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ORIGINAL ARTICLE

Plants containing cardiac glycosides showing antiphytoviral activity against *Potato virus Y* (PVY^{NTN}) on tobacco plants

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Abstract

The tuber necrotic strain of *Potato virus Y* (PVY^{NTN}) causes widespread disease and has severe negative effects on the growth and yields of plants, especially those of the Solanaceae family. The consequences of residual toxicity and non-biodegradation of synthetic chemicals and pollution of the environment has led to investigations into new non-toxic and biological treatments to control plant viral diseases. Ethanolic extracts of *Bowiea volubilis* (bulbs), *Cotyledon orbiculata* (leaves), *Gomphocarpus fruticosus* (leaves), *Merwilla plumbea* (dry and fresh bulbs), *Nerium oleander* (leaves), and the fruits and leaves of *Strophanthus speciosus*, were evaluated against PVY^{NTN} *in vivo* and *in vitro*. At a concentration of 20 mg · ml^{-1}, ethanolic extracts of *Strophanthus speciosus* (leaves) and fruits (50 mg · ml^{-1}) significantly reduced the expression of PVY^{NTN} symptoms on tobacco plants *in vitro* without affecting the normal growth and development of the plant. Similarly, at 50 mg · ml^{-1}, *N. oleander, C. orbiculata* and *B. volubilis* (fresh bulbs) and *S. speciousus* leaves at 20 mg · ml^{-1} extracts showed significant differences in PVY^{NTN} symptoms in the *in vivo* experiment. *Strophanthus speciosus* leaf and fruit extracts showed significant inhibition in the *in vitro* and *in vivo* assays and demonstrated that *S. speciosus* has potential to be used as an antiphytoviral treatment.

Keywords: antiphytoviral, cardiac glycoside, DAS-ELISA, plant extracts, *Potato virus Y* (PVY^{NTN})

Introduction

Potato virus Y (PVY) genus *Potyvirus*, belonging to the largest plant virus family, *Potyviridae,* is one of the most common viruses that infect a wide range of plant species, primarily from the Solanaceae family, including potato, tobacco, tomato, and pepper (Hu *et al.* 2009; Petrov *et al.* 2016b). It also infects other crops such as bean, clover, maize, pea, peanut, soybean, sorghum, tulip and cucurbits (Mohamed 2010). *Potato virus Y* causes a wide range of symptoms such as mosaic, mottling lesions and necrosis leading to death of the plant (Hu *et al.* 2011). However, in some cases it can be symptomless. The appearance of these symptoms depends on the plant species, the cultivar and the virus strain (Hu *et al.* 2011). *Potato virus Y* has a worldwide distribution and has been described as one of the five most important viruses infecting vegetable species worldwide, with *Potato virus Y*, strain NTN (PVY^{NTN}) responsible for systemic vein necrosis (VN) on various host plants including tobacco (Doubnerová *et al.* 2007; Spoustová *et al.* 2015). Currently there is no effective pesticide available to control these viral diseases (Petrov *et al.* 2016a).

This single-stranded positive RNA genome virus consists of approximately 9,700 nucleotides. PVY has

been classified into different strains such as PVY^C, PVY^N, PVY^O, PVY^{NW} and PVY^{NTN} (Visser and Bellstedt 2009). In tobacco plants, PVY isolates are divided into two major groups, namely the ordinary strain, PVY^O, and the necrotic strain, PVY^N, which is further divided into two main groups, namely PVY^{NTN} and $PVY^{N\text{-}Wi}$, which all induce VN in tobacco (Matros *et al.* 2006). Different species of aphids transmit this virus (Tribodet *et al.* 2005; Barker *et al.* 2009).

Symptoms caused by some PVY strains are more cosmetic and the economic impact is visible when potatoes are produced for table use, processing or seed (Boonham *et al.* 2002). It has been difficult for scientists to find a solution to solve plant virus infections because of a lack of effective natural remedies that will systemically cure virus infected plants. Although numerous types of treatments such as chemotherapy, thermotherapy and meristem-tip therapy have been reported to be successful in virus elimination endeavours, they cannot be used on a large scale (Thresh 2003). The most common method is to use pesticides, which are currently largely synthetic with the aim to kill or deter in a destructive way (Petrov *et al.* 2015). Consequently, controlling plant viruses and developing plant virus inhibitors is tremendously complicated. As a result, studies investigating natural products and plant extracts have increased in recent years with various studies showing promising results to control plant virus infections. Some of the plants that have been investigated and which show promising results include *Curcuma longa* (turmeric), *Allium sativum* (garlic), *Hypericum perforatum Tanacetum vulgare, Plectranthus tenuiflorus* and onion (Othman and Shoman 2004; Mohamed 2010; Petrov and Stoyanova 2015; Petrov *et al.* 2016a). Virucides with a biological base, which are developed from living organisms have the advantage of high effectiveness, low toxicity and high biocompatibility (Wang *et al.* 2015). Plant secondary metabolites as potential antipathogen agents have an advantage over chemical pesticides due to environmental compatibility, the possibility of novel specific mechanisms of action against the agent as well as more variety and rich resources (Zhang *et al.* 2007).

