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Flavonoids Extracted from Fruit Pulp of *Cucumis metuliferus* Have Antiviral Properties

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Authors' contributions

This work was carried out in collaboration between all authors. KIA, NNW and HAI designed the study, wrote the protocol and wrote the first draft of the manuscript. LDI and GOC-O managed the literature searches and managed the analyses of the study. All the authors have read and approved the final manuscript.

Research Article

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ABSTRACT

Aim: To investigate the antiviral property of flavonoids from *Cucumis metuliferus* fruit pulp in chicken embryo fibroblast (CEF) cells and embryonated chicken eggs (ECE) induced with infectious bursal disease virus (IBDV).
Study Design: Extraction and administration of bioactive extract.
Place and Duration of Study: Department of Pharmacology, University of Jos, Nigeria and Virology Department, National Veterinary Research Institute, Vom, Nigeria between June, 2011 and August, 2011.
Methodology: The CEF cells were first exposed to 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.782, 0.391 and 0.195 mg/ml of the sterile flavonoids to test for cytotoxicity and the cells monitored visually daily using a light microscope for evidence of cytopathic effects at 24, 48 and 72 hours. Toxicity of flavonoids in embryonated eggs and antiviral assay for flavonoids using IBDV were then carried out. Hemagglutination test for antigenicity of the virus was also performed to confirm antiviral activity.
Results: The flavonoids (100 to 0.195 mg/ml concentrations) were not cytopathic when exposed to CEF cells. After 24 and 48 hours, all the embryonated eggs died at 100 and

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50 mg/ml of the flavonoids respectively, but mortalities were not recorded at other concentrations of the flavonoids. Concentrations of the flavonoids at 100, 50, 25, 12.5 and 6.25 mg/ml were found to be toxic against IBDV, but viral replication was not inhibited from flavonoids concentrations of 3.125, 1.563, 0.782, 0.391 and 0.195 mg/ml.
Conclusion: This investigation revealed that flavonoids from *Cucumis metuliferus* fruit pulp were relatively safe in chickens and possess antiviral activity against IBDV.

Keywords: Flavonoids; virus; cytotoxicity; embryonated eggs; hemagglutination; chicken embryo fibroblast cells.

1. INTRODUCTION

Viral infections are currently an important health issue that has resulted in extensive research to find drugs that are highly effective. It is a well-established fact that medicinal plants and some vegetables like carrots, apple, onions, citrus and tomato contain phytochemicals which have antiviral activity that can help the body fight viral infections [1] [2]. Some of these infections can occur by breathing air contaminated with a virus (for example swine flu- caused by H1N1 virus), by sharing eating utensils, needles, razors, other sharp objects [3]. Unsafe sex is also a means by which viruses are easily transferred from one person to another [4]. It is on record that the pharmacological activities of some active principles of many medicinal plants of Africa have become a vehicle for the discovery of more efficacious and safer drugs [5] [6].

Acquired Immune Deficiency Syndrome (caused by Human Immunodeficiency Virus), bird flu or avian influenza (caused by the H5N1 virus), swine flu (caused by H1N1 virus), Severe Acute Respiratory Syndrome (SARS), mad cow disease, Infectious Bursal Disease or Gomboro (caused by Infectious Bursal Disease Virus- IBDV) and Newcastle disease (ND) are diseases that have wreaked havoc on man and animals alike. Also, the global spread of highly pathogenic avian influenza H5N1 in poultry, wild birds and humans, still poses a significant pandemic threat and a serious public health risk [7].

Cucumis metuliferus belongs to the family Cucurbitaceae, and is a monoecious, climbing, annual vine that can be grown practically anywhere, provided the season is warm [8]. The fruits are ovoid berries of 8-10 cm long and 4-5 cm in diameter with horn-like spines (hence the name horned cucumber), yellow-orange skin and a lime green jelly-like flesh when ripe. The herb flowers from July to September and the fruits ripen from October to December [9]. The fruit of this plant is edible, but it is often used for decoration as well. The seeds are embedded in the mesocarp, which is emerald green and consists of juicy, bland tasting tissue [8]. The horned melon is native to Africa, and it is now grown in California and New Zealand [8]. It is commonly known as African horned cucumber, melano, Jelly melon, and kiwano.

