titrated with allantoic fluid collected from NDV challenged ECE with NDV. After allowing it to stand for 45 min at room temperature (28°C), the end point was recorded for interpretation and calculation of the HA titre. Micro wells with a sharp button of red blood cells at the bottom were considered HA negative, while the ones with a hazy film of red blood cells or no button at the bottom of the V-bottom well were considered HA positive. The endpoints for HA were used in calculation of the HA titre of the test samples and the reduction of viral load due to extracts exposure (Equation 2).

$$\text{Reduced HA (\%)} = \frac{C - E}{C} \times 100$$

where C= the base two logarithmic HA titre of the virus control and E= the base two logarithmic HA titre of the Challenged ECE.

Haemagglutination inhibition (HI) test against NDV antibodies in hatched chicks Ten eggs challenged with the inoculums of NDV with SE1 and 10 eggs EV were left to hatch 12 days post treatment. Serological testing protocol for the HI test as previously described by Allan and Gough (1974) with slight modification was used to detect the presence of antibodies against NDV in chickens on days 0 and 7 post hatching. Sera prepared from hatched chicks blood samples were made into two fold serial dilution with PBS in micro titre plate up to the 11th well, while the 12th wells column were left with neat sera that served as control. The 4 HA units NDV antigen were added up to the 11th well and kept at 28°C for 25 to 30 min. A 1% volume of chicken red blood cells (RBCs) suspension in phosphate buffered saline (PBS) prepared from centrifuged and washed chicken blood was added into each well. The samples forming a button shaped settling of RBCs were recorded as positive and the maximum dilution of each sample causing HI was considered as the end point.

Statistical analysis

Data collected were analyzed using CoStat Version 6.400, (CoHort Software, USA). The weights for different groups were reported in %MEW \pm Standard deviation at 95% confidence interval. The differences of MEW were further analyzed by one-way analysis of variance (ANOVA) and significance was reported at $P \leq 0.05$. Comparison of means was performed by Tukey-Kramer test.

RESULTS

Antiviral activity of extracts obtained by sequential laboratory extraction method

The results on the antiviral activities of the extracts against the NDV in ECE showed that there was a significant difference in activity between extracts ($P \leq 0.05$). The %MEW of treated ECE ranged between 22 and 92% as compared to the unchallenged ECE (Figures 1 to 3). Generally, the embryos treated with ethanolic extracts demonstrated higher %MEW well above 50%, implying more activity from extracts of this solvent as compared to P and D solvents. The results further indicated that

%MEW for SE1 were significantly higher ($P \leq 0.05$) as compared to other ethanolic extracts (Figure 1). There was no significant difference ($P > 0.05$) between the %MEW of the extracts SP3 (18.2%), SP5 (19.1%), SD1 (17.2%), SD4 (22.8%), SE2 (22.7%) and the two positive controls EV+ (12.8%) and EVS+ (18.6). All embryos in two positive control groups died within 30 h, while those treated with the extracts that had similar level of activity demonstrated up to 70% death of embryos in five days of observation. The %MEW of the SE1C had no significant difference with the EV-.

Antiviral activity of extracts obtained by the traditional extraction method

The results from extracts obtained by local extraction method against the ECE challenged with NDV are as shown in Figure 2. The results show that there was a significant difference in activity between extracts ($P \leq 0.05$). Extract ST2 of the root wood demonstrated higher MEW of 91.6% implying higher activity than other extracts. There were no deaths of embryos observed from these extracts. There was no significant difference ($P > 0.05$) among %MEW of ST1 (88.5%), ST3 (84.05%) and ST2 (91.6%) extracts. No significant difference ($P > 0.05$) of %MEW between ST4 (49.8%) and ST5 (49%) was observed. In these groups, 20 to 100% death of embryos was observed from days 2 to 5 and the difference was significant ($P > 0.05$). ST6 demonstrated the least activity with %MEW of 28.4% which was lower and not significant ($P < 0.05$) when compared with the positive control, with 100% death of embryo within 16 h.