The plants selected for this study, namely *Bowiea volubilis* Harv., *Cotyledon orbiculata* L., *Gomphocarpus fruticosus* (L.) W. T. Aiton., *Merwilla plumbea* Planch, *Nerium oleander* L. and *Strophanthus speciosus* (Ward. & Harv.) Reber, all contain cardiac glycosides (Van Wyk *et al.* 2002). Cardiac glycosides are a group of triterpenoids, which are derived from modification of the triterpenes (Prassas and Diamandis 2008; Su *et al.* 2008) with reported activity against human viruses such as *Herpes simplex virus 1* and *2* (HSV-1 and HSV-2) as well as *Human immunodeficiency virus* (HIV) (Prinsloo *et al.* 2010; Bertol *et al.* 2011; Wong *et al.* 2018). The aim of this pilot study was to investigate the antiviral and phytotoxic activities of selected cardiac glycoside containing plants with the potential of being developed as an inexpensive natural preparation for the control of PVY^{NTN}.

Material and Methods

Virus

PVY^{NTN} was obtained from the Agricultural Research Council, Vegetable and Ornamental Plants (ARC-VOP), Roodeplaat, Pretoria, South Africa. The virus was propagated and maintained in tobacco plants. This strain was successfully used to screen commercial cultivars for resistance to PVY^{NTN} (Cloete *et al.* 2013).

Host plants

Nicotiana tabacum (cv. Samsun NN) plants were grown in an insect proof greenhouse at 20–25°C day and 15–20°C night temperatures, with a 16 : 8 h (light : dark) photoperiod. Irrigation was scheduled three times a week. These plants were used as a systemic and local lesion host of PVY^{NTN} virus for *in vivo* and *in vitro* experiments.

Plant collection and extraction

Freshly collected leaves of *C. orbiculata* and *N. oleander*, and fruit and leaves of *S. speciosus* were obtained from the Manie van der Schijff Botanical Garden of the University of Pretoria, Pretoria, South Africa. Leaves of *G. fruticosus* were collected from Faerie Glen Nature Reserve, Pretoria, South Africa. Fresh bulbs of *B. volubilis* and *M. plumbea* were collected from ARC-VOP, Pretoria, South Africa. Some bulbs of *B. volubilis* and *M. plumbea* were left to dry prior to extraction. A representative of each plant was collected and herbarium voucher specimens were deposited in the H.G.W.J. Schweickerdt Herbarium (PRU) of the University of Pretoria (Table 1).

For each plant, 20 g of plant material was soaked in rectified ethanol (96%; 150 ml) on a shaker for 24 h. The extract was then filtered with vacuum filtration. This procedure was repeated twice. Thereafter the filtered extract was concentrated by rotary vacuum evaporation (Büchi R Rotavapor R-200 and Büchi Heating bath B-491). The final mass of the various crude extracts was determined and the percentage yield of all nine plant extracts was calculated. All plant extracts were kept in glass vials in a cold room at 4°C until further use.

Phytotoxicity test

Extracts were initially dissolved in 10% dimethyl sulfoxide (DMSO), and distilled water was added to all

Table 1. Information about plant species selected for the study

Family/species	Plant material used	Month of collection	Voucher specimen number
Apocynaceae			
Nerium oleander	leaves	December/January	117912
Strophanthus speciosus	leaves, fruits	December/January	117913
Asclepiadaceae			
Gomphocarpus fruticosus	leaves	December/January	117914
Crassulaceae			
Cotyledon orbiculata	leaves	December/January	117911
Hyacinthaceae			
Bowiea volubilis	bulbs (dry and fresh)	March	117910
Merwilla plumbea	bulbs (dry and fresh)	March	117909

extracts to yield final concentrations of 20 mg · ml^{-1} (low concentration) and 50 mg · ml^{-1} (high concentration), respectively. Tobacco plants were mechanically smeared with the low and the high concentrations of the extracts at the three-leaf stage of the plants on the upper and the under surfaces of the leaves. Distilled water (dH_2O) and low (5%) and high (10%) concentrations of DMSO were tested as solvent controls. Plants were maintained in the greenhouse at 20–25°C day and 15–20°C night temperatures. Plants were evaluated by observing mechanical damage and local lesions on the leaves after 2 h, 24 h and 7 days.

In vivo: Effect of extracts on the infectivity of PVY^{NTN}

A randomized blocked design experiment was conducted with 60 tobacco plants per treatment. The experiment consisted of three replicates and plants were sprayed with the extracts the day before they were inoculated with PVY^{NTN}. Plants inoculated with PVY^{NTN} but not treated with the extracts and plants not inoculated with the virus and not treated with extracts served as the positive and negative controls, respectively. Inoculations were conducted when tobacco plants were at a three-leaf stage. Extracts were sprayed evenly on the plants until run-off, at concentrations of 20 mg · ml^{-1} and 50 mg · ml^{-1}, using hand-held spray bottles. The experiment was conducted to determine the most effective concentration to control the PVY^{NTN} which is not toxic to the plants. Symptoms induced by PVY^{NTN} were observed and recorded using a scoring rate of 0–5 where: 0 = no symptoms, 1–2 = mild symptoms, 3 = moderate symptoms, 4 = severe to very severe symptoms, and 5 = very severe symptoms. To determine the severity of infection the 0–5 scores were converted to an infection scale of 0–100 where: 0–20 = no symptoms, 20–40 = mild symptoms, 40–60 = moderate symptoms, 60–80 = severe to very severe symptoms and 80–100 = very severe symptoms.

