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In ovo antiviral activity of the constituents of *Artabotrys monteiroae* and *Artabotrys modestus* against Infectious Bursal Disease and Newcastle Disease Viruses

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ABSTRACT

Infectious bursal disease virus (IBDV) and Newcastle disease virus (NDV) are among the avian pathogenic agents of economic importance affecting both subsistence and commercially produced chickens worldwide. With exception of vaccination, there is no treatment of the diseases caused by these viruses to date. The chemical constituents from the two Tanzanian *Artabotrys* species, *A. monteiroae* and *A. modestus* (Annonaceae) were screened for their antiviral activities against IBDV and NDV *in ovo* (in the egg). The nine-day-old embryonated eggs in a set of five were used for testing antiviral efficacy through the allantoic route inoculation assay for each tested compound and controls. For NDV assay, the allantoic fluids from the specimens were further harvested to determine viral infection. Artamonteirine (**1**) from *A. monteiroae* and quebrachitol (2) from *A. modestus* showed significant effective treatment against IBDV. The rest of the tested compounds showed moderate or weak antiviral activity at 360 µg/mL screening concentration. The tested compounds also exhibited *in ovo* antiviral activity against NDV, showing viral titre reduction at a range of 1:128 - 1:256 in the haemagglutination test, indicating 16 and 8 times viral load decline compared with untreated embryo (positive control; 1:2048). Further studies to determine the mode of action and toxicity of the potent compounds need to be undertaken towards the development of such antiviral agents.

Keywords: Antiviral, IBDV, NDV, *Artabotrys monteiroe*, *Artabotrys modestus*, Annonaceae.

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INTRODUCTION

The Infectious Bursal Disease Virus (IBDV, Gumboro) and Newcastle Disease Virus (NDV) are two avian viruses of economic importance in poultry industry. IBDV is highly contagious to young chickens, being characterized by immunosuppression and mortality generally at 3 to 6 weeks of age (O'Connor Jr et al., 2013). The virus destroys the lymphoid follicles in the bursa of fabricius as well as the circulating B-cells in the secondary lyphoid tissues. The disease was first discovered in Gumboro, Delaware in 1962 (van den Berg et al., 2000). In recent years, very virulent strains of IBDV (vvIBDV), causing severe mortality in chicken, have emerged and spread across Europe, Latin America, South-East Asia, Africa, and the Middle East (Müller et al., 2012). Although the virus does not affect humans, and has no public health significance, its economic importance to the poultry industry worldwide is of great concern as it induces chicken susceptibility to other diseases and interferes with effective vaccination.

Similarly, the NDV, a highly contagious zoonotic bird diseases virus affects many domestic and wild avian species. The former category being most affected due to their high susceptibility and potential for severe impacts of an epidemic. Although the disease poses no hazard to human health, the exposure to the infected birds as in most cases in the poultry processing plants can cause mild conjunctivitis and influenza-like symptoms, demonstrating animal-to-human host virus switching mode (https://en.wikipedia.org/wiki/Newcastle_disease). Such phenomenon animal-to-human host switching leading to the emergence of new viral diseases like the recent case of swine and avian flu, has been and will likely continue to be a major source of new human infectious diseases that need continued research to redress the problem.

Currently, there is no therapeutic drug available for treatment of Infectious Bursal Disease (IBD) and Newcastle Disease (ND). However, the diseases can be controlled by vaccination (Müller et al., 2012; https://en.wikipedia.org/wiki/Newcastle_disease), an intervention approach that has been practically inaccessible and expensive to the poor rustics. Consequently, many people in the rural areas practicing predominantly free-range local chickens husbandry resort to the use of the cheap and easily available ethno-veterinary plants (Waihenya et al., 2002; Moreki, 2012; Moreki, 2013 Adejeji et al.,2013; Kisungwe et al., 2013; Rezatofighi et al., 2014; Raza et al., 2015). Such biological resources with demonstrated ethno-veterinary use as well as those not in the ethno-veterinary *per see* have provided evidence for their efficacy at both laboratory and field scales (Waihenya et al., 2002; Moreki, 2012; Bakari et al., 2012; Adejeji et al., 2013; Moreki, 2013; Bakari et al., 2013, Mabiki et al., 2013; Nyandoro et al., 2014; Rezatofighi et al., 2014; Raza et al., 2015). Thus, there is a continued desire for research endeavors to discover not only human drugs, but also drugs of veterinary importance. The prominence of plant-based secondary metabolites in disease control and their potentialities in disease therapeutics have well been documented, the work hereby reported aimed to evaluate antiviral activities of the phytochemicals (Figure 1) from the two Tanzanian *Artabotrys* species, namely *Artabotrys modestus* and *A. monteiroae* against avian viruses by employing the IBDV and NDV *in ovo* model.

