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In vitro Antiviral Activity of *Eucheuma cottonii* against New Castle Disease Virus

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Authors' contributions

This work was carried out in collaboration between both authors. Author SB designed the conceptual frame work of the study, performed the extensive review of the first draft of the manuscript, and wrote the protocol for the study. Author MM wrote the first draft of the manuscript and managed the literature searches and performed the OVO assay and Hemagglutination tests. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: To determine the antiviral activity of *Eucheuma cottonii* against the New Castle disease virus.
Study Design: *In vitro* study.
Place and Duration of the Study: The research was conducted in the Pharmaceutical Chemistry, Drug Design, and Pharmacognosy department at St. John's University of Tanzania and Virology Laboratory at Sokoine University of Agriculture between April 2022 and August 2022.
Methodology: The present study used an OVO assay and hemagglutination test to investigate the *In vitro* antiviral activity of *E. cottonii* collected from Zanzibar, Tanzania, against the Newcastle disease virus (NDV). Ovo assay was assessed using 60 embryonated eggs and a hemagglutination

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test was performed by collecting the allantoic fluid from treated eggs and serum from hatched chicks to detect antigen titer against NDV using 96 well plate diffusion method.
Results: Results of the Ovo assay showed that all the 40 embryonated eggs used as test group were alive and thus confirmed that the extract *of E. cottonii* (0.32 g/mL) is strong enough to destroy the virus. In which the results from the serial dilution assay (hemagglutination test) confirmed a substantial decrease in the high level of NDV as observed in the allantoic fluid of the infected embryonated eggs to range from 10^-3 to 10^-8 serial dilutions.
Conclusion: The OVO assay and hemagglutination results indicate that *E. cottonii* possesses antiviral activity against NDV. Still further studies like isolation and characterization of bioactive compounds are needed to apply *E. cottonii* as an alternative source of therapy to fight against NDV.

Keywords: Red algae; Eucheuma cottonii; antiviral activity; hemagglutination; OVO assay.

1. INTRODUCTION

Aquatic plant production in the marine environment, such as red sea weeds as reported by Food Agriculture Organization (FAO) was found to be significantly important as late as 1989 and over the last 30 years it reached the highest production level of 196,570 tons as recorded in 2015, although a 43% reduction was seen between 2015 and 2018, this was due to Climate-induced outbreaks of seaweed-specific pests and diseases, together with other economic-related impacts on price [1]. The production of marine aquatic plants in Africa is currently concentrated in Zanzibar (Tanzania,102,960 tons) followed by Madagascar (53,370 tons) and South Africa (16,870 tons) in 2018. Other countries like Kenya, Morocco, Mozambique, Namibia and Senegal, are all still either currently producing or have previously produced sea weeds in the past five years according to FAO 2020. Among the macroalgae that are occurring along the coastal regions of Tanzania, specifically, genus Eucheuma (red marine macroalgae) is commercially cultivated and is being exported for its nutritional values and carrageenan properties.

Eucheuma cottonii is a red marine macroalgae belonging to the Family Solieriaceae, which is commonly known as sea moss and locally called “Mwani” in Tanzania. This species is being cultivated in Zanzibar, Bagamoyo, Dar es Salaam, and Pwani regions of Tanzania for its nutritional values and pharmacological properties. *E. cottonii* is an important ethnomedicinal algae which is used in food and medicine worldwide. Furthermore, this macroalgae provides a valuable range of physiological and biological activities, including antiviral, antimicrobial, antitumor, and antidiabetic activities, and it provides strong antioxidant effects and thus could be utilized in treating various communicable and non-communicable diseases [2–5]. The above-mentioned activities may be due to the presence of bioactive compounds like polyphenols, carrageenans, aggarans, and polysaccharides [6]. Furthermore, dietary fibers, minerals, vitamins, antioxidants, phytochemicals, proteins, and polyunsaturated fatty acids isolated from this seaweed possess various pharmacological properties that benefit human health [6–8]. *E. cottonii* has considerable antiviral action against viruses [9,10]. Among the viral diseases, Newcastle Disease virus (NDV) is commonly observed both in avian species and humans.