HA test results for ECE challenged with the inoculum

The HA results for ECE challenged with the inoculum are as shown in Figure 3. The viral load in eggs challenged with NDV mixed with extracts was reduced to 99.6%, which was the highest. The lowest HA titres were recorded in allantoic fluids from ECE treated with ST1, ST2 and ST3 extracts prepared using traditional extraction method, each showing the 2 HA units equivalent to 99.6% reduction of the viral load. SP2, SP4, SE1, SE3 and SE5 demonstrated similar HA titres of 3 HA units equivalent to 99.2% reduction of virus units as compared to the EVS+. The highest viral load was on ECE treated with SP5, SD1, SD2 and SD4 extracts with 512 HA units. The mean antibody titres against NDV in hatched chicks at concentration of 0.2 mg/ml were all negative.

Relationship between percent mean weight of embryo and HA titres

The HA titres correlated well with the %MEW with $R^2 = 0.94$ for extracts extracted using laboratory method and $R^2 = 0.97$ for extracts extracted using the traditional

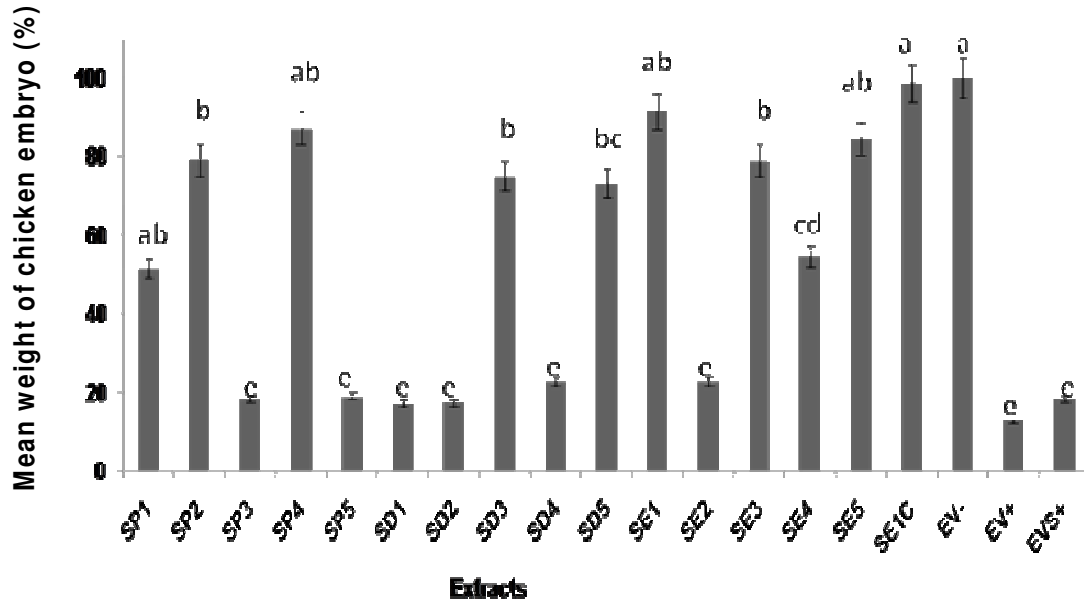


Figure 1. Percentage of embryo weights in eggs infected with NDV and treated with extracts in comparison to the negative control. Where, S= *Synadenium*, P= Petroleum ether, D= Dichloromethane, E= Ethanol, No. 1, 2, 3, 4, 5 = root bark, root wood, stem bark, stem wood and leaves, respectively, EV+ = Positive control group of eggs inoculated with a NDV treated with extracts; EVS+ Positive control group of eggs inoculated with a NDV and DMSO, EV- Negative control group of un-inoculated eggs and SE1C – Egg with Extract SE1 alone. Extracts with the same letter were not significant different (P > 0.05).