In vitro: Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA)

DAS-ELISA, using a Bioreba PVY^{N} monoclonal antibody (Bioreba AG, Reinach, Switzerland), was used to confirm infection by PVY^{N} in the inoculated tobacco plants (Ellis *et al.* 1996). Microtitre plates (F96 cert MaxiSorp Nalge Nunc International, Nunc™, Denmark) were coated with 100 µl of primary antibody [anti-PVY^{N} IgG (Avt. No. 112712 Bioreba)], diluted at 1 : 1,000 in coating buffer (pH 9.6). The plates were incubated for 4 h at 37°C in an incubator [Merck chemicals (Pty) Ltd]. Plates were then washed in 0.02 M phosphate buffered saline (PBS) (pH 7.4), containing 0.05% Tween-20 (PBS-Tween) three times after 3 min incubations between each wash. Leaves were then homogenized in 6 to 10 ml of sample conjugate buffer consisting of 0.02 M PBS buffer (pH 7.4), containing 0.05% Tween-20 (PBS-Tween), 2% polyvinylpyrrolidone (PVP) and 2% egg albumin using a mortar and pestle. One hundred microliter sample extracts were added in duplicate wells of microtitre plates, and incubated for 4 h at 37°C or overnight at 4°C. Following incubation, plates were washed in PBS-Tween three times for 3 min. The third step was carried out by adding PVY^{N} monoclonal antibody conjugated PVY-N IgG (Bioreba 112712) to alkaline phosphatase at a dilution of 1 : 1,000, in sample conjugate buffer as the detecting antibody. This antibody combination was to ensure that PVY^{NTN} was detected and excluded PVY^{O} and PVY^{N} Wilge. One hundred microliters were dispensed in duplicate wells of the microtitre plates. All plates were incubated for 3 to 4 h at 37°C. The positive or negative reactions of the samples were determined by adding 100 µl of 4-nitrophenyl phosphate disodium salt hexahydrate (1 mg · ml^{-1}, pH 9.8) in 10% diethanolamine buffer (substrate solution). After 1 hour of incubation in substrate solution at room temperature, readings were taken at 405 nm using a Flow Titertek Multiskan Plus ELISA plate reader (Labsystems, Finland).

Statistical analysis

All information and data were analysed using statistical software GenStat 64-bit Release 14.1 (PC/ Windows 7) Copyright 2011, VSN International Ltd ($p < 0.05$) (Payne *et al.* 2007). Data processing system and XLstat (Ad-ons on Microsoft Excel 2010) were used for the trial-plan. Statistical analysis was performed by the biometry unit of the Agricultural Research Council, Roodeplaat, South Africa.

Results

Phytotoxicity test

Leaf damage was observed with *M. plumbea, B. volubilis* and *N. oleander* at 50 mg · ml^{-1} when inspection was conducted 2 h after smearing the leaves with plant extracts. The damaged areas noted during the first observation had not spread further when the second observations were conducted after 7 days and can be ascribed to mechanical damage during smearing of the leaves as there was no increase in damage on the plants when the plants were inspected 7 days after treatment with plant extracts. The solvent control (5% DMSO) did not show any toxicity to the plant. However, a high dose of 10% DMSO showed slight damage on the *N. tabacum* leaves. *Nicotiana tabacum* plants treated with distilled H_2O as the control were not affected and showed no sign of damage.

In vivo: Effect of extracts on the infectivity of PVY^{NTN}

After a month, symptoms such as leaf mottling, mosaic lesions and necrosis were observed in some plants, especially the positive control plants, indicating that there was no protection. The infection score for the positive control plants ranged from moderate to severe and very severe symptoms with values ranging from 47–67 and the negative controls at 0. Statistical analysis showed that there was a significant decrease in the infection by PVY^{NTN} with the application of some extracts in comparison to the plants infected with the virus and not treated with extracts (positive control) (Table 2). The downward trend observed with *N. tabacum* plants treated with extracts 1 day before infection with PVY^{NTN} virus illustrated that with an increase in concentration of extracts from 20 mg · ml^{-1} to 50 mg · ml^{-1}, the infection by PVY^{NTN} was generally decreased. High concentrations of extracts were more effective in reducing PVY^{NTN} infection in *N. tabacum* plants. Tobacco plants that served as the positive controls, treated with PVY^{NTN}, with no application of plant extracts, demonstrated a higher infection than plants which were treated with PVY^{NTN} and the extracts (Table 2).

Although the plant extracts could not prevent infection, four out of the nine tested plant extracts [*N. oleander*, *B. volubilis* (fresh bulb), *C. orbiculata* (all at 50 mg · ml^{-1}) and *S. speciosus* leaves (20 mg · ml^{-1})] were successful in significantly reducing infection by PVY^{NTN} *in vivo* (Fig. 1).