In humans, NDV (a member of the paramyxovirus family) causes mild flu-like symptoms and conjunctivitis (an infection of the eye that is also called pink eye) and/or laryngitis. Also like other viruses, NDV infects cells and then uses those cells to replicate. Thus, this virus possesses similar replication phases in its life cycle similar to other viruses that primarily causes infection in humans. The virus is highly contagious and can spread through direct contact, contaminated equipment, and even airborne transmission. Furthermore, this virus poses a great threat to the poultry industry as there is no specific cure despite the vaccines. Likewise, the world population is largely at risk because of the nature of the virus which is highly infectious and can be airborne and cause the same illness in humans. The primary objective of this research was to identify new antiviral drug candidates that have low cytotoxicity and are cost-effective to produce, aiming to assist in managing viral infectious diseases. Thus, this study investigates whether the selected red marine algae occurring in coastal regions of Tanzania possess antiviral activity against NDV which causes viral infections in both avian species and humans.

Fig. 1. Graphical presentation of red marine algae, incubation of eggs, harvested embryos and hemagglutination results

2. MATERIALS AND METHODS

The present study used an OVO assay and hemagglutination test to investigate the in-vitro antiviral activity of *E. cottonii* collected from Zanzibar, Tanzania, against the Newcastle disease virus (NDV). Ovo assay was assessed using 60 embryonated eggs and a hemagglutination test was performed by collecting the allantoic fluid from treated eggs and serum from hatched chicks to detect antigen titer against NDV using 96 well plate diffusion method. These assays were also reported by several studies in literature respectively [11–14].

2.1 Bioassays for Antiviral Activity Determination

2.1.1 OVO assay

60 local chicken eggs from local poultry were used for the OVO assay. Inoculation was performed at 10th day. 60 eggs were candled to see whether the embryonated eggs are living or dead. All the 60 embryos harvested were alive and were used for inoculation. Then the 60 eggs were marked at the edge of the air sac on the shell by using pencil, there after eggs were sterilized at the mark using 70% alcohol. This method was in correlation with the assay procedure reported by Naik et al. and Shoji et al. [12,14] . Forty eggs were inoculated with a mixture of extract (*Eucheuma cottonii*) and Newcastle disease virus (NDV) as a positive control, while 7 eggs received the extract only, 7 received the virus, and 6 received phosphate-buffered saline (PBS) as a negative control, all using sterile insulin syringes. After sealing the inoculated eggs with paraffin wax and incubating for 78 hours, they were candled again, and allantoic fluid was harvested for the hemagglutination (HA) test which is a gold standard tests for assessing hemagglutination inhibition tests in accordance with the studies as reported in literature [11,13]. Furthermore the OVO Assay assisted to look for physical damage by the NDV which was done through observation of embryos growth after inoculation with the drug extracts, virus and PBS,also lesions and feather development as per OVO assay and this was in accordance to studies reported in literature [12,13].

2.1.2 Hemagglutination (HA) titration for newcastle disease virus

Certain viruses possess the capacity to agglutinate red blood cells (RBC) of specific animal species at defined temperatures. Hemagglutination test was conducted in correlation to studies reported in literature [11,13]. 25 uL of PBS was added into each well of a V-96 well plate. The same volume of inactive Newcastle virus was added to the first well. A two –fold dilution of 25 uL of the virus was prepared for each well of the whole plate. 25 uL of the allontoic fluid which was collected by using pippete was added to each well. Then, the solution was mixed gently in each well. The plate was incubated for 40 min in room temperature ready for the assay. Negative Newcastle chicken serum and positive Newcastle chicken serum was used as control. Ascertainment of Hemagglutination test is recorded as shown in Table 1.

Table 1. Ascertainment of Hemagglutination Test

+ve	**Agglutination**
-ve	**No agglutination**

A standard hemagglutination test was conducted on harvested allantoic fluid to calculate NDV HA titer for the sample. 25 uL of Phosphate Buffer Saline (PBS) was added into each well of the 96 well plate. The same volume of inactive NDV was added to the first well. A twofold dilution of 25 uL of virus was prepared for each well of the whole plate. 25 uL of 1% (v/v chicken RBCs was added to each well then, the solution was mixed gently in each well. The plate was incubated for 40 min at room temperature. Negative NDV Chicken serum and positive NDV chicken serum were used as controls.The hemagglutinations test procedure was carried out in the following order ,25 µL of PBS was placed in each well of

the microtitre plate,Then 25 µL of the diluted virus of 10^{-1} to 10^{-8} was added and mixed with extract, virus (NDV) as the positive control, extract and PBS as negative control to the first well of column 1. Mixing and serial dilution was done by transfering 25 µL to the next well and discard 25 µL from the final well. Then 25 µL of 1 ％RBCs was added in each well of the microtitre plate. Finally, plate was shaked and covered and then incubated at room temperature for about 30 minutes and the results were recorded.