The fruits occur in two forms: the bitter and non-bitter, which occur mostly in the wild state. The bitter form contains cucurbitacins (triterpenoids), which is a highly toxic compound [10] and is seldom eaten by humans [11]. Other constituents include lutein, lutein expoxide, 3-epiluttein, myristol, palmitol and dipalmitol lutein [12]. In addition, the plant also contains alkaloids, phenylpropanoids, flavonoids and derpenoids [13].

C. metuliferus is used by traditional medical practitioners in certain parts of Plateau state of Nigeria to treat diseases such as peptic ulcer, diabetes mellitus, hypertension and HIV/AIDS [14]. It was also discovered through personal interaction with poultry farmers and other individuals in Plateau state of Nigeria, that the fruit pulp is used to treat poultry diseases and in the management of Hepatitis B. According to [15], the fruit pulp is used in Plateau state, Nigeria as a remedy to all diseases hence its local name 'Kanda' which means 'stop it before it comes' or 'a local vaccine'.

Since the crude extract of C. metuliferus has been shown to possess antiviral property [16] and it is known that certain constituents of a plant can be largely responsible for its medicinal activities, this study, therefore, investigates whether the flavonoids extracted from the fruit pulp of *C. metuliferus* are responsible for its antiviral activity. The choice of flavonoids for this study is important considering documented evidence by various researchers on the antiviral activities of flavonoids against some viruses like human cytomegalovirus (HCMV), Herpes simplex virus-1, Herpes simplex virus-2 [17] [18] and influenza viruses [19].

2. MATERIALS AND METHODS

2.1 Collection and Identification of Plant

The fruits of *Cucumis metuliferus* were collected from Babale village, Bauchi state, Nigeria, through the assistance of Mr. T.P. Yakubu, of the Department of Pharmacognosy, University of Jos and Jos - Nigeria. The whole plant was identified and authenticated by Professor C.O. Akueshi, of the Department of Botany, University of Jos, Jos – Nigeria where the specimen voucher no. CCM2 was kept in the herbarium for future reference. Approval was sought and given by the authorities of the National Veterinary Research Insititute, NVRI, Vom- Nigeria.

2.2 Laboratory Equipment and Chemical Reagents

Menmert table incubator, drying oven, light microscope, 24 – well tissue culture trays (Sigma UK), Hanks Minimum Essential Medium (HMEM) supplemented with 10% fetal bovine serum (Sigma UK), chicken embryo fibroblast (CEF) cells (NVRI, Vom – Nigeria) and infectious bursal disease virus (IBDV) 100 plague forming units (pfu) in HMEM (NVRI, Vom – Nigeria).

2.3 Extraction of Flavonoids

The method of [16] was used in the preparation of the extract. The mesocarp of the fruits and seeds were carefully scooped out of the pericarp using a spatula. This was then separated using sieves (250 mm screen-size) and sun-dried for 7 days. The resultant mixture was then spread in trays and placed in a drying oven set at 60^{o}C until it was dried. It was then pulverized to coarse powder with the aid of mortar and pestle.

100 g of the dried fruit pulp powder was dissolved in 1000 ml solution of dilute ammonia and distilled water and left to stand for 24 hours with intermittent shaking then filtered. The filtrate was extracted using chloroform and the chloroform layer was evaporated to dryness before reconstituting it with dilute hydrochloric acid and then basified with dilute ammonia. This was re – extracted using chloroform and evaporated to dryness [20]. The extracted flavonoids (33.6g) were stored in air tight container at room temperature prior to use.

