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In-Ovo Antiviral Activity of *Hibiscus sabdariffa* against Newcastle Disease Virus

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Abstract

Newcastle disease is a highly contagious viral infection affecting poultry and wild birds. The causative agent is Avian paramyxovirus 1 (APMV-1), causing significant economic losses despite vaccination efforts due to its high mortality rate. *Hibiscus sabdariffa* was identified at Modibbo Adama University Yola, and laboratory assays were performed at the National Veterinary Research Institute Vom. The study explores the antiviral effects of extracts from *H. sabdariffa* calyx against a virulent strain of Newcastle Disease Virus (NDV) using embryonated chicken eggs (ECE). Standard methods were employed for cytotoxicity assay, embryo infective dose 50 (EID50) determination, and therapeutic antiviral assays. Methanol was used for extraction and phytochemical analysis, revealing various bioactive compounds like cardiac glycosides, alkaloids, flavonoids, terpenes, and phenols. Toxicity assay showed cytotoxicity at concentrations over 25 mg/ml, but therapeutic antiviral assays demonstrated virus replication inhibition at concentrations as low as 5 mg/ml. These findings suggest the potential of *H. sabdariffa* calyx extracts as safe and effective treatments for NDV, with promising therapeutic antiviral properties. Further pharmaceutical research is recommended to explore their use in developing novel Newcastle Disease treatments.

Keywords: *Hibiscus sabdariffa*; Newcastle Disease Virus; Acute toxicity; Egg infectious dose 50 Antiviral assay.

INTRODUCTION

Newcastle disease (ND) is a highly significant infectious disease with a global distribution and is prevalent in the poultry industry. It has the potential to cause substantial economic losses (Haddas, 2023). ND is particularly endemic in numerous developing nations, especially in areas where poultry farming plays a decisive role as a source of livelihood. In such regions, the impact of ND is most pronounced (Al-Natour *et al.*, 2024).

Newcastle disease virus (NDV) is the aetiologic agent of Newcastle disease, which has the remarkable ability to infect more than 240 species of birds. Transmission primarily occurs through direct contact between infected and healthy birds2 (Al-Rasheed *et al.,* 2024). Similar to other viral infections, ND is one of the most highly contagious diseases globally (Megahed *et al.*, 2023).

The mortality and morbidity rates in a poultry flock can vary significantly, ranging from 90% to 100% in poorly vaccinated chickens. Even in well-vaccinated layers, egg production can decline, depending on the specific strains of the Newcastle disease virus (NDV) (Chiwanga *et al.*, 2023).

Newcastle disease is officially recognized as a "list 'A' disease" by the OIE, as outlined in OIE's 2012 documentation. The NDV is a single-stranded RNA-containing virus with helical capsid symmetry of the Paramyxoviridae family and the Avulavirus genus (Chollom *et al.,* 2023). The control of Newcastle disease primarily relies on the rigorous implementation of biosecurity measures on farms, raising public awareness about the disease, and vaccination (Charkhkar et al., 2024). Among these control strategies, vaccination stands out as the foremost effective and efficient approach, capable of providing comprehensive protection against the disease. However, it is worth noting that in some cases, vaccine failures can occur, leading to widespread outbreaks with high rates of mortality and morbidity (Hassanzadeh *et al.,* 2024).

Hibiscus sabdariffa, commonly known as Roselle, is a plant with a rich history of medicinal use in Africa (Omilabu *et al.,* 2010). Roselle, which belongs to the Malvaceae family, is a versatile medicinal plant found in

many tropical and sub-tropical regions worldwide (El-Sagher *et al.,* 2024). This plant is known by various names in English-speaking regions, including Rozelle, Sorrel, Green sorrel, Jamaica sorrel, Guinea sorrel, Indian sorrel, Queensland jelly plant, Sour-sour, lemon bush, Jelly okra, and Florida cranberry (Mohamed, 2021). In Nigerian languages, it is locally termed Yakwua in Hausa, with the calyx drink being popularly known as sobo (Ajoku et al., 2015). It is also known as Amukan in Yoruba and Okworoozo in Igbo (Ajoku et al., 2015).