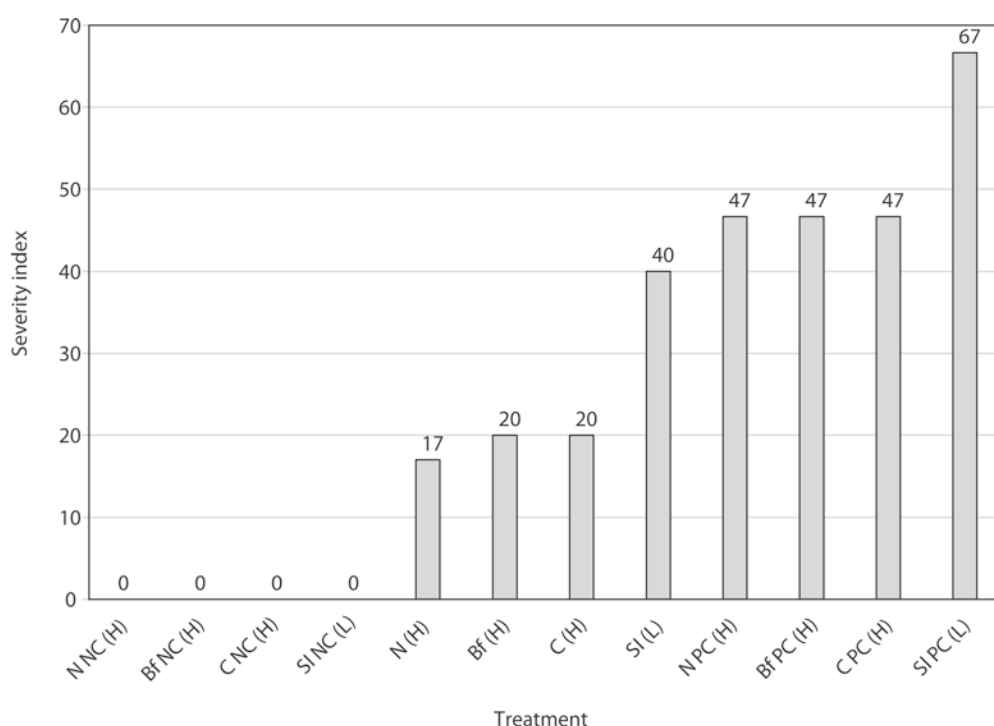

Fig. 1. Statistical significant treatments obtained in the *in vivo* experiment. N = *Nerium oleander*, Bf = *Bowiea volubilis* (fresh bulb), C = *Crassula orbiculata* and Sl = *Strophanthus speciosus* (leaves); NC = negative control, PC = positive control, H = 50 mg · ml^{-1}, L = 20 mg · ml^{-1}

Table 2. *In vivo* tests showing the severity of infection by PVY^{NTN} of tobacco plants treated with a low (20 mg · ml^{-1}) and high concentration (50 mg · ml^{-1}) of plant extracts. Values not followed by the same letter are significantly different. [Bd: *B. volubilis* (dry bulb); Bf: *B. volubilis* (fresh bulb); C: *C. orbiculata*; G: *G. fruticosus*; Md: *M. plumbea* (dry bulb); Mf: *M. plumbea* (fresh bulb); N: *N. oleander*; Sf: *S. speciosus* (fruits); Sl: *S. speciosus* (leaves); PC: positive control; NC: negative control; H: 50 mg · ml^{-1}; L: 20 mg · ml^{-1}]

Treatment	Infection scale score	Description	$LSD_{0.05}$ = 23.89	Treatment	Infection scale score	Description	$LSD_{0.05}$ = 23.89
Sl PC (L)	67	severe to very severe	a	Sf (L)	27	mild	defg
Md PC (H)	60	severe to very severe	ab	Md PC (L)	27	mild	defg
Bd PC (L)	60	severe to very severe	ab	Mf (H)	23	mild	defgh
Bd (L)	47	moderate	abcd	Bf (H)	20	mild	efgh
Bf (L)	47	moderate	abcd	C (H)	20	mild	efgh
Bd PC (H)	47	moderate	abcd	G (H)	17	no symptoms	efgh
Bf PC (H)	47	moderate	abcd	N (H)	17	no symptoms	efgh
C PC (H)	47	moderate	abcd	C (L)	17	no symptoms	efgh
Mf PC (H)	47	moderate	abcd	Md (H)	7	no symptoms	fgh
N PC (H)	47	moderate	abcd	Md NC (H)	3	no symptoms	gh
Sf PC (H)	47	moderate	abcd	Sl NC (H)	2	no symptoms	h
Sl PC (H)	47	moderate	abcd	Bd NC (H)	0	no symptoms	h
G PC (L)	47	moderate	abcd	Bf NC (H)	0	no symptoms	h
Mf PC (L)	47	moderate	abcd	C NC (H)	0	no symptoms	h
N PC (L)	47	moderate	abcd	G NC (H)	0	no symptoms	h
Sf PC (L)	47	moderate	abcd	Mf NC (H)	0	no symptoms	h
Sl (H)	40	moderate	bcde	N NC (H)	0	no symptoms	h
Mf (L)	40	moderate	bcde	Sf NC (H)	0	no symptoms	h
Sl (L)	40	moderate	bcde	Bd NC (L)	0	no symptoms	h
G PC (H)	40	moderate	bcde	Bf NC (L)	0	no symptoms	h
Bf PC (L)	40	moderate	bcde	C NC (L)	0	no symptoms	h
C PC (L)	40	moderate	bcde	G NC (L)	0	no symptoms	h
Md (L)	35	mild	cde	Md NC (L)	0	no symptoms	h
N (L)	33	mild	cde	Mf NC (L)	0	no symptoms	h
G (L)	33	mild	cde	N NC (L)	0	no symptoms	h
Sf (H)	33	mild	cde	Sf NC (L)	0	no symptoms	h
Bd (H)	30	mild	cdef	Sl NC (L)	0	no symptoms	h

In vitro: Effect of extracts on systemic PVY^{NTN} infection

Table 3 shows the results of the DAS-ELISA test conducted with all the plants that were tested *in vitro* to confirm the effect of the tested plant extracts against PVY^{NTN}. Plants treated with the extract at both low and high concentrations, in general reduced the infection of the virus, although not always significantly. Moreover, disease symptoms on the virus treated positive controls were more severe, whereas there was no infection in the negative control. Although plant extracts were not able to prevent PVY^{NTN} infection completely, the DAS-ELISA OD values of the virus infected plants, treated with the plant extracts was generally lower than with the virus infected plants without the plant extract treatment (positive controls), indicating that tested plant extracts were able to reduce virus concentration.