2.4 Tissue Culture Cytotoxicity Assay for Flavonoids

Chicken embryo fibroblast (CEF) cells were cultured in 24-well tissue culture trays (1 ml per well using suspended cells at a concentration of 10^4 cells per ml). The cells were grown using HMEM plus 10% fetal bovine serum (FBS). Cultured cells were incubated under a humidified CO_2 atmosphere (5% CO_2/95% filtered air at 37ºC) until a confluent monolayer was obtained. 1000 mg of the flavonoids were dissolved in 2 ml of sterile distilled water. Two – fold dilutions of the flavonoids were made from the ratios 1:2 to 1:64 in serum-free medium (HMEM), that is, a concentration of 100 mg per ml down to 0.195 mg per ml were prepared. The flavonoids dilutions (0.1 ml each) were tested for cytotoxicity by exposing monolayer of the CEF cultures to dilutions of the sterile flavonoids and incubated for 72 hours at 37ºC. The medium in the cells were first aspirated out using a multi-channel pipette and corresponding dilutions of flavonoids were introduced into the wells containing confluent monolayer. These were done in four replicates. Controls were wells containing plain medium only (cells were without flavonoids dilutions).

The cells were monitored visually (using a light microscope) daily for three days for evidence of any cytopathic effects or pathological changes [21].

2.5 Antiviral Assay for Flavonoids using IBDV

Chicken embryo fibroblast (CEF) cells were cultured in 24-well tissue culture trays (1 ml per well) until confluent monolayer were obtained. In a set of empty bijou bottles, doubling dilutions each of the flavonoids were prepared in HMEM with 0.1 % FBS to give a range of final concentrations from 100 to 0.195 mg/ml. These solutions were transferred, using multi – channel pipette, into the corresponding wells of cell cultures from which the media had been aspirated. Control cultures were set up which included cells with flavonoids but without virus (negative control) and cells without the flavonoids but with the virus (positive control). After one hour incubation at 37ºC, 1 ml of IBDV in HMEM (plus 0.1% FBS), containing 100 pfu were added to 12 wells (1 well for each concentration including the positive and negative controls and each dilution was always tested in quadruplicate). This replication is similar to work done by [22]. When the virus - induced cell cultures started showing the characteristic pathological changes induced in normal infected cells known as cytopathic effects (CPE), all other cultures were examined microscopically and assessed for CPE. In cases where no CPE were evident, the virus was assumed to be completely inactivated or inhibited. Cases of partial inactivation (50% decrease or more in CPE as compared to untreated virus) were also recorded, since these represent inactivation or inhibition of a fraction of the 100 pfu present in the standard virus dose. The minimum antiviral concentration was that dilution of the flavonoids that gave rise to complete or partial inactivation of the virus [21].

2.6 Toxicity of Flavonoids in Embryonated Chicken Eggs (ECE)

1000 mg of the flavonoids was dissolved in 2 ml of sterile distilled water. A modified method of [23] was used for this assay. Two – fold dilutions of the flavonoids were made from the ratios of 1:2 to 1:64 (concentration of 100 to 0.195 mg/ml were prepared). 0.1 ml of each dilution was inoculated into a set of 5 eggs (10 – day old embryonated chicken eggs) each and incubated for 72 hours at 37ºC. The eggs were candled every 24 – hours to check for viability and mortality for a maximum of 72 hours. Dead eggs were recorded and discarded appropriately [21].

2.7 Rapid Hemagglutination (HA) Test of Extracted Flavonoids of C. *metuliferus* on IBDV

500 mg/ml of flavonoids was reconstituted and two – fold dilutions of the flavonoids were made from the ratio of 1:2 to 1:64 dilutions (100 to 0.195 mg/ml concentrations were prepared). 1 ml of IBDV (with median embryo infective dose of $10^{9.7}$ /0.1 ml and hemagglutination titre of 2^9) was then added to 1 ml each of the various dilutions of the flavonoids and incubated at +4ºC and 37ºC for 30 minutes respectively. 0.2 ml of the flavonoids – virus suspension of each dilution was inoculated into a set of 5 eggs (10 – day old embryonated eggs) each and incubated at 37ºC for 72 hours. The embryonated eggs were candled every 24 hours to check for viability and mortality (dead embryonated eggs were chilled at +4ºC and the allantoic fluid harvested for rapid hemagglutination test to check presence or absence of viral activity). After 72 hours incubation, the embryonated eggs were chilled at +4ºC for 24 hours. The eggs were cut open with a pair of scissors and the allantoic fluid aspirated into bijou bottles. A rapid hemagglutination test was carried out on a glass slide using 10% washed chicken red blood cells on the harvested allantoic fluid. Presence of hemagglutination confirmed viral activity and its absence confirmed antiviral activity of the extracted flavonoids against IBDV [24].