Furthermore, *H. sabdariffa* is well-known for its abundance of phytochemical compounds and possesses several beneficial properties, including being an antioxidant, hypotensive, hypocholesterolemic, immune-modulating, hepatoprotective, renoprotective, diuretic, anti-obesity, antiurolithic, antidiabetic, antimicrobial, and anticancer agent. Importantly, it has been found to have no significant genotoxic effects (Sri et al., 2023).

The lack of effective drugs for treating ND and the challenges connected with the reliability of vaccines, particularly in developing countries, continue to pose significant and ongoing challenges for researchers. Consequently, there is a pressing need to explore antiviral compounds derived from plants that are both safe and effective in combating ND. It is essential to recognize that, like other natural plant-based products, antivirals of natural origin have demonstrated their ability to exhibit satisfactory pharmacological and pharmaceutical properties. They have proven effective against a wide range of viral diseases by disrupting the replication cycle of various DNA and RNA viruses (Ohemu *et al.,* 2020).

Given the detrimental impact of viral diseases on poultry production and the global economy as a whole, this research aims to address this urgent need by investigating the antiviral properties of extracts derived from *H. sabdariffa* calyx against the Newcastle disease virus (NDV) using embryonated chicken eggs (ECE). The objective is to identify potential antiviral agents from natural sources that can contribute to the control and management of ND, which has far-reaching economic implications.

MATERIALS AND METHODS

Collection of Plant Material

The dried calyx of *H. sabdariffa* was procured from Yola Market, Adamawa State, Nigeria. Subsequently, the plant material underwent identification and authentication by Botanist U. A Bappa in the Department of Science Laboratory Scientist, and was assigned a voucher number ASP/PLS/0253.

Extraction

The calyx of *H. sabdariffa* underwent a series of preparation steps. Initially, it was meticulously sorted to eliminate any unwanted particles and dead matter. Subsequently, the sorted calyx material was air-dried in the shade. Once thoroughly dried, the calyx was ground into a fine powder. The powdered calyx material was then preserved in a clean, air-tight container.

The bioactive compounds from the dried powdered calyx were extracted by maceration. Methanol was used as the solvent for the extraction. The extracts obtained through maceration were then concentrated to dryness under reduced pressure using a rotary evaporator at 50 ^{o}C. This process enables the isolation and concentration of the desired compounds from the *H. sabdariffa* calyx for further analysis (Ohemu *et al.,* 2020).

Phytochemical analysis

Phytochemical screening of the extracts was conducted following established and standardized methods, as outlined by Doughari (2006).

Acute toxicity assay of plant extract

This procedure was conducted to evaluate the potential harmful effects of the extracts on 9-day-old chicken embryos, following the method outlined by Chollom et al. (2022). The 9-day-old ECE were divided into seven groups, each consisting of five eggs. In groups one through five, the eggs were first sterilized with 70% alcohol using cotton wool and then punctured with an egg puncher. Subsequently, 0.2 ml of the extract, with concentrations of 5, 10, 15, 20, and 25 mg/ml in sequential order, was introduced into the eggs through the allantoic cavity. After injection, the needle was carefully removed, and the puncture site was sealed with molten wax. Group six received an inoculation of 0.2 ml of phosphate buffer saline (PBS) as a positive control. Group seven served as a negative control and did not receive any extract. The eggs were sealed with molten wax and then placed in an incubator at 37 ℃ for 24 h. The survival and condition of the embryos were assessed after 24 h, 48 h, and 72 h; afterwards, the results were documented.

Inoculum preparation (virus/extract mixture)

To achieve the desired extract concentrations in the virus/extract mixture, a 1:2 v/v dilution will be performed using 100 EID50/0.1 ml of the virus. This dilution will be made with predetermined extract concentrations, resulting in final extract concentrations of 5, 10, 15, 20, and 25 mg/ml in the virus/extract mixture. Following the dilution, the virus/extract mixtures will be allowed to react at 4°C for 1 h.