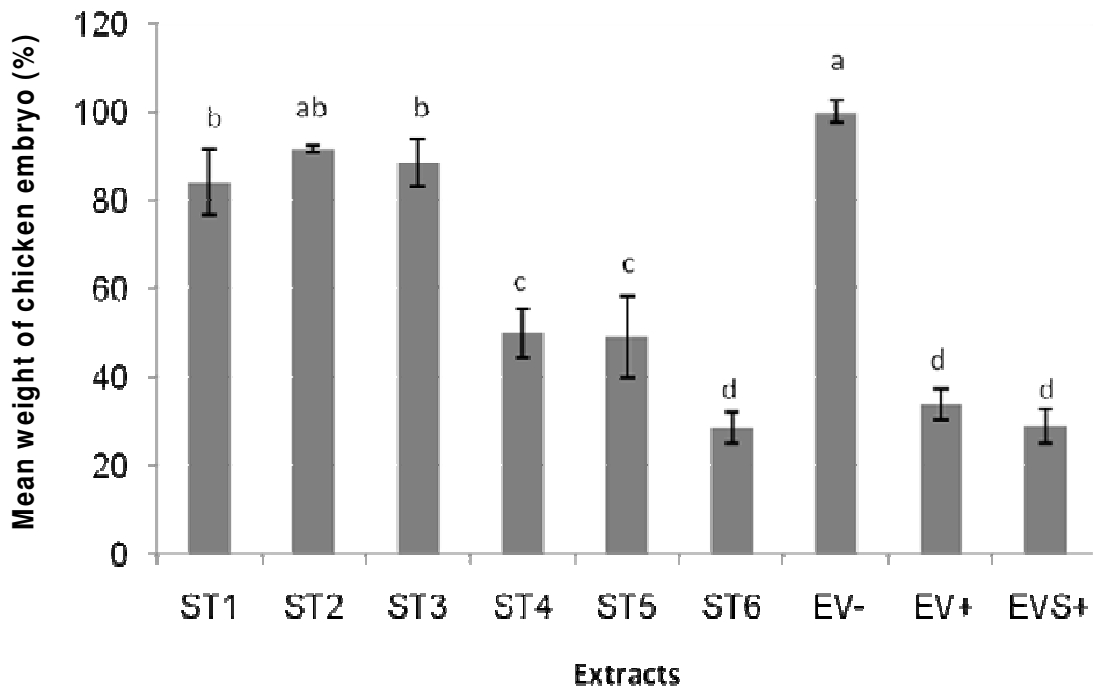


Figure 2. Percentage of embryo weights in eggs infected with NDV treated with extracts extracted using traditional method. Extracts with the same letter were not significant different (P > 0.05). Where, S= *Synadenium*, T= Traditional extraction, No. 1, 2, 3, 4, 5, 6= root bark, root wood, stem bark, stem wood, leaves and Sap, respectively. EV+= Positive control group of eggs inoculated with a NDV treated with extracts; EVS+ Positive control group of eggs inoculated with a NDV and DMSO, EV- Negative control group of un-inoculated eggs. Extracts with the same letter were not significant different (P > 0.05)