In general, the high concentration of plant extracts reduced the plant infection by PVY^{NTN}. This was shown with the decrease in DAS-ELISA OD resulting in a reading closer to the positive controls (Table 3). Moreover, the positive control demonstrated the DAS-ELISA OD values of more than 0.16 indicating a higher infection of PVY^{NTN} in *N. tabacum* plants. The results indicate that only *S. speciosus* extracts (leaves, 20 mg · ml^{-1}; fruits, 50 mg · ml^{-1}) were able to reduce the infection of tobacco plants by PVY^{NTN} virus significantly (Fig. 2).

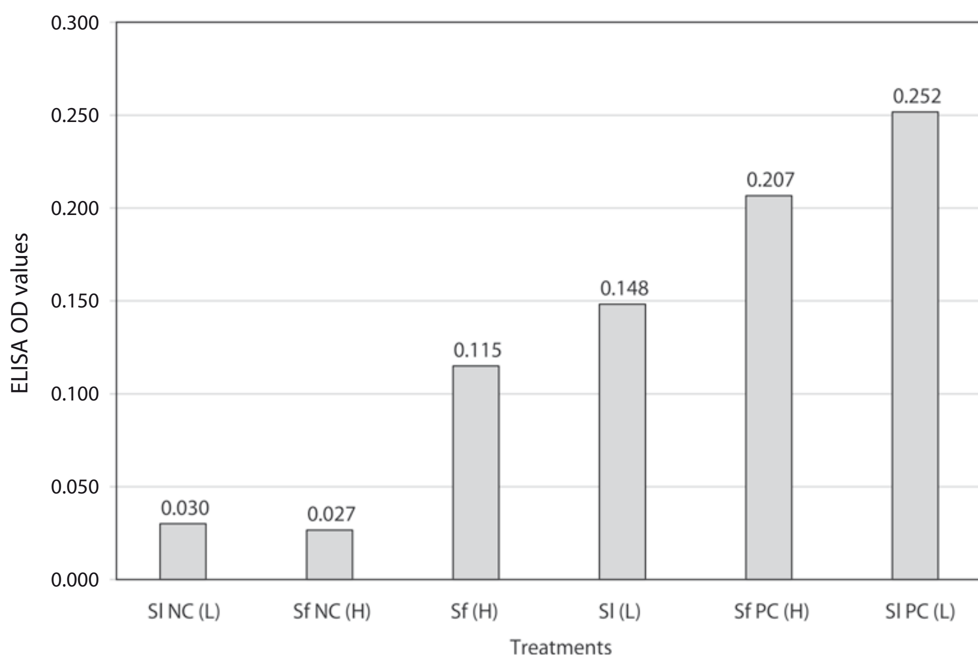

Fig. 2. Statistical significant treatments obtained in the *in vitro* experiment. Sl = *Strophanthus speciosus* (leaves) and Sf = *S. speciosus* (fruits); NC = negative control, PC = positive control, H = 50 mg · ml^{-1}, L = 20 mg · ml^{-1}

Discussion

Numerous crude extracts from plants have been screened to determine their antiviral activity against plant viruses and bioassay guided fractionation of some crude extracts showing inhibitory effect has been extensively studied, which has led to the isolation of many active constituents (Shen *et al.* 2008). The effect of plant extracts on various human viruses such as HIV, HSV and the influenza virus are well known (Husson *et al.* 1991; Lee-huang *et al.* 2003; Schnitzler *et al.* 2008; Prinsloo *et al.* 2010; Dunning *et al.* 2014; Medini *et al.* 2014; Prinsloo *et al.* 2018). Additionally, reports of plant extracts of *Tanacetum vulgare* and *Hypericum perforatum* against plant viruses, specifically *Potato virus Y* show potential for the development of natural based pesticides to control plant viruses (Petrov *et al.* 2015; Petrov *et al.* 2016a). Compounds such as sukomycin have also shown positive results in reducing symptoms of *Potato virus Y* and *Tomato mosaic virus* in tobacco, although there is currently no effective antiviral drug against plant viruses in tobacco (Petrov *et al.* 2016b).

Investigating plant extracts for the control of plant viruses, therefore holds great promise. In this study cardiac glycoside containing plants were selected since cardiac glycosides have shown activity against a variety of viruses. The results of this study also supported previous findings that plant extracts can be effective in reducing virus infection and symptom development in virus infection. The plant extracts did not have any negative effects or cause any systemic damage to the tobacco plants, and it was shown that the plants can reduce infection symptoms and systemic infection.

In both the *in vivo* and *in vitro* experiments, increased concentrations of plant extracts resulted in decreased development of PVY^{NTN} related symptoms on tobacco plants. The results also indicate that the tested plant extracts were more active in controlling the local infection (*in vivo*) and the symptoms than controlling the systemic infection (*in vitro*). A similar assumption was reached by Othman and Shoman (2004), who indicated that the antiphytoviral activity of *Plectranthus tenuiflorus* (Vatke) Agnew on some important viruses such as *Tobacco necrosis virus* (TNV), *Tomato spotted wilt virus* (TSWV) and TMV was more active in suppressing symptom development than systemic infection. According to Othman and Shoman (2004) this can be attributed to several possibilities; such as, faster virus replication than inducing resistance in treated plants, or perhaps having more effect on the virus movement than virus multiplication thereby delaying the start of systemic symptoms.

