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Preliminary Phytochemical Screening and Antiviral Potential of Methanol Stem Bark Extract of *Enantia chlorantha* Oliver (Annonaceae) and *Boswellia dalzielii* Hutch (Burseraceae) against Newcastle Disease *In Ovo*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors TLO, AA and SCC designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors VAO, DGD and PNO managed the analyses of the study and contributed in the writing of the final draft. All authors read and approved the final manuscript.

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ABSTRACT

Aim of the Study: To determine the phytochemicals and the antiviral activity of methanol stem bark extract of *Enantia chlorantha* and *Boswellia dalzielii* against Newcastle disease virus in embryonated eggs.

Materials and Methods: Preliminary phytochemical screening was carried out using standard methods. Investigation on the effect of stem bark of *Enantia chlorantha* and *Boswellia dalzielii*

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methanol extracts against Newcastle disease (ND) virus was carried out using an *in ovo* assay. Nine–day-old embryonated chicken eggs were used. 0.2ml New Castle Disease virus (NDV) pre-treated with methanol extract of *Enantia chlorantha* Oliver and *Boswellia dalzielii* Hutch (Stem bark) at final concentrations of 150, 100, 50, 25, 12.5 mg/ml were administered. Controls were included, embryos were observed daily for survival. Allantoic fluids from treated eggs were collected for spot test and haemagglutination test to detect NDV in the eggs.

Results: Phytochemical analysis carried out on *Enantia chlorantha* Oliv. (stem bark), revealed the presence of alkaloids, reducing sugars, cardiac glycosides, steroid, triterpenes and glycosides, while tannin and flavonoids were found to be absent. *Boswellia dalzielii* Hutch revealed the presence of carbohydrates, steroids, triterpenes, cardiac glycosides, tannins and flavonoids and absence of alkaloid.The result of the antiviral assay showed that the minimum toxic concentration of both extracts is 150 mg/ml. *Boswellia dalzielii* showed the most significant activity against NDV with complete survival of the embryo at all concentration studied and complete clearance of the virus from the allantoic fluid, as compared to *Enantia chlorantha* where mortalities were seen at 150 and 25 mg/ml respectively.

Conclusion: This finding has clearly demonstrated that *Enantia chlorantha* and *Boswellia dalzielii* stem bark extract has antiviral potential against NDV *in ovo.*

Keywords: Enantia chlorantha; Boswellia dalzielii; Newcastle disease virus; phytochemical screening; antiviral.

1. INTRODUCTION

Since the origin of human civilisation on earth, medicinal plants have been used in the treatment of diseases and infection, including viral infections [1].

Viral infections are some of the world's most transmissible diseases; this is because they are almost always followed by a secondary bacterial infection. However available antiviral and vaccines have shown good results [2].

The high cost of available antiviral drugs and their toxic side effects, viral resistance coupled with viral latency and conflicting efficacy in recurrent infection in immunocompromised patients has made viral disease a major and continuous burden for researchers [3].

Newcastle diseases virus (NDV) is of the order, mononegavirales and the family; Paramyxoviridae. Paramyxovirus virions are enveloped, covered with large pepplomer and contain a herringbone- shaped helically symmetrical nucleo- capsid. The genome consists of a single linear molecule of negative – sense, single stranded RNA. The envelop contains two glycoproteins, a hemagglutinin (in most species with neuramindase activity) and a fusion protein [4].

The viruses have only been defined primarily in mammals and birds. Transmission is mainly by aerosol and droplets. A lot of human viruses' falls into this family, for example; human parinfluenza virus1, measles virus, mumps virus, human respiratory syncytial virus [4].

In spite of the various intervention programs including vaccination, New castle disease remain a disease of global concern Most Avian species are susceptible to New castle disease, but chicken are the most susceptible [5].

E. chlorantha Oliver (family- Annonaceae) locally known as Awopa, Oso pupa or Dokita igbo (Yoruba) is an understorey tree of the high rain forest of up to 30m high and 70cm girth, with a long clear bole. This tree grows in dense shade and may be recognised by its bright yellow slash and conspicoius black fruits [6]. Its distribution is limited to South Nigeria, West Cameroons and Fernando Po, however may also be found in Gabon, Angola and Zaire [7].

The plant is mainly used in the treatment of malaria fever in Nigeria [8]. Studies have reported the ethnopharmacological uses of *E. chlorantha* as a hepatoprotective, antiviral, antimalarial, antibacterial and antiulcer agent [9]. The stem-bark has also been shown to possess antiviral activity [10]. Investigations have shown that its curative action on sores can be traced to the presence of alkaloid; the principal alkaloid is berberine [7]. The alkaloid protoberberine from *Enantia chlorantha* has been reported to possess anti-hepatitis A, B, C and D properties [11]. Phytochemical studies of the stem bark of the plant also resulted in the isolation of berberine and protoberberine alkaloids [9].