Therapeutic antiviral assay

The methodology closely followed the guidelines set forth by Chollom et al. (2022), making minor adjustments as necessary. At nine days old, embryos were divided into nine groups, each comprising five eggs, and treated with varying amounts of extract. To

maintain sterility, plastic egg trays were meticulously cleaned with Virkon® solution, and the eggs were swabbed with 70% alcohol prior to handling. Subsequently, the eggs were opened, and the extract was introduced into the allantoic cavity within a biosafety cabinet. Groups 1–5 received mixtures of virus and extract, ranging from 5 to 25 mg/ml, in 0.2 ml doses. Group 6 served as the standard NDV (virus control), Group 7 received only the extract suspension (extract control), Group 8 was administered phosphate-buffered saline (diluent control), and Group 9 remained untreated as a baseline control. Following inoculation, all eggs were sealed with molten wax and placed in an incubator set at 37 °C. Daily assessments were conducted to monitor embryo survival, and allantoic fluid samples from treated eggs were collected for spot and hemagglutination tests to detect NDV presence in the experimental eggs.

Spot Hemagglutination Test

Dead embryos, which had been previously refrigerated, were allowed to equilibrate to room temperature for about 30 min. Following this, the eggs were swabbed and transferred to the biosafety cabinet. The procedure involved carefully opening the shell of each egg to expose the air space, after which a pipette was employed to dispense a drop of 10% washed chicken red blood cells onto a white tile. Subsequently, a wire loop, which had been meticulously sterilized, was used to collect a drop of the allantoic fluid, which was then mixed with the drop of blood. To detect any obvious agglutination, which would suggest viral activity in the sample, the tile was gently rocked. (Murakawa et al. 2003). This process was carried out for all eggs, and detailed observations were systematically recorded.

RESULTS

Phytochemicals result of *H. sabdariffa*

The phytochemical study revealed the presence of compounds with bioactivity with both pharmacological significance and nutritional relevance, namely cardiac glycosides, alkaloids, flavonoids, terpenes, and phenols, as shown in Table 1.

Table 1. Qualitative Phytochemical Analysis of Extracts of *Hibiscus Sabdariffa* calyx. (Key: - = Negative, + = Positive)

Phytochemical	Inference
Glycoside	+
Terpenes	+
Tannin	-
Emulsion Saponins	-
Alkaloid	+
Flavonoid	+
Phenol	+
Steroid	-

Cytotoxicity assay of *H. sabdariffa*

Table 2 presents the findings about how *H. sabdariffa* calyx's maximum toxic concentration affected ECE. According to these findings, the maximum extract concentration (25 mg/ml) was tolerated by the embryonated chicken eggs, showing no significant mortality compared to the control group. However, at concentrations of 25 mg/ml and 20 mg/ml, mild toxicity was observed, with only 20 % mortality recorded for both concentrations in the chick embryos. No mortality was recorded in the group treated with phosphate-buffered saline diluent.

Table 2. Acute toxicity properties of *H. sabdariffa* calyx extracts on Newcastle Disease Virus.

Dilution (mg/mL)	Number of Eggs	Mortality rate			% Mortality
		24 h	48 h	72 h	
25	5	1/5	0/4	0/4	20
20	5	0/5	1/5	0/4	20
15	5	0/5	0/5	0/5	0
10	5	0/5	0/5	0/5	0
5	5	0/5	0/5	0/5	0
DC	5	0/5	0/5	0/5	0
NC	5	0/5	0/5	0/5	0

Keys: DC = Diluent control, NC = Negative control

Egg infectious dose 50 (EID_{50}) of Newcastle disease virus

The findings of the Egg Infective Dose 50 (EID50) of NDV on 50% of infected chick embryos, calculated using the Reed Muench formula, are displayed in Table 3. It was determined that 1EID50/0.1mL corresponded to $10^{-8.5}$, and 100EID50/0.1mL equated to $10^{-6.5}$. This is equivalent to a dilution of 1:100,000.

Table 3. Egg Infectious Dose 50 (EID_{50}) of Newcastle Disease Virus (NDV) at 72 h after inoculation.