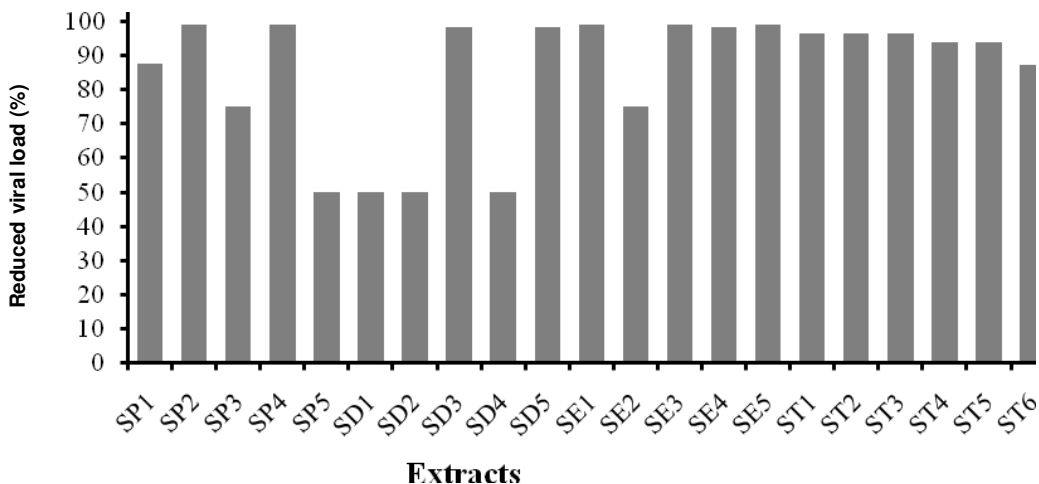


Figure 3. Percentage Reduced Viral Load due to HA test. Where, S= *Synadenium*, T= Traditional extraction, P= Petroleum ether, D Dichloromethane, E= Ethanol; No. 1, 2, 3, 4, 5, 6 = root bark, root wood, stem bark, stem wood, leaves and Sap, respectively

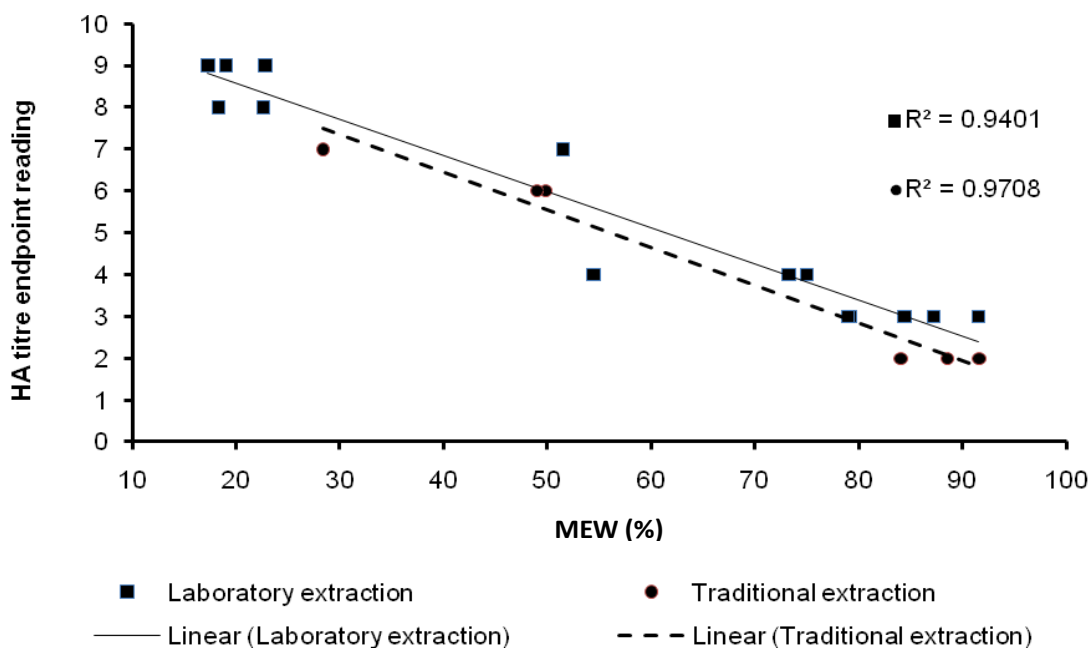


Figure 4. Graph of HA titre endpoint reading against %MEW. Whereas MEW is the mean embryo weight.

technique (Figure 4).

Dose dependent study

The results from the dose dependent study done using SE1 at concentrations ranging from 0.05 to 0.3 mg/ml are indicated in Figure 5. The highest %MEW was observed at concentration of 0.1 mg/ml implying higher activity. Deaths of embryos at 20% were observed after 72 h at 0.3 mg/ml.