Boswellia dalzielii Hutch (Burseraceae) is a tree of the Savanna forest recognisable by its papery bark peeling off in a ragged manner. It is locally abundant in Northern parts of ivory coast and may sometimes be planted as a village stockade on the vocal peak massive of Northern Nigeria as a live fence to bring prosperity ('Ba-samu') or to prevent ('hannu') bad luck, hence the Hausa names [12]. The bark contains whitish fragrant exudates which is burnt alone or with other fragrant resin to fumigate clothing and in room to drive out flies, mosquitoes etc [12]. In Northern Nigeria, the bark is boiled up in large quantities to make a wash for fever, rheumatism and ulcer. The decoction is taken internally for gastrointestinal troubles (stomachic) [13,12]. The methanol extract of the stem bark was reported to be active against *Echis carinatus* (a saw scaled viper) [14]. The aqueous extract of *B. dalzielli* stem bark was reported to have promising and potential benefit as antisnake venom, antidiarrhoeal and broad spectrum antimicrobial activities [15].

This study is aimed to determine the phytochemicals and the antiviral activity of methanol stem bark extracts of *Enantia chlorantha* and *Boswellia dalzielii* against Newcastle disease virus in embryonated eggs.

2. MATERIALS AND METHODS

2.1 Plant Collection and Identification

The fresh stem-barks *Enantia chlorantha* were collected from the Obatedo forest in Akure South L.G.A., Ondo State, Nigeria while the stem barks of *Boswellia dalzielii* were collected from Jos North L.G.A., Plateau State. The plants were first identified on field using keys and description given in the Flora of west Tropical Africa [16] and the useful plants of West Tropical Africa [7]. The botanical identity of *E. chlorantha* was further confirmed and authenticated at the herbarium of the Forestry Research Institute of Nigeria, Ibadan where voucher no FHI 101821 is available for reference. The botanical identity of *B. dalzielii* was also confirmed and authenticated at the herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria where voucher no 1314 is available for reference.

2.2 Extraction

The stem-bark of *E. chlorantha* and *B. dalzielii* were sorted to eliminate unwanted particles and dead matter, after which they were air dried under shed, then ground to powder and preserved according to the methods described in Evans [17].

Extraction was carried out on 300g each of the powdered plant material by maceration, for 48hrs, using Methanol. The extracts obtained were concentrated to dryness using a rotary evaporator, dried and then stored in a suitable container for Phytochemical and biological studies.

2.3 Phytochemical Screening

Phytochemical screening was carried out using methods described in Sofowora [18].

2.4 Antiviral Assay

2.4.1 Source of virus and 9-day old embryonated chicken eggs

A velogenic strain of Newcastle Disease virus was obtained from Viral Research Department while embryonated chickens eggs were obtained from Poultry Division of National Veterinary Research Institute, Vom, Nigeria.

2.4.2 Determination of EID_{50} of the virus

The EID_{50} of the virus was determined as recorded by Young et al. [19]. From this, $100EID_{50}$/0.1ml of the virus stock was made for the experiment.

2.4.3 Acute toxicity assay of plant extracts

This was done to estimate the minimum toxic concentration of the extracts in nine days old embryonated chicken egg (ECE) using 0.2 ml of each concentration to inoculate five viable ECE per extracts concentration of 250, 200, 150, 100, 50, 25, and 12.5 mg/ml; the control group received 0.2 ml PBS. Inoculated eggs were incubated for 2 days at 37°C and monitored daily for mortality by the process of candling. Extracts toxicity was determined by examination of embryo for lesions and hemorrhages and by the percentage mortality of embryos.

2.4.4 Preparation of inoculum (virus/extract mixture)

A 1:1 v/v dilution of the 100 EID_{50}/0.1 ml of virus with predetermined extract concentrations was made to put extract final concentration in the

virus/extract mixture at 150,100, 50, 25 and 12.5 mg/ml. The virus/extract mixtures were kept at 4°C for 1 h to react before inoculation.

2.4.5 Inoculation of eggs

The procedure by Chollom [5] was adopted. Nine-day-old embryonated chicken eggs were divided into eight groups of fives each. The embryonated chicken eggs were labeled according to the extract concentrations used. A set of plastic egg trays were thoroughly cleaned with Virkon ® (a disinfectant), the eggs were swabbed with 70% alcohol in cotton wool and transferred into the cleaned trays.

The swabbed eggs were placed in the micro-safety cabinet where they were punched and immediately inoculated with the extract/virus mixture via the allantoic route. Groups 1 to 5 were inoculated with 0.2 ml of virus/extract mixtures at final concentrations of 150, 100, 50, 25 and 12.5 mg/ml.