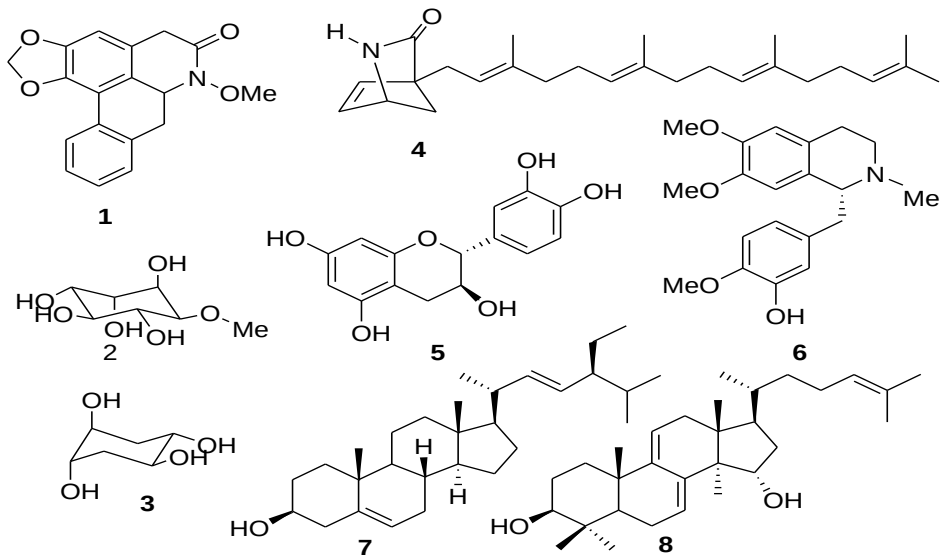

Figure 1: Compounds from *Artabotrys monteiroae* (**1**) and *Artabotrys modestus* (**2-8**).

MATERIALS AND METHODS

Plant materials, isolation and identification of compounds

Information related the collection of plant material and authentication, preparation of plant extract, isolation and identification of the compounds are reported in Nyandoro et al (2013).

Amniotic or allantoic cavity inoculation assay

The embryonated eggs collected from health flocks were used for the evaluation of antiviral activity of the investigated plant constituents against IBDV (KMRG-48 strain) and velogenic NDV strain (13C/SUA). Both viral strains used in the experiment originated from field outbreaks and were supplied by the Department of Microbiology and Parasitology, Sokoine University of Agriculture. Antiviral activity was determined using allantoic cavity inoculation method as previously described (Hsiung, 1973; Nyandoro et al., 2014). The stock solutions containing the tested compounds were prepared by dissolving 2 mg of each sample in 5 ml of DMSO to make 0.4 mg/mL solution concentration. The exterior surface of fertilized eggs were sterilized with 70% ethanol and incubated for 9 days at 37 ºC and 65-70% humidity incubator (Memmert 854, Schwabach, Germany) prior to inoculation. The 9-day-old embryonated eggs were used for the antiviral testing through the amniotic (allantoic) cavity route of inoculation (Hsiung, 1973; Nyandoro et al., 2014). The eggs were candled to confirm their fertility and a puncture made through the shell over the air sac (Grimes, 2002). The eggs were inoculated with 0.1 mL of the specimen [having been prepared by mixing 0.9 ml of tested compound with 0.1 mL of virus in allantoic fluid treated with Gentamycin (antibiotic)], using a sterile needled insulin syringe. The inoculated site of the shell was sealed with paraffin wax, and each egg dated and labeled. For each set of the tested compound, five eggs were used per specimen (compound + virus),

and three sets of controls were employed [egg control (eggs without any inoculums) acting as a negative control, solvent control (DMSO + virus) and virus control (virus only) acting as positive controls. The eggs were then incubated at 37 ºC and 65-70% humidity for 72 and 96 h for IBDV and NDV, respectively. The eggs were candled after every 24 h to monitor embryonic development and survival. The eggshells were then open after 72 or 96 h of incubation to record mortality vs. survivors, size, weight and organ formation. The specimen treated embryos were then compared and contrasted with the control sets in relation to size, weight and completeness in tissue/organ formation. Additionally, InStat 3 statistical package was used to compare the mean weight of the egg control (negative control) with those of the untreated virus-infected-embryos (positive control) and the treated embryos (compound + virus).