DISCUSSION

This study has demonstrated for the first time the activity of *S. glaucescens* extracts against NDV while relating the embryo weight and the viral load. The extract was considered active if it inhibited virus replication in the embryo cells thus allowing embryo growth, also if the extract reduced or decreased the viral load in ECE and thus preventing the death of embryo. The invasion of the NDV into the embryo cell is enabled by the functioning of the fringe of glycoprotein spikes present on the virus

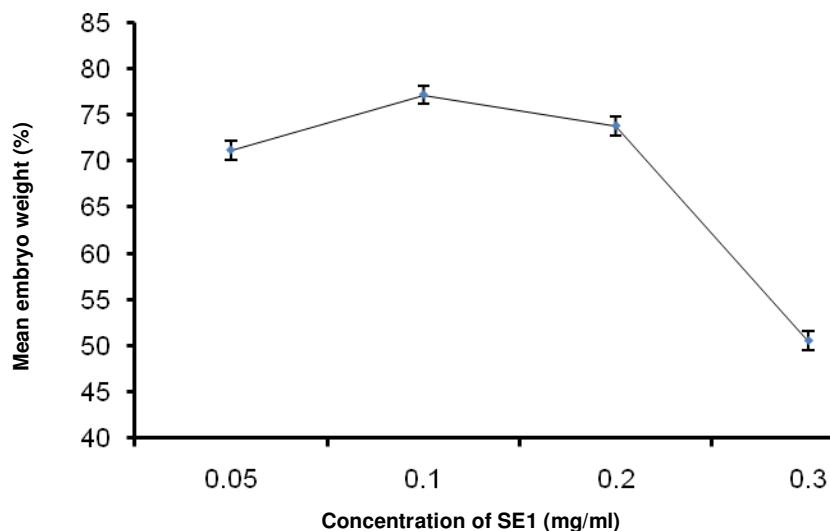


Figure 5. A dose dependent study using the ethanolic extract (SE1). Concentration of extracts with the same letter were not significant different ($P > 0.05$).

envelope (Young et al., 2002) and the replication of viruses inside the cytoplasm of embryo cells causes a disease. Their mechanisms of causing diseases are always based on the replication capacity using the embryo cell metabolic pathways, which mostly leads to cell deaths or suffering to death for multicellular organisms (Hightower and Brant, 1974; Wakamatsu et al., 2006).

The goal of antiviral search is the discovery of antiviral agents that are specific for the inhibition of viral multiplication without affecting normal cell division. The extracts of *S. glaucescens* as presented in this study have demonstrated the ability to inhibit the viral multiplication without significantly affecting normal cell division. The continuation of embryo growth unveiled by increase in weight and organ formation in NDV challenged ECE implies that the extracts could potentially interfere the viral replication cycle either by blocking one point of propagation mechanisms inside the cells, prevent the invasion mechanism or kill the virus in the inoculate. The ability demonstrated by more than 80% of the extracts reducing the HA titre by more than 50% (Figure 3) shows that the extracts can inhibit the replication of virus. The fact that HA titre correlated strongly and negatively with the %MEW (Figure 4) with R^2 of 0.94 and 0.97, indicate that the extracts were highly responsible with inhibiting the multiplication of virus in the embryo without affecting the growing embryo cells. The significant difference in %MEW for different extracts could be attributed to the diversity of compounds in the extracts. Each extract had a different degree of inhibitory activity and specificity against the virus and/or its essential enzymes. The ethanolic extracts prepared by the LSE (SE1, SE3, SE4, SE5) and water extracts from TE method (ST1, ST2 and ST3), were more active as compared to other extracts. Water is a common extraction solvent in traditional