Group 6 was inoculated with 0.2 ml 100 EID_{50}/0.1 ml standard NDV (virus control), Group 7 was inoculated with 0.2 ml phosphate buffered saline (diluents control) while group 8 was not inoculated (uninoculated control). The eggs were sealed with molten wax and incubated at 37°C and embryo survival was observed at 24 hours daily. Allantoic fluids from treated eggs were collected for spot haemagglutination test to detect NDV in the eggs.

2.4.6 Spot haemagglutination test

Dead embryos that had been chilled were brought out of the refrigerator and kept at room temperature for about 30 min. The eggs were swabbed and placed in the biosafety cabinet. The shell of each egg was opened to reveal the air space and a pipette was used to dispense a drop of 1% washed chicken red blood cells on a white tile. A wire loop was thoroughly flamed and used to pick a drop of the allantoic fluid which was mixed with the drop of blood. The tile was gently rocked and observed for visible agglutination, indicating viral activity [20]. This was done for every egg and the observations were recorded.

3. RESULTS AND DISCUSSION

3.1 Preliminary Phytochemical Screening

The result of phytochemical studies of the extract as shown on Table 1 revealed the presences alkaloids, reducing sugars, saponins, steroid and triterpene.

The stem bark extract was found to contain alkaloids, reducing sugars, saponins, cardiac glycoside, steriod, and triterpene. Previous studies on the *Enantia chlorantha* roots and stem-bark respectively, revealed the presence of phenolics, flavonoids, alkaloids, glycosides and saponins while tannins, phlobatannins and steroids were not detected [21,8]. Gill [22] implicated the alkaloid –berberine as the active ingredient of *Enantia chlorantha*. Saponin and tannins were also present. A similar study has isolated, from the stem-bark of *Enantia chlorantha*, berberine and protoberberine alkaloids [9]. Several alkaloids have been shown to possess antiviral activity [23]. The berberine alkaloid has been reported to possess a broad spectrum antiviral activity [24]. Another study revealed that berberine may be useful for the treatment of infections with influenza A [25].

The preliminary phytochemical screening of the stem bark of *B. dalzielli* as shown on Table 1 revealed the presence of carbohydrates, steroids, triterpenes, cardiac glycosides, tannins and flavonoids and absence of alkaloid. These findings are in agreement with those of previous studies [13,26].

3.2 Antiviral Assay

3.2.1 Acute toxicity assay of *E. chlorantha* and *B. dalzielii* methanol extract

The cytotoxicity assay of *E. chlorantha* revealed that the extract is highly toxic at concentration of 250 to 200 mg/ml with 100 % mortality, while mild toxicity was observed at 150 mg/ml with 40 % mortality. 0 % mortality was observed from 100 to 12.5 mg/ml, indicating that these concentrations are safe for the embryo.

100 % mortality was observed with *B. dalzielii* at 250 and 200 mg/ml respectively. 20% mortality was observed at 150 mg/ml, 0 % mortality was seen at concentrations of 100 to 12.5 mg/ml. (Table 2).

The methanol extracts of *E. chlorantha and B. dalzielii* exhibited no acute toxicity against the embryo at the concentration of 100 mg/ml and below, as no death was recorded and embryo appears normal. However, the minimum toxic concentration of both extract was taken as 150

mg/ml, since survival was observed at this concentration.

3.2.2 Antiviral assay of *E. chlorantha* methanol stem bark extract against NDV

Embryos of inoculated eggs with extract at concentration of 150 mg/ml partially inhibited virus growth as 60 % of mortality in the embryos of the eggs were observed.

The growth of the virus in the embryonated eggs was however; completely inhibited by the stem bark extract at concentration of 100, 50 and 12.5 mg/ml. The survival of the entire embryo in the eggs inoculated with concentrations 100, 50 and 12.5 mg/ml respectively, and the negative results of the spot heamagglutination test proves this complete inhibition. 20 % mortality was observed at 25 mg/ml. The diluents control had live embryo throughout the duration of the experiment, while the entire embryo in the virus control died 48 hrs. post inoculation. This result was further confirmed by the results of spot agglutination test. 20 % mortality was observed in the uninoculated control (Table 3).

The stem-bark of *E. chlorantha* had antiviral potential against NDV. Complete inhibition of virus growth was seen at 100, 50 and 12.5 mg/ml. No mortality (0%) was observed in the embryo of all the inoculated eggs at these concentrations. The spot heamagglutination test result also revealed 0% mortality due to virus, implying that the plant inhibited the viral growth. The 20% mortality seen at 25 mg/ml occurred after 24 hours post inoculation which implies it could be due to mechanical injury during inoculation or bacterial infection. These findings support previous studies that the stem-bark has antiviral activity [10].