Viral haemagglutination test

For the NDV assay, the allantoic fluid from the sample treated specimens and control eggs were harvested to assess viral infectivity. The haemagglutination inhibition test was carried out as previously described by Allan and Gough (1974) and as adopted by Nyandoro et al. (2014). A phosphate buffer solution (PBS, 25 µL) was placed in each well of the microtitre plate. Then allantoic fluids (25 µL each) from negative and positive controls and treated specimens (virus + tested compound) were added to the 1st well of column 1, and each thoroughly mixed. A two fold serial dilution was done by transferring 25 µl of the suspension to the next well and the last one discarded. The 25 µl of 1% chicken RBCs in 99% PBS was added to each well of the microtitre plate. The microtitre plate was shaked, covered and incubated at room temperature for about 30 min. The results were observed to see whether there was haemagglutination or not, and the lowest titre values recorded at the end point beyond which there was no further haemagglutination. Unlike NDV, IBDV is inactive in haemaglutination inhibition test; hence no attempt was made to establish the extent of IBDV clearance following infection of the embryos.

RESULTS

Antiviral activity of the phytochemicals **1-8** evaluated for their treatment effectiveness against IBDV and NDV at 360 µg/mL screening concentration are presented in Tables 1, 3 and 5. In that assay system, with the exception of compound **8**, the embryos treated with other compounds **1-7** remained alive, and grew to normal size and completed organ formation demonstrated effective control of viral virulence at different levels of efficacy (Tables 2, 4 and 6). The *N*-methoxyoxoaporphinoid (artamonteirine, **1)** from *A. monteiroae* and the cyclitol (quebrachitol, **2**) from *A. modestus* were the most potent exhibiting significant antiviral activity against IBDV (Table 2). There was no significant difference between the mean weights of the chicken embryos treated with compounds **1** and **2** when compared with the mean weights of uninfected embryos, indicating effectiveness of the treatment (Table 2). The embryos treated with these compounds remained alive, and grew to normal size and completed organ formation (Table 1).

The rest of the compounds (**3-7**), also from *A. modestus* showed antiviral activity against IBDV with low yet varying efficacy. Thus, the embryos treated with each of the compounds **3-7** remained alive and showed complete organ formation, but had medium body size (Table 1). Hence, the difference

between the mean weights of chicken embryos treated with these compounds and that of uninfected embryos were significant, very significant or extremely significant (Table), indicating less effective treatment. On the other hand, all the embryos treated with compound **8** died indicating that the compound was inactive against IBDV (Table 1).

Generally, the compounds **1-8** showed activity against NDV, enabling the treated embryos to grow to normal size and complete organ formation (Table 3 and 5). However, the mean weights of embryos treated with these compounds were either very or extremely significant compared with uninfected ones, indicating that the treatment was less effective (Tables 4 and 6).

For NDV, the haemagglutination inhibition test was further used as an alternative method to examine the antiviral effect of the tested compounds by generating viral titre values, which showed the extent to which the virus was cleared after treatment. The results from the haemagglutination test as observed in microtitre plates are presented in Table 7. Thus, on the basis of viral titre reduction, compounds **1**, **3** and **5** had the lowest titre value (1:128) while the other compounds had viral titre value of 1:256. These values were 16 and 8 times, respectively compared with the positive control (1:2048), indicating efficacy of the compounds against NDV.

Table 1: Antiviral activity (at 360 µg/ml) of compounds from *Artabotrys modestus* and *A. monteiroae* against IBDV.

	Egg 1	**Egg 2**	**Egg 3**	**Egg4**	**Egg 5**	**Embryo size**	**Embryo average weight (g)**	**Embryo organ formation**
Negative control (Eggs without any inoculums)	Alive	Alive	Alive	Alive	Alive	Normal	7.626	Complete
Solvent control (Virus +DMSO)	Dead	Dead	Dead	Dead	Dead	Abnormal	1.670	Incomplete
Positive control (Virus alone)	Dead	Dead	Dead	Dead	Dead	Abnormal	1.647	Incomplete
Virus + **1**	Alive	Alive	Alive	Dead	Alive	Normal	7.845	Complete
Virus + **2**	Alive	Alive	Alive	Alive	Alive	Normal	7.449	Complete
Virus + **3**	Alive	Alive	Alive	Alive	Alive	medium	5.863	Complete
Virus + **4**	Dead	Alive	Alive	Alive	Alive	Normal	6.742	Complete
Virus + **5**	Dead	Alive	Alive	Alive	Alive	medium	5.878	Complete
Virus + **6**	Alive	Alive	Alive	Dead	Alive	medium	6.363	Complete
Virus + **7**	Alive	Alive	Alive	Alive	Alive	medium	4.751	Complete
Virus + **8**	Dead	Dead	Dead	Dead	Dead	Abnormal	1.685	Incomplete