treatment and contains mainly polar compounds unlike the ethanolic extracts which are more refined. In other studies, the phytochemical analysis of the water extracts of the *S. glaucescens* leaves indicated the presence for tannins, triterpenoids and coumarins while the methanol extract showed the presence of steroids, triterpenes and anthocyanins and the ether extract contained carotenoids, steroids, triterpenoids and volatile oils (Neuwinger, 1994). These compounds are reported to have antiviral activity (Jassim and Naji, 2003), and could have shown slightly higher activity due to synergistic effect. Viral infections are usually accompanied by a variety of symptoms not necessarily due to the virus directly, but associated immune functions and other important metabolic pathways, thereby, influencing multiple physiological parameters (Mohamed et al., 2010). It is possible that there are other ingredients in a plant preparation that help to control the virus such as immune modulation and tissue healing among others which justifies why traditional treatments involves preparation of concoction of different plants to attain the maximum beneficial effect of a medicinal plant preparations (Gessler et al., 1994). However, the water extract is hard to handle in isolating pharmacologically active compounds for drug discovery research, because it needs more sophisticated instrumentation not available in many developing laboratories (Rukunga et al., 1990).

The ethanolic extracts from the LSE method are more purified with specific concentration; thus, present a better candidate for further research on drug discovery as perceived from this study. The root barks extract SE1 have shown activity at minimum concentration of 0.1 mg/ml. The extract was denoted as ST1 in water extraction and presented higher activity (88.9% and reduced HA titre of 99.6%) despite the different methods of

preparation. Preliminary phytochemical screenings of SE1 indicated the presence of polyphenolic and sugar moiety compounds (unpublished data). The presence of compounds with sugar moiety is not new within the *Synadenium* genus and these could have contributed to activity of the extracts. Rukunga et al. (1990) isolated the glucoside compound from the stem and the leaves of *S. glaucescens* and glucopyranoside compound was isolated from *Synadenium pereskiiifolium* (Kerstin et al., 1991), both compounds were pharmacologically active. Though the mechanism of action of SE1 is not known, the mechanisms of the nucleosides in preventing viral replication are known (Jassim and Naji, 2003). The sugar moiety is the main building block of nucleoside analogues which are used as antiviral drugs of the nucleoside reverse transcriptase inhibitor groups. These interrupt the formation of viral DNA from RNA by substituting a look-alike analogy that resembles nucleosides (building blocks) used by the virus to synthesize DNA thus blocking the viral replication. The polyphenols act principally by binding to the virus and/or the protein of the host cell membrane and thus arrest absorption of the virus (Van den Berghe et al., 1986). The high activity of the ether extracts SP4 and SP2 could have been attributed by the presence other antiviral active compounds such as steroids, triterpenes and anthocyanins carotenoids, triterpenoids which have been reported present in this plant species (Neuwinger, 1994; Jassim and Naji, 2003).

Antiviral drug specificity to the pathogen is very vital in searching for antiviral drugs. The selectivity of the extracts SP3, SP5, SD1, SD2, SD4 and SE2 was poor and thus they demonstrated toxicity towards both the virus and embryo. The %MEW due to their treatment was not significant to the positive control ($P > 0.05$) (Figure 1) and could reduce the viral by 50% (Figure 3). Higher death rate of embryo up to 70% in four days of observation were contributed with mainly the D extracts which imply being more toxic than other extracts. The toxicity of these extracts could be attributed by the presence of phorbol esters reported in the family Euphorbiaceae and the genus *Synadenium* (Gunjan et al., 2007). The fact that these were toxic to the virus they can be used in biosecurity measure as natural disinfectants for decontamination purposes. With the LSE method, these toxic components were separated from the safer and active extracts, unlike the water extraction which is common among traditional treatments which gives a credit to the method especially when considering safety issues.

Conclusion

This study has demonstrated for the first time the antiviral potential of the extracts of *S. glaucescens*. The results indicate clearly that the plant contains antiviral chemical constituents against ND virus which was shown by the strong negative correlations between the %MEW of the

harvested embryo and their HA titres. The polar root bark extracts have indicated more activity despite the extraction method used. The laboratory sequential extraction was able to separate the toxic and the safer content of the compounds. These findings validate the ethnoveterinary uses of the plant and demonstrate a high potential and feasibility of using *S. glaucescens* extracts for treatment and control of ND especially in rural areas where conventional disease management options are limited. This study stands as a stepping stone towards further research on antiviral drug search from *S. glaucescens*.

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