Virus Dilution	Number of dead	Number of Alive	Cumulative Dead	Cumulative Alive	Proportion Dead total	Percentage % Mortality
10^{-6}	5	0	15	0	15/15	100
10^{-7}	5	0	10	0	10/10	100
10^{-8}	4	1	5	1	5/6	83
10^{-9}	1	4	1	5	1/6	16.6
10^{-10}	0	5	0	10	0/10	0

Antiviral assay of *H. sabdariffa* against Newcastle Disease Virus

Table 4 shows the therapeutic potential of the methanol extract from H. sabdariffa calyx. At higher concentrations (25, 20, and 15 mg/mL), the extract effectively inhibited viral activity without causing mortality. However, at lower concentrations (5 and 10 mg/mL), mortality was observed. Controls had no mortality except for the extract control, which had a 20% mortality rate. The virus control group experienced complete mortality and agglutination. Additionally, the assay indicated a decrease in HA viral titer with increasing extract concentrations.

Table 4. Antiviral activity of *H. sabdariffa* calyx extracts on Newcastle Disease Virus (NDV).

Extracts	Concentration (mg/ml)	Number of Egg	Mortality			Spot Test		Mortality %
			24 h	48 h	72 h	+ ve	- ve	
MEC	25	5	0/5	0/5	0/5	0	5	0
	20	5	0/5	0/5	0/5	0	5	0
	15	5	0/5	0/5	0/5	0	5	0
	10	5	0/5	0/5	1/5	1	4	20
	5	5	0/5	1/5	1/4	2	3	40
VC	0.2 ml	5	0/5	1/5	4/4	5	0	100
EC	25 mg	5	1/5	0/4	0/4	0	5	20
DC	0.2 ml	5	0/5	0/5	0/5	0	5	0
NC	-	5	0/5	0/5	0/5	0	5	0

Key: VC = Virus control, EC = Extract control, DC = diluent control, NC = Negative control
MEC= Methanol Calyx extract, -ve = Negative, +ve = Positive

DISCUSSION

Recently, there has been a growing interest in the use of medicinal plants to treat various ailments with herbal preparations being increasingly integrated into healthcare practices for both humans and animals. The use of extracts derived from *H. sabdariffa* in the treatment of various conditions has been well-documented by Albadri et al. (2023). These findings align with those reported by Okereke (2015). The variation in the levels of these bioactive compounds may be attributed to the ability of different solvents to extract specific active ingredients or substances from the plant calyx, depending on their polarity (Mohammed, 2020).

These bioactive substances are thought to be involved in the antiviral activity seen against NDV and to be the source of the traditional use of *H. sabdariffa* as a herbal remedy. They exert their effects by either killing the virus or interfering with its replication, as demonstrated in previous research (Hegazy et al., 2023). The quest for bioactive lead compounds that might be utilized as precursors for the production of more potent drugs may benefit significantly from the utilization of these active ingredients (Abubakar *et al.*, 2020).

Furthermore, the nutritional supplements derived from these natural sources, although crude, are generally considered safer for consumption by animals and humans compared to commercially formulated supplements. Commercial supplements may involve genetic modifications that can lead to health concerns such as cancer cell formation and obesity in the long run (Chollom et al., 2023).

The observation that ECE displayed tolerance to the extract in a dose-dependent manner aligns with the inherent characteristics of biological cells. Living cells possess certain tolerance levels when exposed to biochemical substances. They can thrive and multiply efficiently when the concentrations of these substances are compatible with their metabolic and physiological well-being. However, when exposed to exogenous products that significantly disrupt their normal physiology, cells may begin to exhibit pathological changes and ultimately succumb, indicating toxicity at those concentrations (Ohemu *et al.*, 2020).

The findings of the toxicity assay performed on the *H. sabdariffa* methanol extract showed that the extracts showed deficient levels of toxicity. The *H. sabdariffa* calyx extracts were well-tolerated by embryonated eggs at concentrations of 25 mg/ml and below, which underscores their suitability for research purposes at these tolerable levels. This finding is critical to the efficient and well-researched use of H. sabdariffa in the treatment of Newcastle disease in poultry. To ensure optimal outcomes for farmers, it becomes crucial for researchers to establish appropriate dosage guidelines based on the body weight of the birds. This ensures that lower doses, which may be ineffective against the virus but tolerated by host cells, are not administered. In contrast, very high doses, exceeding the tolerance threshold of host cells and potentially harmful, are avoided. Therefore, it is imperative to develop a scientifically derived formula that guarantees both the efficacy of *H. sabdariffa* products against the virus and their safety for host cells.