3. RESULTS

Below are the results of the haemaglutination test done to identify the antiviral activity of *Eucheuma cottonii* which was supported by physical observation of anatomical structure damage as per OVO Assay Observations.

3.1 OVO Assay Results (Harvested Embryos from Incubated eggs)

Among the total 60 embryonated eggs after incubation, 40 embryonated eggs out of 40 were all alive giving 100% survival rate making successfulness of the *E. cottonii drug* extracts where by 5 out of 7 embryonated eggs inoculated with the virus only were dead making 71.42% death rate showing the potential virulence of the Virus. Conclusively, 7 out of 7 Embryonated eggs inoculated with the drug extracts were alive giving 100% survival rate and from OVO Assay this indicates that *E. cottonii* possess viral inhibition with the almost zero mortality. The remaining 6 embryonated eggs inoculated with the PBS only were all alive confirming that the solvent media used was not affecting the experiment.

Fig. 2. The picture show that 40 embroynated eggs which were inoculated with the mixture of extract of *Eucheuma cottonii* and PBS, and NDV the result showed that all the embryonated eggs were alive as the extract *of Eucheuma cottonii* was strong enough to destroy the virus

Table 2. Shows the viability of inoculated as per OVO assay

Conc of virus	Viable Embryo	Non-viable Embryo
10^{-1}	5	0
10^{-2}	5	0
10^{-3}	5	0
10^{-4}	5	0
10-4	5	0
10-5	5	0
10-6	5	0
10-7	5	0
10-8	5	0
Extract	7	0
PBS	6	0
Virus	3	4

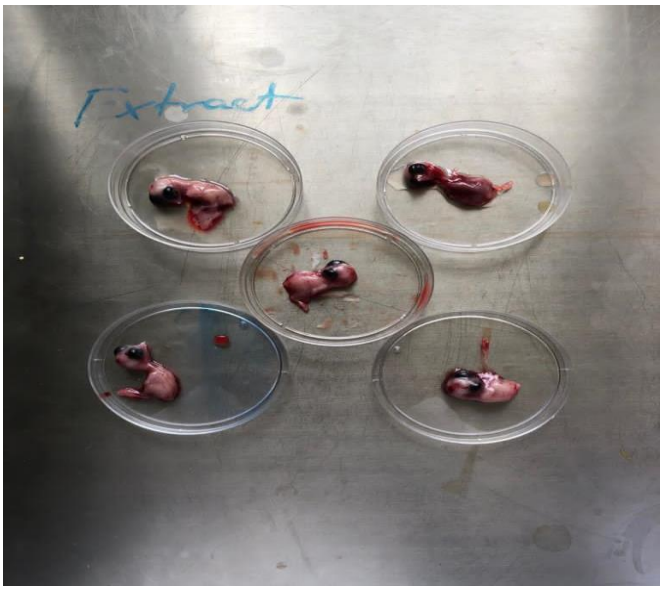

Fig. 3. The embryonated eggs as looked it revealed of no anatomical structure damage and no hemolysis of embryo observed indicating that *Eucheuma cottonii* extract did not harm the embryo hence confirmed safeness of the extract material

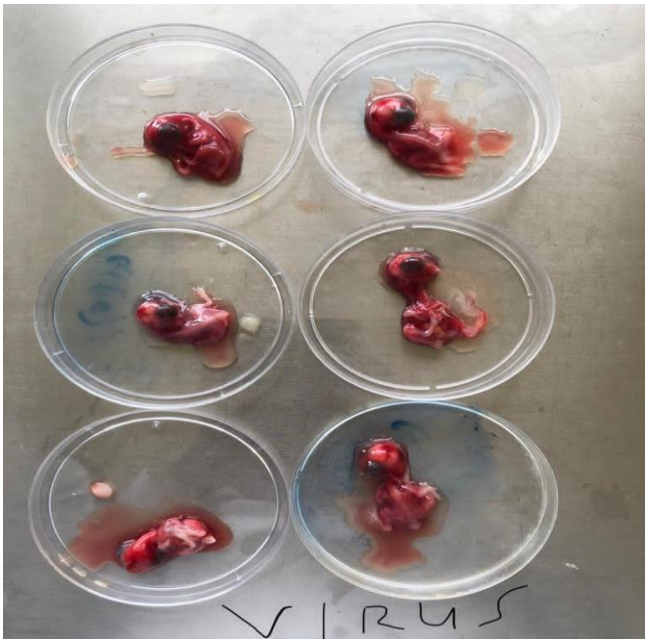

Fig. 4. The embryonated eggs as it looked it showed massive hemolysis of the embryo and massive damage of anatomical structure as to when compared with the embryonated eggs inoculated with drug extract alone this revealed massive destruction by the virus