1 = Artamonteirine, **2** = Quebrichitol, **3** = Tetrol, **4** = Artamodamide, **5** = Catechin, **6** = Laudanine, **7** = Stigmasterol, **8** = Polycarpol

Table 2: Comparison of the mean weight of untreated (infected with IBDV), treated (with compounds from *Artabotrys monteiroae & A. modestus*) vs. uninfected embryos (Mean+_SEM = 0.882 ± 0.004).

Treatment	Mean ± SEM	T-value	P-value	95 % CL	Comment
IBDV only	0.215 ± 0.020	30.618	< 0.0001	0.159-0.270	Extremely significant
Virus + **1**	0.892 ± 0.022	0.4431	0.3347	0.830-0.955	Not significant
Virus + **2**	0.872 ± 0.008	1.099	0.1519	0.848-0.895	Not significant
Virus + **3**	0.760 ± 0.041	2.941	0.0093	0.799-0.924	Very significant
Virus + **4**	0.825 ± 0.029	1.965	0.0425	0.746-0.905	Significant
Virus + **5**	0.744 ± 0.023	5.833	0.0002	0.679-0.809	Extremely significant
Virus + **6**	0.802 ± 0.016	4.687	0.0008	0.757-0.848	Extremely significant
Virus + **7**	0.676 ± 0.152	13.093	< 0.0001	0.634-0.718	Extremely significant
Virus + **8**	0.226 ± 0.011	55.266	< 0.0001	0.196-0.257	Extremely significant

Table 3: Antiviral activity (at 360 µg/ml) of artamonteirine (**1**), a compound from *Artabotrys monteiroae* against NDV.

	Egg 1	Egg 2	Egg 3	Egg 4	Egg 5	Embryo size	Embryo average weight (g)	Embryo organ formation
Negative control (Eggs without any inoculums)	Alive	Alive	Alive	Alive	Alive	Normal	9.438	Complete
Positive control (Virus +DMSO)	Dead	Dead	Dead	Dead	Dead	Abnormal	3.837	Incomplete
Positive control (Virus alone)	Dead	Dead	Dead	Dead	Dead	Abnormal	3.204	Incomplete
Virus + **1**	Alive	Alive	Alive	Dead	Alive	Normal	6.958	Complete

Table 4: Comparison of the mean weight of untreated (infected with NDV), treated with artamonteirine (**1**, a compound from *Artabotrys monteiroae*) vs. uninfected embryos (Mean ± SEM = 0.972 ± 0.026).

Treatment	Mean ± SEM	T-value	P-value	95 % CL	Comment
NDV only	0.505 ± 0.015	15.573	< 0.0001	0.463-0.546	Extremely significant
Virus + **1**	0.846± 0.003	4.938	0.0006	0.835-0.850	Extremely significant

Table 5: Antiviral activity (at 360 µg/ml) of compounds from *Artabotrys modestus* against NDV.

	Egg 1	Egg 2	Egg 3	Egg 4	Egg 5	Embryo size	Embryo average weight (g)	Embryo organ formation
Negative control (Eggs without any inoculums)	Alive	Alive	Alive	Alive	Alive	Normal	6.708	Complete
Positive control (Virus +DMSO)	Dead	Dead	Dead	Dead	Dead	Abnormal	3.359	Incomplete
Positive control (Virus alone)	Dead	Dead	Dead	Dead	Dead	Abnormal	2.595	Incomplete
Virus + **2**	Alive	Alive	Alive	Alive	Alive	Normal	5.293	Complete
Virus + **3**	Alive	Alive	Alive	Alive	Alive	Normal	5.213	Complete
Virus + **4**	Alive	Dead	Alive	Alive	Alive	Normal	4.959	Complete
Virus + **5**	Dead	Alive	Alive	Alive	Alive	Normal	5.799	Complete
Virus + **6**	Alive	Alive	Alive	Dead	Alive	Normal	5.490	Complete
Virus + **7**	Alive	Alive	Alive	Alive	Alive	Normal	5.224	Complete
Virus + **8**	Alive	Alive	Alive	Alive	Dead	Medium	4.608	Complete

Table 6: Comparison of the mean weight of untreated (infected with NDV), treated (with compounds from *Artabotrys modestus*) vs. uninfected embryos (Mean ± SEM = 0.826 ± 0.013).