In a similar study, the water extract of *E. chlorantha* showed significant antiviral activity against yellow fever virus (YFV) as it completely inhibited the infectivity of YFV as evident in complete absence of Cytopathic effects (CPEs) [27].

3.2.3 Antiviral assay of *B. dalzielli* methanol stem bark extract against NDV

The bark extract at 150, 100, 50, 25 and 12.5 mg/ml completely inhibited virus growth in embryonated eggs as revealed by survival of embryos of the inoculated eggs. The diluent and uninnoculated controls had live embryo throughout the duration of the experiment, while the entire embryo in the virus control died 48hr post inoculation. This result was further confirmed by the results of spot agglutination test (Table 4).

Table 1. Result of phytochemical screening of the stem bark extracts

Constituents	*B. dalzielli* Bark	*E. chlorantha* bark
1. Carbohydrates	+	+
2. Unsaturated steroid & triterpene	+	+
3. Cardiac glycoside	+	+
4. Tannins	+	-
5. Flavonoids	+	-
6. Alkaloids	-	+

Key = + present, -Absent

Table 2. Acute toxicity assay of *E. chlorantha* and *B. dalzielii* methanol extracts

Extract dilutions (mg/ml)	No of Eggs	% Mortality after 72 hours	
		Methanol extract *Enantia chlorantha*	Methanol extract *Boswellia dalzielii*
250	5	100	100
200	5	100	100
150	5	40	20
100	5	0	0
50	5	0	0
25	5	0	0
12.5	5	0	0
Vc	5	100	100
Dc	5	0	0
Uc	5	0	0

Table 3. Antiviral activity of *Enantia chlorantha* methanol Stem-bark extract against NDV

		Mortality (Pi)				HA		
Extract Dilutions (mg/ml)	**No of Eggs**	**24hr**	**48hr**	**72hr**	**%Mortality**	**Agglutination (+ve)**	**No Agglutination (-ve)**	**% Agglutination due to virus**
150	5	2/5	0/3	1/3	60	0	5	0
100	5	0/5	0/5	0/5	0	0	5	0
50	5	0/5	0/5	0/5	0	0	5	0
25	5	1/5	1/5	1/5	20	0	5	0
12.5	5	0/5	0/5	0/5	0	0	5	0
Vc	5	0/5	5/5	-	100	5	0	100
Dc	5	0/5	0/5	0/5	0	0	5	0
Uc	5	0/5	1/5	0/4	20	0	5	0

KEY: Vc:Virus control, Dc: Diluent control, Uc: Uninoculated control, Pi: Post inoculation, HA: Heamagglutination

Table 4. Results of antiviral activity of *B. dalzielli* methanol stem bark extract against NDV

		Mortality (Pi)				HA		
Extract Dilutions (mg/ml)	**No of Eggs**	**24hr**	**48hr**	**72hr**	**%Mortality**	**Agglutination +ve**	**No Agglutination -ve**	**% Agglutination due to virus**
150	5	0/5	0/5	0/5	0	0	5	0
100	5	0/5	0/5	0/5	0	0	5	0
50	5	0/5	0/5	0/5	0	0	5	0
25	5	0/5	0/5	0/5	0	0	5	0
12.5	5	0/5	0/5	0/5	0	0	5	0
Vc	5	0/5	5/5	-	100	5	0	100
Dc	5	0/5	0/5	0/5	0	0	5	0
Uc	5	0/5	0/5	0/5	0	0	5	0

KEY: Vc:Virus control, Dc: Diluent control, Uc: Uninoculated control, Pi: Post inoculation, HA: Heamagglutination

The results of the antiviral assay of the stem bark of *B. dalzielli* confirm that the plant has antiviral potential against NDV. This was revealed by the complete inhibition of the virus growth *in ovo* at 150, 100, 50 and 12.5 mg/ml. No mortality (0%) was observed in the embryo of all the inoculated eggs at these concentrations. The stem bark of *B. dalzielli* has been found to contain phenolic compounds such as protocatechuic acid, gallic acid and ethylgallate as well as a diterpeniod-incensole and triterpeniods- boswellic acid derivatives [15]. These compounds may be responsible for the antiviral activity seen.

4. CONCLUSION

This study has demonstrated for the first time that the methanol extracts of *Enantia chlorantha* Oliv. and *Boswellia dalzielii* Hutch possess antiviral activity against NDV, particularly at concentrations, of 100 mg/ml and below. Further investigations are required to evaluate the mechanism through which these plants mediate its antiviral activity and the phytochemical responsible for the antiviral activity.

ETHICAL APPROVAL

It is not applicable.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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