3.2 Hemagglutination Test Result

Allontoic fluid was collect by using pippete and placed on the microtiter plate Vshape, 25 uL of PBS in each well of the microtiter plate, then 25 uL of +ve and –ve control (allontoic fluid /antigen) to the first well of column was to serial dilution by transfer 25 uL of suspension to the next well 10^{-1} up to 10^{-8}, also hemagglutination by using allontoic fluid of the embroynated eggs which inoculated by extract only, PBS only and Extract only.

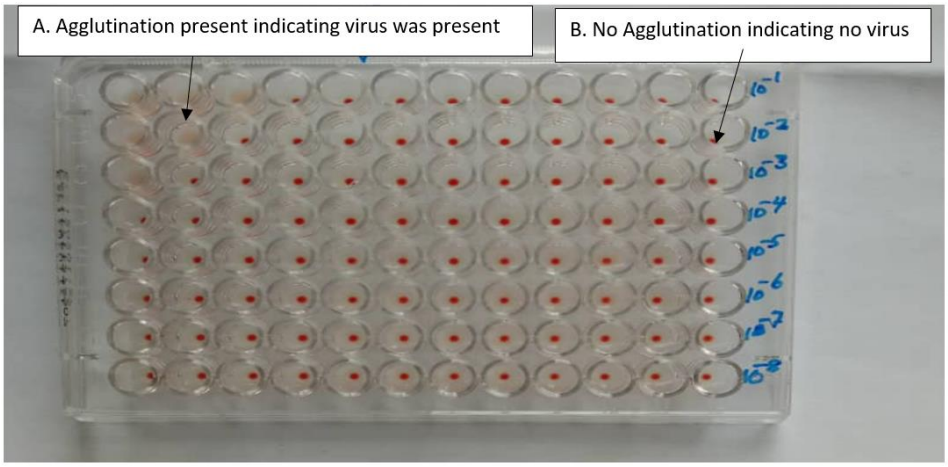

Fig. 5. Hemaglutination plate Showing the results of the allantoic fluid inoculated with the mixture of the drug extract (*E. cottonii*) and the New castle Virus)

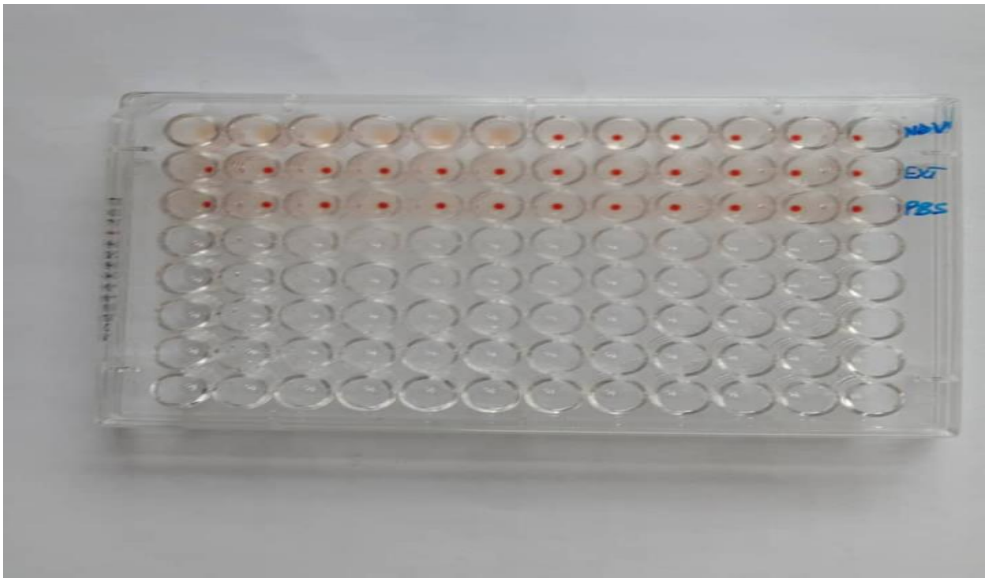

Fig. 6. Haemaglutination plate showing the results of the allantoic fluid inoculated only with PBS, Extracts and Virus which were used as positive and Negative controls Respectively