	Mean ± SEM	T-value	P-value	95 % CL	Comment
NDV only	0.389 ± 0.076	5.675	0.0002	0.178-0.599	Extremely significant
Virus + **2**	0.724 ± 0.006	7.057	< 0.0001	0.706-0.740	Extremely significant
Virus + **3**	0.684 ± 0.035	3.844	0.0025	0.587-0.779	Very significant
Virus + **4**	0.671± 0.032	4.544	0.0009	0.583-0.758	Very significant
Virus + **5**	0.763 ± 0.003	4.616	0.0009	0.754-0.773	Extremely significant
Virus + **6**	0.738 ± 0.016	4.243	0.0014	0.694-0.782	Very significant
Virus + **7**	0.717 ± 0.012	5.997	0.0002	0.683-0.752	Extremely significant
Virus + **8**	0.662 ± 0.017	7.613	< 0.0001	0.855-0.881	Extremely significant

Table 7: NDV Haemagglutination assay results.

Specimen	+ve/-ve	Well #	Titre
Negative control (Eggs without any inoculums)	-ve	-ve	-ve
Positive control (NDV alone)	+ve	11	1:2048
Positive control (NDV +DMSO)	+ve	10	1:1024
Virus + **1**	+ve	7	1:128
Virus + **2**	+ve	8	1:256
Virus + **3**	+ve	7	1:128
Virus + **4**	+ve	8	1:256
Virus + **5**	+ve	7	1:128
Virus + **6**	+ve	8	1:256
Virus + **7**	+ve	8	1:256
Virus + **8**	+ve	8	1:256

DISCUSSION

The results of the antiviral assays presented above indicated that the tested compounds from *A. monteiroae* and *A. modestus* had antiviral activity against the two tested avian viruses, albeit with unequal efficacy. Whereas the oxoaporphine alkaloid artamonteirine (**1**) from *A. monteiroae* and quebrachitol (**2**) from *A. modestus* exhibited the strongest activity against IBDV, other compounds demonstrated moderate to weak activity. The observed antiviral activity of compound **1** could be ascribed to the presence of the oxo- and methylenedioxy functionalities as reported for similar aporphine alkaloids (Sondergaad et al., 2005; Mohamed et al., 2010). The compound has previously been reported to possess potent activity against *Staphylococcus aureus* comparable to the standard antibiotic Ampicillin as well as an acute lethal effect against mosquito larvae (Nyandoro et al., 2013). Such potencies are further corroborated in the present studies. On the other hand, antiviral activity observed for quebrachitol (**2**) couldn't be surprising since the compound and other related cyclitols are well known for their medicinal importance including antiviral properties due to their ability to phosphorylate or aminate with cellular molecules (Harit and Ramesh, 2016). Therefore, similar targets against the tested avian viral strains can be envisaged.

It is worth noting that the tested compounds were more potent to IBDV than NDV. The observed differences in activity against the two assayed viruses and between various tested compounds could probably be attributed to differences in the mode of action of the compounds against the tested viruses. The compounds could have virucidal,

prophylactic, inhibitory, or antiretroviral effects, or combinations thereof. Although the viral load was reduced as indicated by titre values for NDV, the mode of action and elucidation of the exact target sites of the active compounds was not determined. Indeed, this is a subject of future research. While the viral load was somehow reduced and virulence control to some extent was apparent as shown by titre values (for NDV) and embryo survival, normal size, complete organ formation, and mean weights of chicken embryos treated with some compounds (**1** and **2**) showing no significant difference as compared with uninfected embryos, indicating their effectiveness.

Conclusion

Of the tested compounds, artamonteirine (**1**) from *A. monteiroae* and quebrachitol (**2**) from *A. modestus* demonstrated significant effectiveness against IBDV *in ovo* at 360 µg/mL screening concentration while the rest showed moderate or weak antiviral activity. The tested compounds also exhibited *in ovo* antiviral activity against NDV, showing viral titre reduction at a range of 1:128 - 1:256 in the haemagglutination test, indicating 16 and 8 times viral load decline compared with untreated embryo (positive control; 1:2048). Further studies to determine the mode of action and toxicity of the potent compounds need to be undertaken towards the development of such antiviral agents.

COMPETING INTERESTS

The author declares that he has no competing interests.

AUTHORS' CONTRIBUTIONS

SSN carried out the research and produced the manuscript.

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