However, it is noteworthy that a minor level of toxicity was noted at lower concentrations, namely at 25 mg/ml (20 % mortality rate) of the extract. This observation aligns with the findings of previous studies conducted by Ohemu et al. (2020) and Abubakar et al. (2020), which investigated the acute toxicity potential of certain plant extracts in Ovo. Similar to the findings of the current study, earlier investigations showed that methanol extracts were less toxic in the 5 to 25 mg/ml range. Nonetheless, it is crucial to emphasize that studies have shown that the *Moringa oleifera* seed extract has reduced toxicity at greater concentrations of 250, 200, and 100 mg/ml (Chollom *et al.*, 2012). Variations in plant toxicity are attributable to elements such as their chemical makeup, evolutionary strategies for protection, toxin concentrations, genetic diversity, environmental pressures, developmental phases, and the absorption of toxins from their environment.

The dilution concentration at which the virus had infected 50 % of the embryos was determined by calculating the Egg infectious dose (EID50) of NDV. The calculation of EID50 followed established standard procedures. Based on the Reed-Muench formula, the Egg infective dose (EID) of NDV on 50 % of the infected chick embryos is shown in Table 3. The findings indicate that the EID50 was observed at a dilution of 10^{-8}, while a 100 % infection rate was recorded at dilutions of 10^{-5} and 10^{-6}. In contrast, dilutions of 10^{-9} and 10^{-10} yielded infection rates of 16.6% and 0%, respectively.

The Egg Infective Dose (EID50) test for NDV was conducted to ascertain the concentration of dilution at which the virus infected 50 % of the embryos. In this study, the EID50 was determined to be at a dilution of 10^{-8}. It is noteworthy that this result slightly differed from those obtained in previous studies conducted by Chollom et al. (2012) and Abubakar et al. (2020). These variations might be attributed to differences in the inoculation procedures within the growth medium. Additionally, the results of the spot hemagglutination test, conducted to confirm the presence of NDV in the inoculated eggs, revealed varying degrees of agglutination at different extract concentrations. Determining this value is crucial to ensure that there are adequate viable viruses in the challenge viral suspension to induce disease in the experimental birds. It also helps assess the inhibitory potential of the plant extract, mirroring the virulence of a field virus in the event of natural NDV infection. Variations in EID50 values can arise due to factors like the specific strain or genetic makeup of the pathogen under study, the condition and age of the host organisms (embryonated chicken eggs), fluctuations in environmental conditions during the experiment, and variances in the techniques employed for both inoculation and assessment.

Previous research has highlighted the antiviral potential of Hibiscus sabdariffa calyx extract against various viruses, including different strains of avian influenza, hepatitis A virus, Aichi virus, murine norovirus-1, herpes simplex virus-2, feline calicivirus, and measles virus. Additionally, this study investigated the therapeutic effects of methanolic extracts from H. sabdariffa against Newcastle Disease Virus (NDV), consistent with the findings of Mohammed et al. (2023), attributing the inhibitory effects to its bioactive components such as glucoside hibiscus, anthocyanin, and gossypetin.

Furthermore, *H. sabdariffa* exhibited antiviral activity, suggesting that the herb may bind to specific cell receptors, preventing the virus from adhering to the cells. The ongoing growth of chicken embryos, as evidenced by increased organ development in NDV-challenged ECE, indicates that the extracts may disrupt the cycle of viral replication. This interference could occur by blocking certain stages of viral propagation inside the cells, preventing viral invasion mechanisms, or directly killing the virus in the inoculate.

CONCLUSION

Empirical findings from the study demonstrate the antiviral potentials of *H. sabdariffa* against ND, attributed to various bioactive compounds present in its calyx extracts, including alkaloids, flavonoids, tannins, phenols, steroids, glycosides, and saponins. Importantly, toxicity evaluation revealed the extracts to be relatively non-toxic. To optimize effectiveness while ensuring safety, researchers must determine appropriate dosage levels based on bird body weight. This requires a scientifically derived formula to strike a balance between virus efficacy and host cell safety. The administration of *H. sabdariffa* extract presents a promising and healthier approach to mitigating NDV infection, positioning it as a potential therapeutic agent for Newcastle disease treatment.

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