3. RESULTS AND DISCUSSION

Cell cultures exposed to varying concentrations of the flavonoids (100 to 0.195 mg/ml) showed no cytopathic effects as presented in Table 1. The control which was the CEF cells alone also showed no cytopathic effect.

Cell cultures exposed to IBDV and then treated with flavonoids showed cytopathic effects at 0.195-3.125 mg/ml, with no effects observed at higher concentrations of 6.25-100 mg/ml (Table 2). Cytopathic effects which were observed in the positive control administered IBDV were absent in the group administered the flavonoids only (negative control).

The toxicity of the flavonoids in embryonated eggs was noted to be time and concentration dependent. After 24 hours, 5 embryonated eggs died at concentration of 100 mg/ml of the extracted flavonoids, but mortalities were not recorded at other concentrations of the extracted flavonoids (Table 3). After 48 hours, 5 embryonated eggs died at concentration of 50 mg/ml of the extracted flavonoids, but mortalities were not recorded at other concentrations of the flavonoids. The 5 embryonated eggs not treated with the extracted flavonoids (control) all survived after 24 and 48 hours.

The confirmatory antigenicity test showed that at concentrations of 100 to 6.25 mg/ml, zero out of five embryonated eggs was positive and five were negative for the virus, but at concentrations of 3.125 to 0.195 mg/ml, five of the embryonated eggs were positive and zero negative for the virus (Table 4). Five of the embryonated eggs exposed to the virus alone (control) under temperatures of +4 and 37ºC were positive and none was negative for the virus.

Table 1. *In vitro* screening of cytotoxic activity of flavonoids of *C. metuliferus* in CEF cells

Concentration (mg/ml)	Cytopathic effect
100	-ve
50	-ve
25	-ve
12.5	-ve
6.25	-ve
3.125	-ve
1.563	-ve
0.781	-ve
0.391	-ve
0.195	-ve
Control	-ve

n = 4; -ve = no cytopathic effect

Table 2. *In vitro* screening of anti-viral activity of flavonoids of *C. metuliferus* in IBDV infected CEF cells

Concentration (mg/ml)	Cytopathic effect
100	-ve
50	-ve
25	-ve
12.5	-ve
6.25	-ve
3.125	+ve
1.563	+ve
0.781	+ve
0.391	+ve
0.195	+ve
Positive control	+ve
Negative control	-ve

n = 4; +ve = cytopathic effect; -ve = no cytopathic effect

Table 3. *In vivo* screening of flavonoids of *C. metuliferus* for anti-IBDV activity in ECE

Dose (mg/ml)	Mortality/Viability 24 hours	Mortality/Viability 48 hours	Mortality/Viability 72 hours	Mortality/ Viability Total
100	5/0	-	-	5/0
50	0/5	5/0	-	5/0
25	0/5	0/5	0/5	0/5
12.5	0/5	0/5	0/5	0/5
6.25	0/5	0/5	0/5	0/5
3.125	0/5	0/5	0/5	0/5
1.563	0/5	0/5	0/5	0/5
0.781	0/5	0/5	0/5	0/5
0.391	0/5	0/5	0/5	0/5
0.195	0/5	0/5	0/5	0/5
Control	0/5	0/5	0/5	0/5

n = 4

Table 4. In *vivo* screening of flavonoid of *C. metuliferus* for anti-IBDV activity in ECE employing spot HA on allantoic fluid

Dose (mg/ml)	Temperature (ºC)	Temperature (ºC)	Hemagglutination test +ve/-ve for virus
100	37	+4	0/5
50	37	+4	0/5
25	37	+4	0/5
12.5	37	+4	0/5
6.25	37	+4	0/5
3.125	37	+4	5/0
1.563	37	+4	5/0
0.781	37	+4	5/0
0.391	37	+4	5/0
0.195	37	+4	5/0
Control	37	+4	5/0