Table 3. The Result of Antiviral Activity of *Eucheuma cottonii* Extract Derived from Hemagglutination Plate in Fig. 5 & 6

Serial dilution	Embroynated eggs allontoic fluid											
Plate	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
10-1	+	+	+	-	-	-	-	-	-	-	-	-
10-2	+	+	-	-	-	-	-	-	-	-	-	-
10-3	+	-	-	-	-	-	-	-	-	-	-	-
10-4	-	-	-	-	-	-	-	-	-	-	-	-
10-5	-	-	-	-	-	-	-	-	-	-	-	-
10-6	-	-	-	-	-	-	-	-	-	-	-	-
10-7	-	-	-	-	-	-	-	-	-	-	-	-
10-8	-	-	-	-	-	-	-	-	-	-	-	-
Extract only	-	-	-	-	-	-	-	-	-	-	-	-
PBS only	-	-	-	-	-	-	-	-	-	-	-	-
Virus (NDV) only	+	+	+	+	+	+	-	-	-	-	-	-

Interpretation from the haemaglutination test results:

1. **Dilutions**: We tested dilutions from 10^{-1} to 10^{-8}

2. **Results**:

- **Positive Reactions (+)**: The highest dilutions showing positive reactions (presence of NDV) were from 10^{-1} to 10^{-8} indicating the virus was present in these concentrations.

- **Negative Reactions (-)**: From 10^{-4} to 10^{-8} onward, there were substantial decrease in positive reactions, indicating that the virus concentration is below detection limits at these dilutions.

3. **Control sample**:

- **Extract only**: No positives, confirming there is no contamination from the extraction process.

- **PBS only**: No positives, confirming the PBS is not affecting the results.

- **Virus only**: Confirmed that the virus is present in the expected concentration

4. DISCUSSION

Macroalgae, especially red algae, are reported as strong antiviral agents for which they either prevent viral particle fusion into host cells or help decrease the viral load by amplifying host response [15,16]. It is also worth mentioning that a few species of green and brown macroalgae tend to exhibit antiviral activity including Sargassum and Ulva species, which showed activity against HSV-1 and measles virus [17,18]. The red seaweed, *Eucheuma cottonii*, has received recognition for its rich biochemical composition that consists of polysaccharides, phenolic compounds, and other phytochemicals. Additionally, polysaccharides are known to stimulate the immune response, which reduces the viral load in the infected host [19,20].These compounds have been investigated for their numerous biological activities, including antiviral properties [4,21–23].

The results of the present study demonstrated the substantial decrease in NDV detected in the allantoic fluid of the infected embryonated eggs, making it a promising medicinal macroalgae to fight against the New Castle Disease Virus. The serial dilutions result from hemagglutinations in a test group sample showed a decrease in NDV with significant reductions of antigen titer occurring at 10^{-4}& 10^{-8}, confirming that *E. cottonii* substantially inhibited the viral replication. Furthermore, in the embryos which were infected with the virus and treated with drug extract, there was no hemolysis of the embryos when compared to those which were not treated with the drug extract. The available evidence supports the hypothesis that seaweeds are useful natural resources in the treatment of viral infections [24–28]. However, there is no research reported regarding the Newcastle Disease Virus against macroalgae. Thus, *E. cottonii* may pave the way as an alternative source of therapy to fight against NDV.

5. CONCLUSION

The results of the research study indicate that *E. cottonii* possesses antiviral activity and holds as a promising antiviral agent against NDV. Additionally avian flu caused by the New Castle Virus poses a serious illness to most of the population around the world mostly in low- and middle-income countries, also most of them opt for antibiotics use to alleviate these infections which further increases the challenge of antibiotics misuse leading to antimicrobial resistance which is the major catastrophe the world is facing. Additionally E.cottonii is an edible species that bears more potential for antiviral and nutritional properties and hence could enable mankind and society at large to rise above such challenges. These findings will also contribute to the advancement of our understanding regarding how marine resources can be used in developing potent antiviral medications, which is important in light of increased interest in alternative treatments for viral diseases.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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