Comparing both the *in vivo* and *in vitro* experiment results, with increased concentrations of extracts, the symptoms of PVY^{NTN} virus on the tobacco plant decreased. In the *in vivo* experiment, plant extracts, including *N. oleander, C. orbiculata* and *B. volubilis* (fresh bulb) showed significant inhibition of PVY^{NTN} at 50 mg · ml^{-1} and *S. speciosus* leaves at 20 mg · ml^{-1}. These extracts were able to limit the lesion development with no, or only mild symptoms (infection scale of 17, 20, 20 and 40) as compared

Table 3. DAS-ELISA OD values of PVY^{NTN} infection after plant extract treatment. Values not followed by the same letter are significantly different. [Bd: *B. volubilis* (dry bulb); Bf: *B. volubilis* (fresh bulb); C: *C. orbiculata*; G: *G. fruticosus*; Md: *M. plumbea* (dry bulb); Mf: *M. plumbea* (fresh bulb); N: *N. oleander*; Sf: *S. speciosus* (fruits); Sl: *S. speciosus* (leaves); PC: positive control; NC: negative control; H: 50 mg · ml^{-1}; L: 20 mg · ml^{-1}]

Treatment	DAS ELISA OD value	$LSD_{0.05} = 0.085$	Treatment	DAS ELISA OD value	$LSD_{0.05} = 0.085$
Sl PC (L)	0.252	a	C (H)	0.130	d-r
Bd PC (L)	0.242	ab	Bf (L)	0.128	d-r
Mf (L)	0.213	abcd	Sf (L)	0.127	e-r
Sf PC (H)	0.207	a-e	Mf (H)	0.125	e-r
Mf PC (H)	0.198	a-f	Bd (H)	0.117	h-r
Bd (L)	0.193	a-f	Sf (H)	0.115	h-r
N PC (L)	0.188	a-g	N PC (H)	0.115	h-r
Mf PC (L)	0.177	a-h	N (L)	0.103	g-r
C (L)	0.172	a-j	Md (H)	0.093	h-r
Sf PC (L)	0.170	a-j	Md NC (H)	0.030	r
C PC (H)	0.168	a-j	Sl NC (L)	0.030	r
Md PC (H)	0.168	a-j	C NC (H)	0.028	r
C PC (L)	0.163	b-k	Sf NC (H)	0.027	r
Bd PC (H)	0.160	b-l	N NC (L)	0.027	r
Bf PC (H)	0.157	b-m	Sl NC (H)	0.027	r
Bf PC (L)	0.155	c-h	C NC (L)	0.027	r
G PC (H)	0.155	c-h	Sf NC (L)	0.027	r
G PC (L)	0.150	c-q	Bd NC (L)	0.025	r
Sl (L)	0.148	c-q	Bf NC (H)	0.023	r
G (L)	0.147	c-q	Md NC (L)	0.023	r
Md PC (L)	0.147	c-q	Bd NC (H)	0.025	r
Md (L)	0.142	d-r	G NC (H)	0.023	r
Sl (H)	0.135	d-r	Mf NC (H)	0.023	r
G (H)	0.135	d-r	G NC (L)	0.020	r
Sl PC (H)	0.133	d-r	Bf NC (L)	0.017	r
N (H)	0.130	d-r	Mf NC (L)	0.017	r
Bf (H)	0.130	d-r	N NC (H)	0.020	r

to the positive control treatments with moderate to severe and very severe symptoms (infection scale values 47 and 67) (Fig. 1). Jing *et al.* (2012) examined the antiviral potential of 126 Chinese plants, including *C. orbiculata*. Similar to the results of this study, *C. orbiculata* strongly inhibited infection and viral replication. In the *in vitro* ELISA tests, *S. speciosus* was the most effective plant extract to inhibit the infection of PVY^{NTN} at both concentrations tested, although with different extracts of the plant. Even though the DAS-–ELISA OD values were in the low range, statistically significant values could still be obtained, indicating that there was indeed a significant decrease in the systemic infection, even at a low infection rate. The ethanolic extracts of *S. speciosus* (leaves) at the lower concentration (20 mg · ml^{-1}) significantly reduced the expression of PVY^{NTN} symptoms on tobacco plants *in vitro*, as well as the fruit extract at 50 mg · ml^{-1} (Fig. 2) without affecting the normal growth and development of the plant. Therefore, this also supports the findings of the *in vivo* experiment with *S. speciosus* showing activity in both experiments.

Even though the study confirmed and supports the development of natural based antiviral treatments, a higher concentration of extracts would probably yield even better results and could be tested in further studies, as well as spraying at intervals and in combination, especially after infection. It is suggested that the increased concentration of the tested plant extracts and the application of plant extracts before and after inoculation when conducting *in vivo* experiments may be more successful in controlling the virus. This should be investigated further. Subsequently, the success in the use of plant extracts as antiphytoviral agents needs to be confirmed in field trials where natural infections occur.