The preliminary investigation of the crude fruit extracts of *C. metuliferus* as documented by [16] [25] revealed the presence of some secondary metabolites including flavonoids, alkaloids, cardiac glycosides and tannins. Secondary metabolites have been documented to be highly biologically active providing protection against diseases like cancer, coronary heart disease, chronic and degenerative diseases, carcinogenesis, neurodegenerative disorders, diabetic pregnancy, rheumatic disorder, DNA damage and ageing [26] [27], though most may be toxic physiologically [28]. In addition, alkaloids (caffeine, theobromine and theophylline), anthocyanins (cyanidin), carotenoids (beta-carotene, lutein and lycopene), coumestans and flavan-3-ols have been shown to have been shown to have anti-oxidant activity [29]. Flavonoids extracted from different plants have been documented to exhibit various pharmacological activities *in vivo* and many have served as leads for the production of some drugs [30].

The results obtained in this study revealed that flavonoids extracted from the fruit pulp of *C. metuliferus* have no cytopathic effects when exposed to CEF cells. The activities of plant extracts in effecting any therapeutic or biological changes in diseased animals or living tissues are direct functions of the chemical constituents inherent in them after extraction [31]. As can be seen in this study, the flavonoid constituent of the fruit of *C. metuliferus* inhibited the cytotoxic effects of IBDV in CEF cells.

Cell cultures of varying diluted concentrations of the flavonoids (100 to 6.25 mg/ml) exposed to IBDV showed no cytopathic effects denoting inhibition of viral replication. In varying two – fold concentrations of 100 to 6.25 mg/ml of the flavonoids exposed to cell cultures and IBDV, IBDV replication was inhibited. The extracted flavonoids were non-lethal in 10 – day old embryonated eggs except at concentrations of 100 mg/ml and 50 mg/ml after 24 and 48 hours respectively. Exposure of the flavonoids to IBDV – infected embryonated eggs showed antiviral activities from varying two – fold diluted concentrations between 100 to 6.125 mg/ml as there was inhibition of IBDV replication. This was confirmed by the hemagglutination test which is used for definitive detection of virus antibody. The test is based on the principle of adsorbing out the cross - reacting antibodies to IBDV – antigen. As described by [16], negative hemagglutination response seen denoting that there was no viral replication in the embryonated eggs suggests the absence of antibodies production in response to the viral antigen. Other varying two – fold diluted concentrations between (3.125 – 0.195 mg/ml)

where hemagglutination test was positive imply the presence of antibodies production in response to antigen as is seen with the control, where viral replication was not inhibited. This study which revealed the antiviral activity of the flavonoids against IBDV supports earlier work done by [32], who demonstrated the inhibitory activity of some flavonoids against the human immunodeficiency virus (HIV).

Infectious Bursal Disease (IBD), an acute, contagious viral infection causes immune suppression in young chickens and disease and mortality in 3–6-week-old chickens [33] [34]. Currently, IBDV has a worldwide distribution occurring in all major poultry producing areas [35]. Presently, there is no known treatment, though increased fluid intake and multivitamins have been known to reduce symptoms and severity of the infection among poultry [36]. Maintaining a high degree of sanitation and routine vaccination helps control the emergence and spread of disease.

Apart from IBDV, other viruses like Human Immunodeficiency Virus (that causes AIDS), H5N1 virus (that causes bird flu or avian influenza), H1N1 virus(causes swine flu) and Newcastle disease virus (NDV) that cause Newcastle disease in poultry are examples of viruses that cause diseases which have wreaked havoc on animals and man causing eye infections (conjunctivitis), flu-like symptoms (e.g., fever, cough, sore throat, muscle aches) as well as severe respiratory illness (e.g. pneumonia, acute respiratory distress, viral pneumonia), nausea, diarrhea and vomiting are present [37].

4. CONCLUSION

Flavonoids from fruit pulp of *C. metuliferus* can, therefore, be said to have antiviral properties which justify the use of the plant parts especially the fruit in prevention/management of viral infections in chickens.

Further study is needed to properly isolate and identify the specific flavonoid exhibiting antiviral activity as well as evaluate its activity against other viruses.

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COMPETING INTERESTS

Authors have declared no competing interests exist.

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