Conclusions

The aim of this pilot study was to determine the effect of plant extracts containing cardiac glycosides on PVY^{NTN} as a new approach to antiphytoviral treatment. The need for development of more plant based antiviral treatments for plant viruses motivated the investigation of plant extracts containing antiviral compounds, such as cardiac glycosides, to control the plant virus PVY^{NTN} since the virus results in considerable economic losses of potato, tobacco, tomato and pepper. Of the six plants evaluated in this study, ethanolic extracts of *N. oleander, C. orbiculata* and *B. volubilis* (fresh bulb) showed significant inhibition of PVY^{NTN} *in vivo* at 50 mg · ml^{-1} and *S. speciosus* leaves at 20 mg · ml^{-1}. *Strophantus speciosus* was the most promising plant with significant inhibition in both the *in vivo* and the *in vitro* assays with the leaf extract at 20 mg · ml^{-1} and the fruit extract at 50 mg · ml^{-1}, with the potential of being used as an antiphytoviral treatment. The difference in active plant extracts in the two experiments might also indicate different modes of action since having a better inhibitory effect on the virus movement than virus multiplication would result in a delay in systemic symptoms. The plants showing better results in symptom development might be more active in reducing viral load and therefore lower infection was achieved *in vivo*. This study thus also shows that combining plants with different modes of action, might also be an alternative approach to developing natural plant based antiviral treatments. Since there are very few remedies available to treat viral infections in plants, this could be an indication of a very promising field of study in the future.

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References

Barker H., McGeachy K.D., Toplak N., Gruden K., Zel J., Browning I. 2009. Comparison of genome sequence of PVY isolates with biological properties. American Journal of Potato Research 86 (3): 227–238. DOI: https://doi.org/10.1007/s12230-009-9076-0

Bertol J.W., Rigotto C., De Padua R.M., Kreis W., Barardi C.R.M., Braga F.C., Simoen C.M.O. 2011. Antiherpes activity of glucoevatromonoside, a cardenolide isolated from a Brazilian cultivar of *Digitalis lantana*. Antiviral Research 92 (1): 73–80. DOI: https://doi.org/10.1016/j.antiviral.2011.06.015

Boonham N., Walsh K., Preston S., North J., Smith P., Barker I. 2002. The detection of tuber necrotic isolates of *Potato Virus Y* and the accurate discrimination of PVY^{O}, PVY^{N}, PVY^{C} stains using RT-PCR. Journal of Virological Methods 102 (1–2): 103–112. DOI: https://doi.org/10.1016/S0166-0934(02)00008-3

Cloete M., Mabasa K., Mulabisana J., Visser A., Rakuambo J., Netshifhehe S. 2013. Evaluation of commercial potato cultivars for resistance to the tuber necrotic strain of *Potato Virus Y*. Acta Horticulturae 1007: 255–259. DOI: https://doi.org/10.17660/ActaHortic.2013.1007.26

Doubnerová V., Janošková M., Synková H., Subr Z., Čeřovská N., Ryšlavá H. 2007. Effect of *Potato virus Y* on the activities of antioxidant and anaplerotic enzymes in *Nicotiana tabacum* L. transgenic plants transformed with the gene for P3 protein. General and Applied Plant Physiolgy 33: 123–140.

Dunning J., Baillie J.K., Cao B., Hayden F.G. 2014. Antiviral combinations for severe influenza. Lancet Infectiious Diseases 14 (12): 1259–1270. https://doi.org/DOI:10.1016/S1473-3099(14)70821-7

Ellis P., Stace-Smith R., Bowler G., Mackenzie D.J. 1996. Production of monoclonal antibodies for the detection and identification of strains of *Potato Virus Y*. Canadian Journal of Plant Pathology 18 (1): 64–70. DOI: https://doi.org/10.1080/07060669609500657

Hu X., Meacham T., Ewing L., Gray S.M., Karasev A.V. 2009. A novel recombinant strain of *Potato virus Y* suggests a new viral genetic determinant of vein necrosis in tobacco. Virus Research 143 (1): 68–76. DOI: https://doi.org/10.1016/j.virusres.2009.03.008

Hu X., Nie X., He C., Xiong X. 2011. Differential pathogenicity of two different recombinant PVY^{NTN} isolated in *Physalis floridana* is likely determined by the coat protein gene. Virology Journal 8: 207–211. DOI: https://doi.org/10.1186/1743-422X-8-207

Husson G.P., Vilagines P., Sarrette B., Vilagines R. 1991. Antiviral effect of *Haemanthus albiflos* leaves extract on herpes virus, adenovirus, vesicular stomatitis virus, rotavirus and poliovirus. Annales Pharmaceutiques Françaises 49 (1): 40–48.

Jing B., Ma Z., Feng J., Liang H., Li C., Zhang X. 2012. Evaluation of the antiviral activity of extracts from plants grown in the Qinling region of China against infection by tobacco mosaic virus (TMV). Journal of Phytopathology 160 (4): 181–186. DOI: https://doi.org/10.1111/j.1439-0434.2012.01880.x

Lee-huang S., Zhang L., Huang L., Chang Y., Huang P.L. 2003. Anti-HIV activity of olive leaf extract (OLE) and modulation of host cell gene expression by HIV-1 infection and OLE treatment. Biochemical and Biophysical Research Communications 307 (4): 1029–1037. DOI: https://doi.org/10.1016/S0006-291X(03)01292-0

Matros A., Amme S., Kettig B., Buck-Sorlin G.H., Sonnewals U., Mock H.P. 2006. Growth at elevated CO_2 concentrations leads to modified profiles of secondary metabolites in tobacco cv. Samsun NN and to increased resistance against infection with *Potato virus Y*. Plant, Cell and Environment 29 (1): 126–137. DOI: https://doi.org/10.1111/j.1365-3040.2005.01406.x

Medini F., Legault J., Pichette A., Abdelly C., Ksouri R. 2014. Antiviral efficacy of *Limonium densiflorum* against HSV-1 and influenza viruses. South African Journal of Botany 92: 65–72. DOI:10.1016/j.sajb.2014.02.003

Mohamed E.F. 2010. Antiviral properties of garlic cloves juice compared with onion bulbs juice against *Potato virus Y* (PVY). Journal of American Science 6: 302–310.

Othman B.A., Shoman S.A. 2004. Anti-phytoviral activity of the *Plectranthus tenuiflorus* on some important viruses. In-

ternational Journal of Agriculture and Biology 6: 844−849. DOI: 1560−8530/2004/06−5−844−849

Payne R.W., Murray D.A., Harding S.A., Baird D.B., Soutar D.M. 2007. GenStat for Windows®. 10th ed. VSN International, Hemel Hempstead, UK.

Petrov N., Stoyanova M., Valkova M. 2016a. Antiviral activity of plant extract from *Tanacetum vulgare* against *Cucumber mosaic virus* and *Potato virus Y*. Journal of Bioscience and Biotechnology 5 (2): 189–194.

Petrov N., Tishkov S., Teneva A., Dimitrova I., Bojilova M. 2016b. Antiviral activity of sukomycin against *Potato virus Y* and *Tomato mosaic virus*. Journal of Bioscience and Biotechnology 5 (3): 203–207.

Petrov N.M., Stoyanova M., Valkova M. 2015. The antiviral activity of extract from St. John's Wort against *Potato Virus Y*. Proceedings of the Union of Scientists – Ruse 7: 229−232.

Prassas I., Diamandis E.P. 2008. Novel therapeutic applications of cardiac glycosides. Nature Reviews Drug Discovery 7: 926−935. DOI: https://doi.org/10.1038/nrd2682

Prinsloo G., Meyer J.J.M., Hussein A.A., Munoz, E., Sanchez, R. 2010. A cardiac glycoside with *in vitro* anti-HIV activity isolated from *Elaeodendron croceum*. Natural Products Research 24 (18): 1743−1746. DOI: https://doi.org/10.1080/14786410903211912

Prinsloo G., Marokane C.K., Street R.A. 2018. Anti-HIV activity of southern African plants: Current developments, phytochemistry and future research. Journal of Ethnopharmacology 210: 133−155. DOI: https://doi.org/10.1016/j.jep.2017.08.005

Schnitzler P., Schneider S., Stintzing F.C., Carle R., Reichling J. 2008. Efficacy of an aqueous *Pelargonium sidoides* extract against herpesvirus. Phytomedicine 15: 1108−1116. DOI: https://doi.org/10.1016/j.phymed.2008.06.009

Shen J.G., Zhang Z.K., Wu Z.J., Ouyang M.A., Xie L.H., Lin Q.Y. 2008. Anti-phytoviral activity of bruceine-D from *Brucea javanica* seeds. Pest Management Science 64: 191−196. DOI: https://doi.org/10.1002/ps.1465

Spoustová P., Hýsková V., Müller K., Schnablová R., Ryšlavá H., Čeřovská N., Malbeck J., Cvikrová M., Synková H. 2015. Tobacco susceptibility to *Potato virus Y* (NTN) infection is affected by grafting and endogenous cytokinin content. Plant Science 235: 25–36. DOI: https://doi.org/10.1016/j.plantsci.2015.02.017

Su C.T., Hsu J.T.A., Hsieh H.P., Lin P.H., Chen T.C., Kao C.L., Lee C.N., Chang S.Y. 2008. Anti-HSV activity of digitoxin and its possible mechanisms. Antiviral Research 79: 62−70. DOI: https://doi.org/10.1016/j.antiviral.2008.01.156

Thresh J.M. 2003. Control of plant virus diseases in sub-Saharan Africa: the possibility and feasibility of an integrated approach. African Crop Science Journal 11: 199−223. DOI: https://doi.org/10.4314/acsj.v11i3.27571

Tribodet M., Glais L., Kerlan C., Jacquot E. 2005. Characterization of *Potato Virus Y* (PVY) molecular determinants involved in the vein necrosis symptom induced by PVY^{N} isolated in infected *Nicotiana tabacum* cv. Xanthi. Journal of General Virology 86: 2101−2105. DOI: https://doi.org/10.1099/vir.0.80926-0

Van Wyk B.E., Van Heerden F., Van Oudtshoorn B. 2002. Poisonous Plants of South Africa, 1st ed. Briza Publications, Pretoria, South Africa, 48, 54, 76, 156, 188, 202 pp.

Visser J.C., Bellstedt D.U. 2009. An assessment and molecular variability and recombination patterns of South African isolates of *Potato Virus Y*. Archives of Virology 154: 1891−1900. DOI: https://doi.org/10.1007/s00705-009-0525-3

Wang Q.Y., Deng L., Liu J.L., Zhang J.X., Hao X.J., Mu S.Z. 2015. Schisanhenol derivatives and their biological evaluation against tobacco mosaic virus (TMV). Fitoterapia 101: 117−124. DOI: https://doi.org/10.1016/j.fitote.2015.01.006

Wong R.W., Lingwood C.A., Ostrowski M.A., Cabral T. 2018. Cardiac glycoside/aglycones inhibit HIV-1 gene expression by a mechanism requiring MEK1/2-ERK1/2 signaling. Scientific Reports 8: 1−17. DOI:10.1038/s41598-018-19298-x

Zhang Z.K., Ouyang M.A., Wu Z.J., Lin Q.Y., Xie L.H. 2007. Structure-activity relationship of triterpenes and triterpenoid glycosides against tobacco mosaic virus. Planta Medica 73: 1457−2463. DOI: https://doi.org/10.1055/s-2007-990263